Normal self proteins as targets for tumour specific cytotoxic T lymphocytes (CTL)

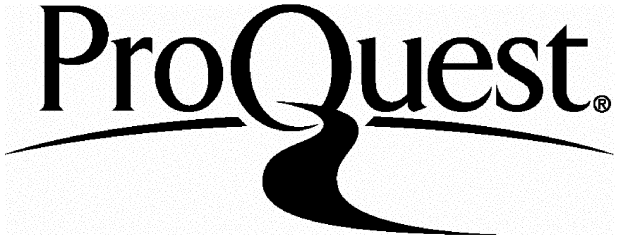
==========

Astrid Maria Dahl

January 1996

This thesis is submitted in fulfilment of the requirements for the degree of doctor in philosophy (PhD).

The work was sponsored by Imperial Cancer Research Fund.



Abstract

Many tumour cells express high levels of proteins which are present in normal cells at lower levels. Such proteins might be targets for immunotherapy if lymphocytes with specificity for these proteins are present in the repertoire and can discriminate between cells expressing high or low levels of the target protein. The first step in exploring the possibility of targeting immunotherapy to normal self proteins is therefore to determine whether lymphocytes specific for molecules overexpressed in tumours exist and can be activated to recognise specifically cells expressing high levels of the proteins.

It was possible to stimulate murine cyclin D1 or mdm2 specific cytotoxic T lymphocytes (CTL) by *in vivo* priming of mice with recombinant vaccinia virus expressing the proteins. The *in vitro* conditions used for restimulation of *in vivo* primed CTL were found to have a profound effect on the magnitude of the CTL response observed *in vitro*. In addition, *in vitro* priming of CTL from naive spleens with peptide pulsed dendritic cells identified a mdm2 derived peptide, mdp441, which could stimulate CTL capable of recognising endogenously processed mdm2. These peptide specific CTL were of high avidity in contrast to most of the peptide specific CTL stimulated with other self peptides. The mdp441 peptide was not recognised by mdm2 specific CTL generated by *in vivo* immunisation with mdm2 protein.

The results show that tolerance to self proteins is not absolute and that it is possible to stimulate CTL to recognise endogenously processed self protein either by *in vivo* immunisation with recombinant vaccinia virus expressing the self protein or by *in vitro* priming of naive responder cells with peptide pulsed antigen presenting cells. These results have implications for immunotherapy of human cancers.

Acknowledgements

I would like to address a very warm thank to my two supervisors, Dr. Hans Stauss and Professor Peter Beverley. I would also like to thank all the people in the Tumour Immunology Unit as well as Dr. Gavin Wilkinson, Cardiff and Dr. Vera Fantl, ICRF for unconditional help and support at various stages during the past three years.

Above all, I would like to express me deep and profound gratitude to Mark Handley, Dr.Torben Lund and Dr. Elma Tchillian, the three most exceptional, extraordinary, intelligent and inspiring people I have encountered and had the great fortune to attract to the Dahl nebula and cyber space think tank.

Table of contents

List of abbreviations 10

1. Introduction 13
1.1. The function and role of the immune system 13
1.2. The mechanisms determining the identity of the antigens presented to CTL 18
1.2.1. Polymorphic MHC class I molecules bind peptides and present them to CTL 18
1.2.2. The synthesis and assembly of peptide/MHC class I complexes 20
1.3. The mechanisms determining the identity of the CTL able to be activated by presented antigens 26
1.3.1. The molecular interaction between T cell receptor and MHC/peptide complexes 26
1.3.2. Mechanisms of T cell activation 28
1.3.3. Mechanism of execution of effector function by CTL 34
1.3.4. Mechanisms by which the T cell repertoire is influenced by processes taking place during T cell development in the thymus and in the periphery 37
1.4. Tumours as potential targets for CTL 41
1.4.1. The contribution of altered protein expression in tumour cells to the generation of novel tumour antigens 41
1.4.2. Potential benefits and disadvantages of using normal self proteins as targets for tumour reactive CTL 44
1.5. Properties of selected recombinant vaccinia virus and adenovirus expression systems 50
1.5.1. Biology of vaccinia virus 50
1.5.2. The immune response to recombinant vaccinia virus 53
1.5.3. Vectors used to construct recombinant vaccinia virus 55
1.5.4. Biology of human adenovirus type 5 (Ad5) 57
1.5.5. The immune response to recombinant adenovirus 60
1.5.6. Vectors used to generate recombinant adenovirus. 61

2. Materials and methods 65
2.1. Molecular biology and protein chemistry techniques 65
2.1.1. Growth and handling of bacteria 65
2.1.2. General procedures for handling of DNA 66
2.1.3. Transfection of mammalian cell lines 72
2.1.4. Isolation and analysis of genomic DNA from mammalian cells 74
2.1.5. Isolation and analysis of mRNA from mammalian cells 78
2.1.6. Isolation and analysis of cellular proteins 83
2.2. Tissue culture 88
2.2.1. Chemicals, animals and cell lines 88
2.2.2. Culture of immortalised tumour cell lines and transfectants 95
2.2.3. General methods for analysis of cells 96
2.2.4. Induction of cytotoxic T lymphocytes 110
2.3. Generation of recombinant vaccinia virus and adenovirus 115
2.3.1. Construction, amplification and analysis of recombinant vaccinia virus 115
2.3.2. Construction, amplification and analysis of recombinant adenovirus 118

3. Generation of expression systems for the production of mdm2, cyclin D1, fibroblast growth factor receptor I and wild type p53 in mammalian cells 120
Introduction 120

3.1. Generation of mdm2 expression systems 124
3.1.1. Generation of recombinant vaccinia virus expressing mdm2 124
3.1.2. Generation of recombinant adenovirus expressing mdm2 132
3.1.3. Generation of cell lines with stable overexpression of mdm2 138
3.2. Generation of cyclin D1 expression systems 145
3.2.1. Generation of recombinant vaccinia virus expressing cyclin D1 145
3.2.2. Generation of recombinant adenovirus expressing cyclin D1 153
3.3. Generation of fibroblast growth factor receptor I expression systems 156
3.3.1. Generation of recombinant vaccinia virus expressing the fibroblast growth factor receptor I (FRI) 156
3.3.2. Generation of transfectants expressing FRI 163
3.4. Generation of p53 expression systems 166
3.4.1. Generation of recombinant vaccinia virus expressing p53 166
3.4.2. Production of recombinant adenovirus expressing p53. 173
Discussion 176

4. Stimulation of cytotoxic T lymphocytes specific for self proteins by *in vivo* immunisation with recombinant vaccinia virus expressing self proteins. 180
Introduction 180
4.1. Induction of CTL specific for cyclin D1 182
4.2.Induction of CTL specific for mdm2 189
4.3. Induction of CTL specific for FRI 196
4.4.Induction of CTL specific for p53 198
Discussion 199

5. **Peptide stimulation of autoreactive high avidity CTL which can recognise naturally processed self protein** 211
Introduction 211
5.1. Use of class I binding motifs to identify class I binding self peptides 212
5.2. Induction of CTL to self peptides 222
5.2.1. A cell population enriched for dendritic cells and purified by a simple two step procedure efficiently primes CTL to self peptides *in vitro* 222
5.2.2. Several class I binding self peptides stimulate self peptide specific CTL from naive spleens *in vitro* 230
5.3. Characterisation of self peptide specific CTL 233
5.3.1. CD8 dependency and class I restriction of self peptide specific CTL lines 233
5.3.2. Recognition of endogenously processed self proteins by self peptide specific CTL 237
5.3.3. mdp441 specific T cells recognising endogenously processed protein are of high avidity 243
5.3.4. The concentration of peptide used to prime CTL does not affect the avidity of the resulting CTL population 256
5.3.5. Induction of mdp441 peptide specific CTL by *in vivo* immunisation with mdp441 peptide 258
Discussion 259

6. Conclusion 271

Appendix I : Commonly used buffers and stock solutions 277
Appendix II: List of reagents 281
References 285

List of tables and figures

1. Introduction:

Tables:

Table 1.4.1. Properties of the murine self proteins cyclin D1, mdm2, fibroblast growth factor receptor I (FRI) and wild type p53 (WT p53). 49

Figures:

Figure 1.2.1. Summary of the major processes involved in the assembly of MHC class I/peptide complexes. 21
Figure 1.5.1 Outline of the replication cycle of vaccinia virus 52
Figure 1.5.2. Schematic representation of the vaccinia virus expression vector pSC11 56
Figure 1.5.3. Schematic representation of the cloning strategy used to generate the recombinant adenovirus plasmid pMV60 -X. 62
Figure 1.5.4. The eukaryotic CMV-IE expression cassette present in vector pMV100 64

2. Materials and methods:

Tables:

Table 2.1.1. Solutions used for making competent E. coli cells. 71
Table 2.1.2. Buffers used for transfection of DNA via calcium precipitation. 72
Table 2.1.3. Conditions used for transfection of mammalian cells by electroporation 73
Table 2.1.4. Solutions used for labelling of DNA probes for DNA-DNA hybridisation on nylon membranes. 76
Table 2.1.5. DNA probes used in hybridisations. 77
Table 2.1.6. Oligonucleotides used as primers in PCR. 80
Table 2.1.7. Antibodies used for immunoprecipitations. 85
Table 2.2.1. Murine cell lines. 92
Table 2.2.2. Human cell lines. 93
Table 2.2.3. Monkey and hamster cell lines. 93
Table 2.2.4. Antibodies directed against murine determinants. 97
Table 2.2.5. Antibodies directed against human determinants 98
Table 2.2.6. Conjugated antibodies or other molecules used for FACS analysis 99

3. Generation of expression systems for the production of mdm2, cyclin D1, fibroblast growth factor receptor and wild type p53 in mammalian cells:

Tables:

Table 3.1. Expression systems for priming, stimulation and analysis of CTL *in vivo* and *in vitro* 123
Table 3.2. DNA, mRNA and protein production obtained with different mammalian expression systems 176

Figures:

Figure 3.1.1. Outline of the procedure used to generate recombinant vaccinia virus expressing murine mdm2 125
Figure 3.1.2. Presence of mdm2 cDNA in Tk$^-$143 cells infected with recombinant vaccinia virus mdm2 127
Figure 3.1.3. Production of murine mdm2 mRNA in Tk$^-$143 cells infected with recombinant vaccinia virus mdm2 129
Figure 3.1.4. Production of mdm2 protein in CHO cells infected with recombinant vaccinia virus mdm2, clone 3.2.1. 131
Figure 3.1.5. Outline of the procedure used to generate recombinant adenovirus expressing murine mdm2 133
Figure 3.1.6. Presence of mdm2 cDNA in CHO cells and EL4 cells infected with recombinant adenovirus mdm2 (Ad mdm2) 135
Figure 3.1.7. Production of mdm2 mRNA in CHO cells infected with recombinant adenovirus mdm2 136
Figure 3.1.8. Production of endogenous and exogenous mdm2 mRNA transcripts in adenovirus mdm2 infected CHO cells 137
Figure 3.1.9. Outline of the procedure used to generate transfected cells expressing murine mdm2 139
Figure 3.1.10. Integration of the entire mdm2 cDNA in the EL4 mdm2 "2.2." clone 141
Figure 3.1.11. Integration of the entire mdm2 cDNA in 4 different P1.HTR mdm2 clones 142
Figure 3.1.12. Transcription of exogenous murine mdm cDNA cloned into the EcoRI site of pDO-R neo and transfected into EL4 cells or P1.HTR cells 144
Figure 3.2.1. Outline of the procedure used to generate recombinant vaccinia virus expressing cyclin D1 146
Figure 3.2.2. Presence of cyclin D1 cDNA in Tk$^-$143 cells infected with recombinant vaccinia virus.cyclin D1 148
Figure 3.2.3. Production of murine cyclin D1 messenger RNA in RMA cells infected with vaccinia virus cyclin D1 (VVCD1) clone 22.1.1.1 150
Figure 3.2.4. Production of cyclin D1 protein in Tk$^-$ 143 Cells infected with recombinant vaccinia virus cyclin D1, clone 22.1.1.1. (VV CD 22.1.1.1) 152
Figure 3.2.5. Outline of the procedure used to generate recombinant adenovirus expressing cyclin D1 154
Figure 3.2.6. Presence of correctly orientated cyclin D1 cDNA in the pMV60 plasmid 155
Figure 3.3.1. Outline of the procedure used to generate recombinant vaccinia virus and transfectants expressing the murine fibroblast receptor I (FRI) 157
Figure 3.3.2. Presence of murine fibroblast growth factor receptor I (FRI) cDNA in Tk$^-$143 cells infected with recombinant vaccinia virus FRI (VV FRI) 159
Figure 3.3.3. Production of murine FRI mRNA in RMA cells infected with recombinant vaccinia virus containing the FRI cDNA, clone VV FRI 1.1.1.1 160
Figure 3.3.4. Production of FRI protein in Tk$^-$143 cells infected with recombinant vaccinia virus FRI, clone 1.1.1.1. (VV FRI 1.1.1.1.) 162
Figure 3.3.5. Presence of FRI cDNA in EL4 cells transfected with pDO-R FRI. 164
Figure 3.4.1. Outline of the procedure used to generate recombinant vaccinia virus expressing murine wild type p53 167

Figure 3.4.2. Presence of WT p53 cDNA in Tk⁻143 cells infected with recombinant vaccinia virus p53 169
Figure 3.4.3. Production of p53 mRNA in p53 negative cells infected with recombinant vaccinia virus p53 170
Figure 3.4.4. Production of murine WT p53 mRNA in human Tk⁻143 cells infected with recombinant vaccinia virus p53 172
Figure 3.4.5. Outline of the procedure used to generate recombinant adenovirus expressing murine wild type p53 174
Figure 3.4.6. Presence of correctly orientated p53 cDNA in pMV60 175

4. Stimulation of cytotoxic T lymphocytes specific for self proteins by immunisation *in vivo* with recombinant vaccinia virus expressing self proteins:

Tables:
Table 4.1.1. Conditions used for *in vivo* and *in vitro* stimulation of cyclin D1 (CD1) specific CTL lines 183
Table 4.2.1. Conditions used for *in vivo* and *in vitro* stimulation of mdm2 specific CTL lines 190

Figures:
Figure 4.1.1. Induction of cyclin D1 specific CTL by *in vivo* immunisation with recombinant vaccinia virus cyclin D1 (VV CD1) 185
Figure 4.1.2. Dendritic cells infected with VV CD1 can be used to restimulate a potent cylin D1 specific CTL response from mice immunised with VV CD1, but can not be used to generate a long term stable cell line.. 187
Figure 4.2.1. Immunisation of C57BL/6 mice with vaccinia virus mdm2 stimulate mdm2 specific CTL 192
Figure 4.2.2. *In vivo* primed mdm2 specific CTL can be restimulated *in vitro* with transfectants expressing mdm2 and dendritic cells infected with vaccinia virus mdm2 193
Figure 4.2.3. *In vivo* primed mdm2 specific CTL can be restimulated *in vitro* exclusively with transfectants expressing mdm2 195
Figure 4.3.1. FRI specific CTL can be generated by *in vivo* immunsation with recombinant vaccinia virus FRI (VV FRI) and *in vitro* restimulation with transfectants expressing FRI 197

5. Peptide stimulation of autoreactive high avidity CTL which can recognise naturally processed self protein:

Tables:
Table 5.1.1. Stabilisation of cell surface expression of murine K^b or D^b class I molecules by murine self peptides containing known K^b or D^b binding motifs. 219
Table 5.1.2. Summary of class I binding properties of motif containing peptides..... 220
Table 5.2.1. CTL induction by self peptides containing class I binding motifs..... 232
Table 5.3.1. Blocking of lytic activity of self peptide specific CTL with anti CD8 antibodies. 235
Table 5.3.2. Avidity scores for peptide specific CTL lines stimulated with murine self peptides or control CTL epitopes 254
Table 5.3.3. Effect of different peptide concentrations used for CTL stimulation on the avidity of the resulting CTL 257

Figures:
Figure 5.1.1. MHC class I expression on RMA cells, RMA-S cells and temperature induced RMA-S cells 213
Figure 5.1.2.a. Stabilisation of surface expression of murine class I molecules K^b or D^b by murine mdm2 peptides 215

Figure 5.1.2.b. Stabilisation of surface expression of murine class I molecules K^b or D^b by murine cyclin D1 peptides 216
Figure 5.1.2.c. Stabilisation of surface expression of murine class I molecules K^b or D^b by murine WT p53 peptides 217
Figure 5.1.2.d. Stabilisation of surface expression of murine class I molecules K^b or D^b by OVAp257 and SVp324 (SV9) peptides 218
Figure 5.2.1.a.and b. FACS analysis of a mouse splenic non-adherent cell population purified by centrifugation on a metrizamide gradient 223
Figure 5.2.1.c. FACS analysis of a mouse splenic non-adherent cell population purified by centrifugation on a metrizamide gradient, continued 224
Figure 5.2.2. Splenic dendritic cells are potent inducers of peptide specific CTL *in vitro* 226
Figure 5.2.3. Peptide specific CTL are efficiently stimulated using a two weekly restimulation cycle. 227
Figure 5.2.4. Comparison of CTL lysis of peptide pulsed RMA cells or RMA-S cells. 229
Figure 5.2.5. CTL induction in vitro by self peptides selected on the basis of class I binding motifs 231
Figure 5.3.1. The effector function of self peptide specific CTL is dependent on the CD8 molecule. 234
Figure 5.3.2. Class I restriction of self peptide specific CTL. 236
Figure 5.3.3.a. mdp100 peptide specific CTL do not lyse cells infected with recombinant vaccinia virus mdm2 238
Figure 5.3.3.b. Repeated stimulations of naive spleen cells with mdp441 peptide stimulate mdp441 specific CTL which specifically lyse cells infected with vaccinia virus mdm2. 239
Figure 5.3.3.c. CTL lines specific for cyclin D1 peptides cdp20 or cdp41 do not lyse cells infected with recombinant vaccinia virus cyclin D1. 240
Figure 5.3.3.d. CTL lines specific for p53 peptides p53p227, p53p232 or p53p240 do not lyse target cells infected with recombinant vaccinia virus p53. 241
Figure 5.3.4. Some mdm2p441 specific CTL lines lyse EL4 cells 242
Figure 5.3.5.a. Lytic activity of mdp100 peptide specific CTL line against RMA-S target cells coated with different concentrations of mdp100 peptide 245
Figure 5.3.5.b. Lytic activity of mdp441 peptide specific CTL line against RMA-S target cells coated with different concentrations of mdp441 peptide 246
Figure 5.3.5.c. Lytic activity of cdp20 peptide specific CTL line against RMA-S target cells coated with different concentrations of cdp20 peptide 247
Figure 5.3.5.d. Lytic activity of cdp41 peptide specific CTL line against RMA-S target cells coated with different concentrations of cdp41 peptide 248
Figure 5.3.5.e. Lytic activity of p53p227 peptide specific CTL line against RMA-S target cells coated with different concentrations of p53p227 peptide. 249
Figure 5.3.5.f. Lytic activity of p53p232 peptide specific CTL line against RMA-S target cells coated with different concentrations of p53p232 peptide. 250
Figure 5.3.5.g. Lytic activity of p53p240 peptide specific CTL line against RMA-S target cells coated with different concentrations of p53p240 peptide. 251
Figure 5.3.5.h. Lytic activity of OVAp257 peptide specific CTL line against RMA-S target cells coated with different concentrations of OVAp257 peptide. 252
Figure 5.3.5.i. Lytic activity of SV9 peptide specific CTL line against RMA-S target cells coated with different concentrations of SV9 peptide 253

List of abbreviations

A	adenine or adenosine
A_{260}	optical absorbance at 260 nm
A_{280}	optical absorbance at 280 nm
aa	amino acid
Ab	antibody
Ad	adenovirus
Ad5	adenovirus type 5
AP	alkaline phosphatase
APC	antigen presenting cell
ATP	adenosine triphosphate
ATTC	The American Type Culture Collection
bp	basepairs
Bq	becquerel (1 disintegration/sec)
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
C	cytosine or cytidine
$CaCl_2$	calcium chloride
CD1	cyclin D1
cDNA	complementary DNA
Ci	curie (3.7×10^{10} Bq)
Con A	concanavalin A
cpm	counts per minute
Cr	chromium
CTL	cytotoxic T lymphocyte
Da	Dalton
dATP	deoxyadenosine triphosphate
DC	dendritic cell
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EBV	Epstein-Barr virus
ECACC	The European Collection of Animal Cell Cultures
EDTA	ethylenediamine tetraacetic acid
E/T ratio	effector to target cell ratio

EtBr	ethidiumbromide
EtOH	ethanol
F	farad
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FMoc	N-(9-fluorenylmethoxycarbonyl)
FRI	fibroblast growth factor receptor I
g	gram
x g	(number of times) the gravitational force
HCl	hydrochloric acid
HEL	hen's egg white lysozyme
Hepes	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HLA	human leukocyte antigen
HPLC	high-performance liquid chromatography
HPV	human papillomavirus
ICRF	Imperial Cancer Research Fund
IDC	interdigitating dendritic cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kb	kilobase
KCl	potassium chloride
kDa	kilodalton
l	liter
LB	Luria Bertani
LC	Langerhans cell
LCMV	lymphocytic choriomeningitis virus
m	meter
M	molar
mAb	monoclonal antibody
MB_{50}	Peptide concentration required to induce half maximal stabilisation of class I molecules in a whole cell binding assay
2-ME	β-Mercaptoethanol
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
Mit. C	mitomycin C
ML_{50}	peptide concentration required to induce half maximal lysis by CTL in a chromium release assay
mM	milimolar
M-MLV	Moloney murine leukemia virus

Mops	3-(N-morpholino) propanesulphonic acid
mRNA	messenger RNA
Mw	molecular weight
NaAc	sodium acetate
NaCl	sodium chloride
NK	natural killer
NP	nucleoprotein
NP-40	nonidet P-40
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
pmole	picomole
rIL-2	recombinant interleukin 2
RNA	ribonucleic acid
RNAse	ribonuclease
R-PE	R-Phycoerythrin
rpm	rounds per minutes
RT	reverse transcriptase
S	Svedberg
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
STE	saline tris- EDTA buffer
SV	Sendai virus
T	thymine or thymidine
TCR	T cell receptor
TE	tris-EDTA buffer
TEMED	N,N,N',N', tetramethylethylenediamine
TFA	trifluoroacetic acid
Th	T helper
TIL	tumour infiltrating lymphocyte
TIU	Tumour Immunology Unit
TNF	tumour necrosis factor
V	volt
vol	volume
VV	vaccinia virus
WT p53	wild type p53
X-Gal	5-bromo-4-chloro-3-indolyl-β-galactopyronoside.

N.B. All amino acids were abbreviated using standard abbreviations.

1. Introduction

The immune system consists of a combination of cells and molecules which enable vertebrae including humans to detect and eliminate cellular or molecular components which are abnormal. The immune system is the body's defence against pathogens including viruses, bacteria and parasites. During recent years it has become clear that the immune system is able to recognise and eliminate tumour cells using mechanisms also involved in the control of pathogens. This thesis will deal with the identification of molecules which are expressed in tumours and which can stimulate the immune system to react to these tumours.

1.1. The function and role of the immune system

The immune system is able to provide a state of protection from disease, immunity. Immunity can be provided by two different mechanisms. Innate immunity is provided via polyspecific mechanisms, which do not improve on repeated contact with the same substance. Adaptive specific immunity is provided via specific mechanisms enabling the host to respond with increasing efficiency to repeated contact with the same substance (1; 2).

The innate immune system is based on cellular as well as soluble factors. The main mechanisms involve phagocytosis of abnormal cells by phagocytic cells or lysis of abnormal cells. Professional phagocytic cells such as the polymorphonuclear neutrophils and to a lesser extent eosinophils residing mainly in the blood stream and the macrophages residing in the tissues can recognise foreign particles and phagocytose them (1). In the innate immune system, lysis of abnormal cells can occur via several mechanisms. Tumour cells or cells infected with microorganisms can be lysed by natural killer cells (NK cells). NK cells are bone marrow derived and thymus independent and constitute 5-10% of the peripheral blood lymphocytes in humans (2). The cells are operative in normal healthy individuals but can be further activated by interferons and IL-2. The cells use integrins for target cell adhesion and probably operate via several receptors and target ligands in the recognition phase (3). Abnormal cells can also be lysed by the effects of soluble proteins such as the complement proteins or by secreted interferons derived from a variety of different cell types such as macrophages or NK cells. Interferons are particularly important to prevent viral replication (2).

The innate immune system is based on the recognition of a limited number of components shared between different types of abnormal cells. These components include microbial polysaccharides, which stabilise the complement enzyme C3bBb on the surface of the microorganism or trigger immediate bactericidal activities of macrophages.

The innate immune system has three main characteristics (4). Firstly, the innate immune system is polyspecific due to the use of multiple receptors to recognise core components of common microbial constituents associated with a range of pathogens. Secondly, the system offers a certain degree of self non-self distinction. Finally, the innate system is characterised by rapid response kinetics in that all cells able to recognise a given microbial pattern rapidly become effectors.
Receptors on cells of the innate immune system are non-clonal. The limitations of innate immunity are mainly due to the limited number of different receptors resulting in restricted specificity. This becomes problematic once microorganisms change and evolve strategies to escape existing components of the innate immune system.

The limitations of innate immunity are overcome by specific acquired immunity. Specific acquired immunity is characterised by specificity and memory and is based on cellular components as well as soluble components. The specificity of acquired immunity is due to the involvement of two different cell types, B lymphocytes and T lymphocytes, carrying clonally expressed antigen receptors. Specific acquired immunity is to a certain degree influenced by cells and molecules also important for the development of innate immunity.

B lymphocytes mature in the bone marrow and traffic between tissues, the blood stream and lymph glands following maturation. B lymphocytes contain membrane bound antibodies which are able to interact with extracellular components found in the tissues or blood stream. When antigen is bound specifically to surface bound antibodies, the lymphocytes are triggered to develop into a population of antibody secreting plasma cells or non-dividing memory cells over a period of 4 to 5 days (1; 2). All clonal progeny from a given B cell secrete antibody molecules with the same specificity. The clonal proliferation of antigen specific B cells form the basis of the specificity and rapidity of the antibody response upon subsequent encounters with antigen.

T lymphocytes mature in the thymus and will be described in more detail in later sections. As with B lymphocytes, T lymphocytes circulate between tissues, the blood stream and lymph glands after maturation. T cells do not recognise whole cells or molecules, but recognise peptides presented on the surface of cells in the context of major histocompatibility complex (MHC) class I or class II molecules. Like B lymphocytes, T

lymphocytes mediate the antigen specific interaction with antigen presenting cells via specific receptors known as T cell receptors (1; 2).
There are two major T lymphocyte subsets, CD4 positive T cells and CD8 positive T cells. T helper cells are mostly CD4 positive and recognise peptides derived from exogenous or membrane bound proteins in the context of MHC class II molecules. T helper cells interact with antigen presenting cells such as macrophages, dendritic cells and B lymphocytes. This interaction can result in the production of cytokines by the T helper cell. The production of cytokines stimulate effector function and proliferation of a wide variety of cell types including B lymphocytes and cytotoxic T lymphocytes (CTL). CTL are mostly CD8 positive. CTL recognise peptides in the context of MHC class I molecules. Interaction of CTL with MHC class I/peptide complexes on target antigen presenting cells can result in differentiation and proliferation of the CTL and lysis of the target cell (1; 2).

Experimental evidence indicate that acquired specific immunity can contribute to tumour recognition and elimination *in vitro* and *in vivo* (5; 6; 7). In contrast, the role of innate immunity in tumour recognition is still ill defined, but will be briefly summarised below prior to outlining the reasons for primarily focusing on acquired specific immunity and CTL in particular in a tumour immunotherapy context.

Granulocytes and NK cells are cell types involved in innate immunity which are thought to play some role in tumour elimination *in vivo*. Firstly, macrophages can autonomously lyse some tumour cells, but activation by T cell secreted cytokines and IFN–γ can augment the process (8). Secondly, granulocytes and in particular eosinophils have been found to infiltrate and be important for rejection of tumours in several murine experimental systems involving the use of tumours transfected with cytokines (9; 10). Thirdly, NK cells recognise and lyse tumour cells in several different ways one of which is dependent on low or absent MHC class I expression on the target cell (2; 3; 10). Certain MHC class I molecules present on target cells appear to deliver an inhibitory signal to NK cells via the interaction with specific receptors on the NK cells including the Ly49 family in the mouse and the p58 family in humans (11). In vivo, NK cells are thought to be important in the defence against tumours which are poorly immunogenic and unable to elicit a strong T cell response due to low levels of MHC expression.

NK cells as well as neutrophils and monocytes can also participate in tumour recognition by interacting with components of the specific acquired immune system, namely antigen specific antibodies. These cells possess receptors for antibodies, Fc receptors, on the cell surface. Binding of tumour specific antibodies to these receptors can mediate antibody dependent cell- mediated cytotoxicity of tumours (2; 12). To mention one example, NK

cells and antibodies were recently found to be required for recognition and elimination of gp75 expressing melanoma cells in mice (13).

Antibodies specific for oncogenes or other tumour specific proteins have been found at high levels in several cancer patients and the use of labelled and unlabelled antibodies is currently being investigated as an alternative to chemotherapy in humans (12; 14). Passive antibody based therapies represent a way of specifically targeting tumours. Problems with access to tumour sites as well as unwanted immune responses to components of the antibodies used might limit the use of passive antibody based immunotherapy. Active antibody based immunotherapy based on *in vivo* immunisation has also been problematic due to problems with inducing strong antibody responses *in vivo* (5). However, there are examples of immunisation protocols, such as immunisation with purified or modified glycolipid antigens or antiidiotypic antibodies against the melanoma specific ganglioside (cell surface glycolipid) GD2, which appear to be associated with prolonged disease-free survival in patients with metastatic melanoma (15).

Activation of CTL against tumour specific antigens would overcome some of the limitations of some of the antibody based therapies. There is overwhelming evidence that CTL specific for a wide range of tumour antigens can recognise and destroy tumour cells *in vitro* and *in vivo* (5; 16; 17; 18). Results from clinical trials in humans suggest that activation of tumour specific CTL is involved in the specific recognition and destruction of tumours in humans *in vivo* (19; 20). The use of CTL to target tumours requires that the CTL can get into contact with the tumour or tumour derived antigens and specifically recognise and destroy tumour cells without simultaneously destroying surrounding healthy tissue. To achieve these goals, it will be important to profoundly understand a wide range of issues dealing with the nature of CTL, the nature of tumours and the nature of the interaction between CTL and tumours. These issues include:-

I) The mechanisms determining the identity of the antigens presented to CTL, including:-
a) mechanisms of peptide binding to MHC class I molecules
b) mechanisms of synthesis and assembly of peptide/MHC class I complexes.

II) The mechanisms determining the identity of the CTL being able to be activated, such as:-
a) mechanisms of T cell recognition of antigen.
b) mechanisms of T cell activation.
c) mechanisms of execution of T cell effector function.
d) mechanisms by which the T cell repertoire is influenced by T cell development in the thymus and in the periphery

III) Tumours as potential targets for specific CTL, such as:-

a) the contribution of altered protein expression in tumour cells to the generation of novel tumour antigens.

b) the potential benefits and disadvantages of using normal self proteins as targets for tumour specific CTL.

1.2. The mechanisms determining the identity of the antigens presented to CTL

The identity of the peptide antigens presented on the cell surface to CTL is determined by several factors. These factors include the nature of the MHC class I molecules on the cell surface and the mechanisms involved in the synthesis and assembly of MHC class I/peptide complexes.

1.2.1. Polymorphic MHC class I molecules bind peptides and present them to CTL

Cytotoxic T lymphocytes (CTL) are mostly CD8 positive and recognise target cells sharing MHC class I molecules with the CTL (21). The discovery of the principle of MHC restricted CTL recognition was important for the later discovery that CTL do not recognise whole proteins in a manner analogous to the recognition of antigen by B cells, but instead recognise MHC class I molecules in association with small peptides (22). The interaction between T cell receptors on the CTL and MHC class I/peptide complexes on antigen presenting cells trigger a series of intercellular signals which lead to CTL differentiation, proliferation and execution of effector function.

MHC class I molecules are heterodimers composed of a membrane-anchored heavy chain (with extracellular domains $\alpha 1$, $\alpha 2$ and $\alpha 3$) non-covalently linked to soluble β_2 microglobulin (β_2m) (23). MHC class I heavy chains are encoded by the A, B and C loci on chromosome 6 in humans and by the K, D and L loci on chromosome 17 in mice and are extremely polymorphic (23). As an example, it has been estimated that there are between 50 and 100 alleles at both the K and D loci (24). Each individual can express a maximum of 6 different class I molecules and it is estimated that between 10^5 to 10^6 class I molecules are present on the cell surface (25).

A particular combination of class I alleles is denominated the haplotype. Certain inbred mice strains have been designated as prototype strains, and the haplotype expressed by these strains is designated by a particular superscript. Mice of the H-2^b haplotype such as C57BL/6 mice express only two different class I molecules, K^b and D^b, on the cell surface, whereas mice of the H-2^d haplotype, such as Balb/c mice, express three different class I molecules, K^d, L^d and D^d (2). Most of the amino acid substitutions between the products of different class I alleles are localised to the $\alpha 1$ and $\alpha 2$ domain with the $\alpha 3$ domain being relatively conserved and β_2m invariant. As an example there are approximately 50 amino acid differences between K^b and D^b molecules.

The first crystal structure of a MHC class I molecule with bound peptide was solved in 1987 (26) and to date the crystal structures of five different MHC class I molecules, human HLA-A2, HLA-Aw68 and HLA-B27 and murine K^b and D^b molecules have been solved (for review see (27)). All these class I molecules were crystallised together with

peptide. The crystallographic data has been supplemented by sequence data of peptides naturally bound to class I molecules (28; 29; 30; 31). It appears that peptides bound to class I molecules are usually 8 to 9 amino acids long although there are some longer exceptions. The peptides are bound in an extended conformation along a 30 Å long cleft formed by the α1 and α2 helix of the class I heavy chain. The peptide backbone forms a number of hydrogen bonds and van der Waal's bonds with the MHC molecule. These interactions are independent of the sidechain of the peptide and will stabilise any peptide which can adopt the required backbone conformation (27).

While the network of hydrogen bonds and van der Waal's contacts connects the peptide main chain to complementary MHC side chains, further interactions involve a few peptide binding side chains. These interactions impose sequence requirements on the peptides that can bind, a so-called peptide binding motif. The presence of these motifs is due to a number of pockets in the peptide binding cleft of the class I molecules. Many of the MHC side chains that contribute to these pockets are polymorphic, so the stereochemistry of the pockets differ between class I molecules. The size and charge of these pockets will determine the amino acid sequence of the peptide being able to bind stably in the MHC class I cleft. As an example, elution of peptides bound from the murine D^b molecule has shown that the bound peptides preferably contain asparigine at position 5 (31). Crystallographic studies have shown that the pocket accommodating this amino acid contains amino acids with polar and large side chains including Glu9, Gln70, Gln97 and Tyr156 and these residues can form hydrogen bonds with the smaller P5 Asn anchor side chain (32). Amino acids which are very specific for certain pockets in the class I molecules are called primary anchors. However, a few binding peptides are found which do not contain these primary anchor residues. In addition to the strict primary side chain binding pockets, there are further interactions with side chains that do not have a strict preference for individual side chains but can nevertheless modulate the affinity of peptide MHC complexes. These amino acids are referred to as secondary anchors (27).

Certain MHC class I molecules such as HLA-A2.1 and HLA-B7 have been found to be associated with thousands of different peptides. It appears that more than 90% of peptides eluted from cells of different origins but expressing the same class I molecule are identical (25). The identities of several peptides eluted from class I molecules have been revealed (25). The peptides bound to MHC class I molecules originate from a variety of different sources including proteins involved in the regulation of the cell cycle such as cyclin D2 and cyclin B, ribosomal proteins, heat shock proteins, proteins involved in metabolism such as fructose-6-phosphate amino transferase and proteins involved in translation such as elongation factor 2 (33; 34). Peptides derived from abundant proteins such as elongation factor 2 involved in house keeping as well as peptides derived from less abundant proteins making up as little as 0.01-0.02% of total cellular protein such as the

regulatory subunit of phosphatase 2a have been found in association with MHC class I molecules on the cell surface (34). This means that other factors than the relative abundance of a protein influences whether the peptide is presented. One of these factors includes the ability of protein derived peptides to bind to MHC class I molecules as outlined above. The ability to be processed and presented for binding to a given MHC class I molecule is equally important and is a major factor contributing to the stable assembly of MHC class I molecules.

1.2.2. The synthesis and assembly of peptide/MHC class I complexes

At physiological temperatures, MHC class I molecules can not remain in a stable conformation without peptide (35; 36) and intracellularly supplied peptides able to bind to class I molecules are essential for the formation of stable class I molecules in the first place (37; 38). Peptides are generated in the cytosol and transported into the endoplasmic reticulum (ER) by a set of transporter molecules, TAP1 and TAP2. In the ER, peptides associate with newly synthesised class I molecule/β_2m dimers that are retained there by the p88/IP90 chaperone calnexin (39). Calnexin is an abundant calcium binding ER membrane protein. It can bind to several conformations of the MHC class I molecules and seems to be important in stabilising the newly synthesised and unfolded class I molecule in both mouse and man (40). However, human cell lines deficient in calnexin have been found to have normal transport and surface expression of class I molecules (41) and altogether the role of molecular chaperones in the MHC class I assembly process is still unclear.

From the ER, the heavy chain class I/peptide complex is transported to the cell surface via the Golgi apparatus (39), Figure 1.2.1.

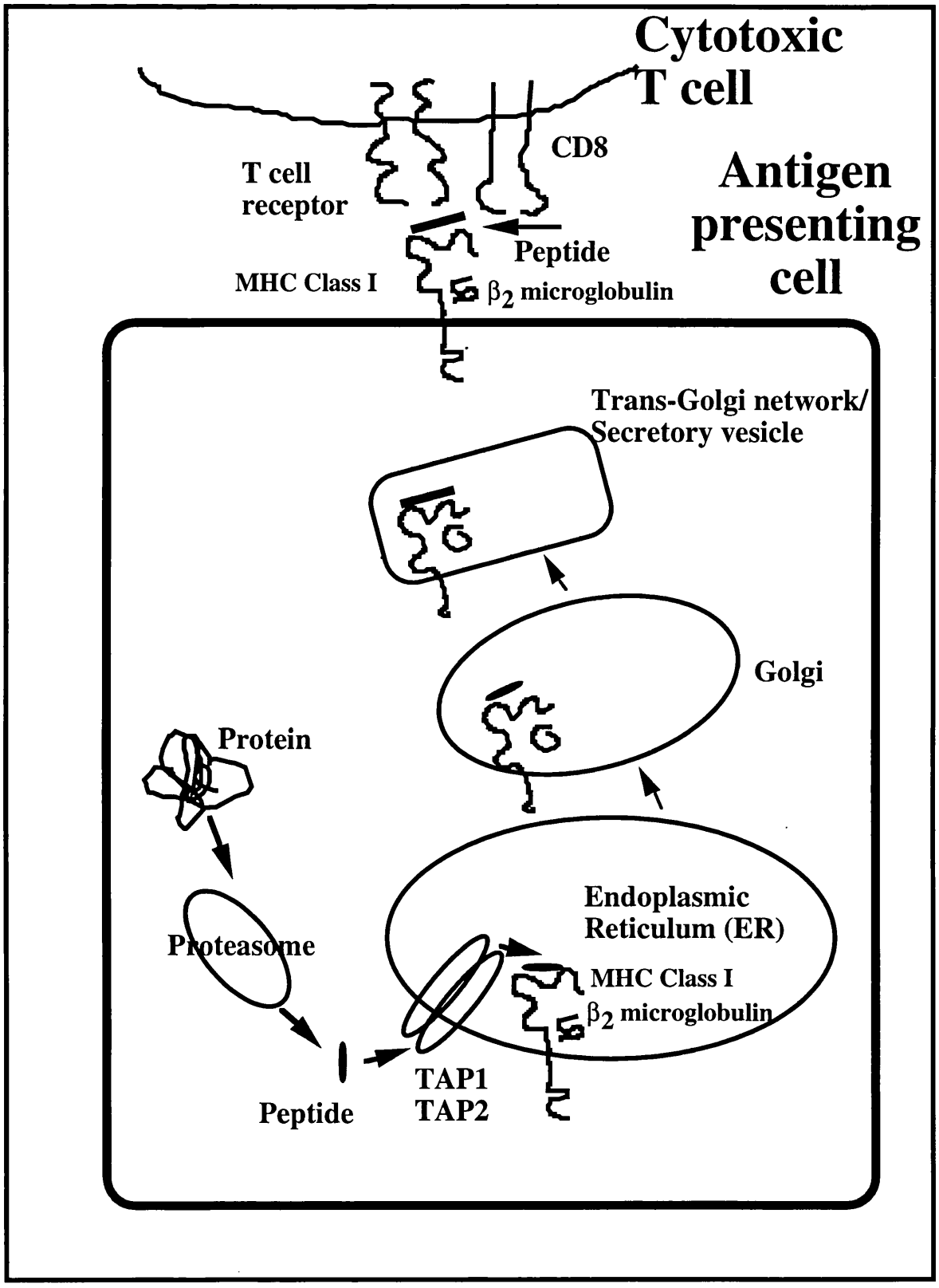


Figure 1.2.1.
Summary of the major processes involved in the assembly of MHC Class I / peptide complexes. See text for details.

The MHC class II pathway resulting in the presentation of peptides to CD4 positive T helper cells is different. In the ER, newly synthesised MHC class II molecules are assembled together with a transmembrane glycoprotein, the so-called Invariant chain (Ii). Association with Ii prevents binding of peptides to MHC class II molecules. Ii contains an endosomal/lysosomal targeting motif which is responsible for the delivery of the newly synthesised class II Ii complexes to a prelysosomal compartment called an early MHC class II compartment (early MIIC). Ii is degraded by proteases such as cathepsin B and D in the early MIIC. A fragment of Ii, CLIP, remains bound to the class II molecule. Proteins or partially degraded proteins which are derived from the cell membrane or taken up by endocytosis from the extracellular fluid are also present in this compartment. The proteins will gradually be completely degraded and travel together with the class II /CLIP complexes to the MHC class II compartment (MIIC). In this compartment, CLIP is removed by the DM molecule and peptide will be able to bind (42). From the MIIC, the peptide MHC class II complexes are transported to the cell surface.

The differences in the way class I and class II molecules load peptides explain why class II restricted T cells mainly recognise peptides derived from exogenous proteins whereas class I restricted CTL mainly recognise peptides derived from endogenously produced proteins present in the cytosol. There is increasing evidence that exogenous proteins can also get access to the class I pathway (43; 44; 45; 46; 47), but the physiological relevance of this finding is unclear (47). The mechanism by which exogenous proteins get access to the class I pathway is still not clear, but the pathway could be important for priming of CTL responses to proteins released from dying cells such as necrotic tumour tissue. In this respect, complexing of proteins with heat shock protein gp96 has been found to provide a way of linking exogenous protein uptake to the class I pathway (48).

Peptides presented by MHC class I molecules are mostly derived from proteins cleaved in the cytosol by proteases. There is now ample evidence that the protease responsible for the generation of at least some epitopes is the multi-catalytic protease complex, the proteasome. The proteasome is involved in the ubiquitin dependent degradation of the bulk of cellular proteins. The catalytic core of the complex is the 20S particle. This particle comprises about 1% of total cellular protein and is located in the cytosol and the nucleus of all eukaryotic cells (49). The 20S particle associates with a 19S particle. The 19S particle has a binding site for ubiquitin and appears to function as a chaperone unfolding polypeptides and injecting them into the 20S complex, which contains up to 5 different peptidase activities (50). The 20S proteasome contains about 14 distinct, but related subunits of which HC7-I, HC10-II and HN3 are responsible for tryptic, chymotryptic and V8-protease-like actions respectively (51). In vitro transfection studies have shown that the LMP7 subunit increases the capacity of the proteasome to cleave after hydrophobic and basic residues and decreases the ability to cleave after acid residues

whereas the LMP2 subunit increases the cleavage after hydrophobic residues and decreases the ability to cleave after acidic residues (52). Interferon-γ (IFN-γ) affects the proteolytic activity of the proteasome, enhancing the activity for endoproteolytic cleavage of peptide bonds on the carboxyl side of basic and neutral residues, while decreasing their activities for peptides containing acidic amino acids. IFN-γ does not affect HC7-I, HC10-II and HN3, but does up-regulate LMP2 and LMP7. These subunits appear to replace two other subunits, the X and Y subunits, and thereby influence the specificity of the complex (51).

The evidence of the role of the ubiquitin/proteasome system in the generation of antigenic epitopes is based on several lines of evidence. Firstly, inhibitors of the proteasome such as peptide aldehydes (53; 54) or lactacystein (55) prevent the generation of certain peptide epitopes. Secondly, purified proteasome 20S particles have been shown to produce certain immunodominant CTL epitopes exactly such as a hepatitis B virus e-protein epitope from a longer peptide precursor (56) or the ovalbumin OVAp257 epitope from whole ovalbumin (57). In the latter case, a subdominant ovalbumin epitope was cleaved by the proteasome at internal sites with the cleavage depending on the amino acid sequences surrounding the peptides in question (57). Thirdly, LMP2 knock-out mice and LMP7 knock-out mice are deficient in the presentation of certain CTL epitopes (58; 59) although *in vitro* experiments have questioned the importance of these subunits in the generation of peptide epitopes in general (60). Finally, the rate of antigen degradation by the ubiquitin-proteasome pathway has been found to correlate with the ability to present a certain antigen (ovalbumin) to a class I restricted T cell hybridoma (61), but ubiquitin might not be required for the generation of other epitopes (56).

Peptides from the cytosol are transported into the ER via an ATP dependent mechanism involving the translocation of the peptides through a channel in the ER membrane formed by the interaction of two subunits of the TAP transporter complex, TAP1 and TAP2 (62; 63). The TAP molecules are able to physically interact with the MHC class I/β_2m complex in the ER (64; 65) through binding of the TAP2 subunit to the complex (63). The physical interaction between TAP molecules and the MHC class I molecules appears to be essential for peptide binding to MHC class I molecules, as human cell lines with genetic defects preventing this association have defective binding of peptides to class I molecules (66).

The human TAP molecule has been found to transport peptides of 8-12 amino acids in a rather promiscuous way, but transport of peptides as long as 28 amino acids has also been reported (67). Human TAP molecules preferentially select peptides with a hydrophobic and basic C terminal residue (68; 69), and with hydrophobic residues in position 3 and hydrophobic or charged residues in position 2 (68). The mouse TAP

molecule has been shown to preferentially transport peptides with a minimum of 9 amino acids with a hydrophobic C terminus (69; 70). Interestingly, several immunodominant peptides have been found to be very inefficiently transported by mouse (71) or human TAP molecules (68). Certain amino acids in certain positions have been found to have a deleterious effect on transport (68; 71) but the transport can be improved by introducing certain amino acid substitutions in the sequence or by including naturally flanking amino acids to the peptide sequence (71). The later finding may suggest that some peptides are transported into the ER having a size optimal for class I binding, whereas others are transported as longer fragments which potentially could be trimmed in the ER (68; 71). Proteases able to produce antigenic peptides are present in the ER (72; 73). This would also explain how class I molecules such as HLA-B7 bind peptides which have motifs differing significantly from the TAP motifs. In one case, the lack of TAP transport of an immunogenic self peptide was shown to be the restriction point preventing the endogenously produced peptide to be presented to CTL able to be activated by exogenously added peptide (74). There is a limited degree of sequence polymorphism in human and murine TAP molecules, but this polymorphism does not appear to influence the set of peptides transported (75; 76).

In summary, the identity of the antigens available for presentation to CTL is strongly influenced by four main factors:-

1) The abundance of the proteins in the cells;
2) The nature of the peptides generated by proteolytic processing of individual proteins in the cell;
3) The efficiency with which the peptides are translocated into the endoplasmic reticulum;
4) The efficiency of the binding of peptides to MHC class I molecules.

These four factors are all involved in determining the number of peptides derived from an individual protein which is presented on the cell surface by MHC molecules. In addition, for a given protein able to be processed for presentation by MHC class I molecules, there appears to be proportionality between the level of protein produced by the cell and the level of peptides presented on the surface. This has been shown by analysis of the presentation of peptide epitopes from the Listeria monocytogenes protein p60 (77). According to this, a normal cell expressing low levels of a house keeping self protein such as cyclin D1 or mdm2 would be expected to have less MHC class I complexes containing a particular cyclin D1 or mdm2 derived peptide on the cell surface than a tumour cell expressing high levels of cyclin D1 or mdm2, providing that other components of the MHC class I synthesis and assembly pathway are unchanged.

The presence of MHC/peptide complexes on the cell surface is an absolute requirement for the activation of CTL. The availability of T cells able to recognise the MHC/peptide complexes with sufficient avidity to allow activation is equally important and will be dealt with in the next section.

1.3. The mechanisms determining the identity of the CTL able to be activated by presented antigens

1.3.1. The molecular interaction between T cell receptor and MHC/peptide complexes

T cells recognise antigens via the T cell receptor (TCR). Two different types of T cell receptors exist, the αβ TCR made up a α chain and a β chain, and the γδ TCR made up of a γ chain and a δ chain. The αβ complex or the γδ complex are non-covalently associated with five other invariant molecules including the TCR ζ chain. The whole complex is called the CD3 complex. The αβ receptor is the most common receptor and is present on 95% of peripheral blood T cells (78). γδ T cells are implicated in the recognition of certain bacterial or virus infected cells including mycobacteria infected cells. The recognition is peptide independent and involves non-peptidic antigens such as isopentenyl pyrophospate (79).

T cell receptor genes are split in the germline DNA into a number of gene segments comprising V, D, J and C segments. These gene segments recombine in a developing T cell to produce a contiguous V-(D)-J exon. This exon is spliced together with the C region at the level of RNA. There are approximately 50 Vα segments, over 70 Jα segments, one single Cα segment, nearly 60 Vβ segments and two Cβ segments which each possesses a set of Jβ segments and one Dβ segments, the recombination of which allows a high degree of diversity in the T cell receptor repertoire. In addition to the diversity created by the combination of the V, D, J and C segments, non-germline encoded nucleotides can be added at the V-(D)-J and Jα-Cα. Altogether, more than 10^{16} junctional combinations may exist for the αβ heterodimers (78). Within the αβ TCR there are three hypervariable regions, CDR1, CDR2 and CDR3. CDR3 is located at the VJ and VDJ junctions of the α and β chains respectively. It seems likely that CDR3 regions will make direct contact with the antigenic peptide presented by the MHC molecule (80).

The crystal structure of the TCR/peptide/MHC complex has not been solved yet, but mutational analysis as well as structure data obtained from the crystal structures of MHC class I/peptide complexes indicate that the T cell receptor makes direct contact with certain amino acids. The overall orientation of the TCR on the MHC target has been found to be identical for different TCR/MHC/peptide complexes with the T cell receptor contacting residues in the C-terminal region of the α helices on the class I molecule and having an orientation that is parallel to the β-pleated strands and diagonal to the α helices (81). The use of different class I specific antibodies has shown that MHC class I molecules adopt different conformations depending on which peptide is bound. It has been suggested that this peptide related conformational change in the MHC molecule might affect T cell recognition (82; 83). The crystal structure of different peptides bound to the same class I

molecule does confirm that different MHC class I side chains are in different conformations depending on which peptide is bound (84). Individual peptide/MHC complexes seem to be able to be contacted by different T cell receptors in several different ways. As an example, three different T cell clones specific for HLA-A1 in association with a peptide derived from a melanoma specific antigen MAGE-1 were found to have different T cell receptors which were dependent on different amino acids within the peptides bound to the class I molecule for recognition of the complex (85).

The affinity of the T cell receptor for the peptide/MHC class I complex is low compared with the affinity of other receptor ligand interactions including most antibody-antigen interactions or growth factor-growth factor receptor interactions. The binding of ^{125}I trace labelled soluble peptide MHC class I complexes to TCR molecules on intact cells has been used to measure the equilibrium constant for the interaction between TCR and MHC/peptide complex. The equilibrium constant was estimated to be 1×10^7-2×10^7 M^{-1} when a particular TCR was interacting with peptide in association with allogeneic MHC molecule and 1.5×10^6 M^{-1} when a different TCR was interacting with the same peptide in association with syngeneic MHC molecule (86).
Despite being of low affinity, the discriminatory power of certain T cell receptors for particular peptide/MHC class I complexes is impressive. A CTL clone specific for a self peptide 2Ca in the context of the murine class I molecule K^b was found to be able to distinguish between peptides which differed in only a single oxygen atom (87). Even a few MHC/peptide complexes on antigen presenting cells can stimulate CTL. The number of complexes needed to sensitise T cells to lysis vary between 10 and several thousands, and the fraction of surface class I molecules occupied by the epitope varies from less than 1 per mille as in the case of the ovalbumin derived CTL epitope OVAp257 to as much as 67% for some peptides (88). Serial triggering of many T cell receptors by a single peptide-MHC complex could be responsible for the low number of complexes which can activate a T cell (89).

Despite the high discriminatory power of the T cell receptor, the same receptor is able to bind to many different MHC class I/peptide complexes, although in some cases with different affinity. The concept of so-called molecular mimicry or cross reactivity of different peptide ligands is extremely important for the understanding of the generation of the T cell receptor repertoire and maintenance of T cell memory. For class II restricted T cells, it has been found that peptides with as little as 1 amino acid in common could stimulate specific T cell responses (90) and T cell clones derived from multiple sclerosis patients and specific for myelin basic protein were found to cross react with several virally and bacterially derived peptides (91). Molecular modelling of MHC class II/peptide complexes has shown that molecular mimicry is not based on sequence

similarities between different peptides but rather on similarity between the antigenic surfaces seen by the T cell receptor (92).

1.3.2. Mechanisms of T cell activation.

Peripheral T cells are generally thought of as being in one of three states: naive, memory or effector (93). A naive T cell is a circulating precursor which has not encountered antigen, and hence been activated, since exit from the thymus. A memory T cell is derived from a naive cell after encountering antigen in the context of antigen presenting cells at least once, but has subsequently returned to a resting state. Memory T cells exist at a higher frequency than naive T cells and are long lived as a population. Effector cells are short-lived cells which arise from either naive or memory cells several days after restimulation. A proportion of effectors appear to die shortly after restimulation, whereas some survive and go to the memory pool. Memory and effector T cells respond well to TCR stimulation, but naive T cells respond weakly or not at all (93).

T cell activation (proliferation and secretion of cytokines) is brought about by a combination of TCR and co-stimulatory signals. These interactions include the interactions between CD28/CTLA-4 and B7-1, 2 or 3 as well as the interactions between CD8 and MHC class I, LFA-I and ICAM-I, CD2 and LFA 3, CD5 and CD72 and VLA integrins and their respective ligands. The importance of some of these interactions in mediating adhesion and signalling to activation seems to depend on the affinity of the TCR for the MHC/peptide complex. Of all these interactions, B7-1, 2 and 3 appear to be the principal costimulatory molecules while the other molecules mostly seem to function in increasing the avidity of the interaction between the T cell and the antigen presenting cell (93). The combined action of the TCR with the MHC class I/peptide complex and other adhesion molecules contributes to the overall avidity of the interaction of a T cell with an antigen presenting cell.

The CD8 molecule is disulphide linked heterodimer of two unrelated α and β chains. Most class I restricted CTL express this molecule whereas class II restricted T helper cells express the CD4 molecule. The interaction of CD4 and CD8 molecules with MHC molecules during T cell development and response to antigen is very important (94). CD8 is involved in both adhesion and signalling. The CD8 molecule interacts with both the $\alpha 2$ and $\alpha 3$ domain of the MHC class I molecule (95; 96). Basal affinity of CD8 for class I is insufficient to mediate sufficient adhesion, but engagement of the TCR activates CD8 possibly by phosphorylation of the tyrosine kinase $p56^{lck}$. When activated CD8 binds to class I, $p56^{lck}$ undergoes dephosphorylation. This results in increased activity of the enzyme and activation of other enzymes further down the signalling pathway (93; 97;

98). Most allo-specific CTL lines do not need any cell surface molecular interaction to trigger degranulation other than the interaction between the T cell receptor and the class I molecule (98). Allo-specific CTL generally bind to APCs with high avidity due to high affinity of the T cell receptor for the MHC class I /peptide complex (86). CTL which can engage in high avidity interactions with a target cell, due to the interactions between TCR and MHC/peptide complexes, are less dependent of CD8 than CTL only being able to engage in similar low avidity interactions (99; 100).

CD28 is expressed on most $CD4^+CD8^+$ thymocytes and peripheral $CD4^+$ or $CD8^+$ T cells. CTLA-4 is expressed on activated $CD4^+$ and $CD8^+$ T cells and is regulated by signals from CD28. The abundance of CTLA-4 is lower than the abundance of CD28, but the molecule binds with higher affinity to B7-1 than CD28 does (101). B7-1 (CD80) and B7-2 (CD86) appear to have different roles. B7-2 is the dominant costimulatory ligand during primary immune responses whereas B7-1 which is up-regulated later in immune responses may be critical in prolonging primary T cell responses or costimulating secondary T cell responses. B7-1 is expressed at low levels on dendritic cells, macrophages and thymic epithelial cells, but is up-regulated on these cells following activation. B7-2 is constitutively expressed on dendritic cells and macrophages and is up-regulated upon activation. On B cells, B7-2 is rapidly expressed after activation, whereas B7-1 expression appears significantly later (102).

Binding of CD28 is required for antigen specific T cell responses and absence of costimulation via this molecule leads to functional inactivation or clonal anergy of T helper clones. This state is characterised by failure to produce IL-2 and can be corrected by the addition of exogenous IL-2 (101). Blocking of CD28 function during primary T cell activation does not induce anergy as with T helper clones, but the interaction between CD28 and B7-1 or B7-2 can clearly facilitate the initiation and progression of T cell responses. With respect to T helper cells, it has been suggested that, depending on how well the T cell bind to the antigen presenting cell via TCR/MHC/peptide interactions, the CD28/B7 interaction might not be absolutely required for primary T cell activation, but it might be essential for clonal expansion *in vitro* and perhaps *in vivo* (102). For T helper cells, the dependency of the CD28 mediated signal is thought to relate to the strength of the TCR engagement by the antigen, with CD28 signalling being essential under conditions of low antigen density but not necessary under some conditions of high antigen density, such as during a virus infection. CD28 signalling might even contribute to down-regulation of immune responses via clonal exhaustion under conditions of extremely high levels of TCR occupancy (102).

With respect to CTL, naive CTL are less responsive to TCR mediated signals than memory CTL and naive CTL require more costimulation for maximal proliferation and cytokine production than memory CTL do. Effector CTL have the lowest requirement for costimulation (93). Altogether, the CD28-B7-1 interaction is required during the inductive phase of a $CD8^+$ CTL response leading to the generation of effector CTL but is not required during the effector phase. With a sufficient signal mediated by the TCR/CD8/MHC/peptide interaction and CD28/B7 interaction, help provided by CD4 T helper cells is not needed (103; 104; 105).

Most human tumours studied by immunohistology do not bind monoclonal antibodies specific for B7-1 and B7-2 (106). This could explain why many tumours which do indeed express antigens able to be recognised by CTL do not induce immune responses. This was initially demonstrated with a B7-1 negative melanoma cell line unable to induce CTL despite expressing high levels of a defined tumour antigen, the human papillomavirus protein E7. Transfection of B7-1 into the cell line resulted in the induction of CTL able to recognise both the B7-1 positive and B7-1 negative tumour. The presence of B7-1 was required to induce the immune response but not to serve as a target (107). This work has since been confirmed using several other tumour cell lines. Some tumours are not able to stimulate CTL to proliferate and exert effector function even in the presence of antigen and B7-1/B7-2 -CD28 interaction (106). Additional costimulatory signals provided by ICAM-1 were necessary to stimulate a primary immune response for some of the tumours falling into that category (10). A subset of NK cells also express CD28 and the B7-1/CD28 interaction is thought to be important in NK mediated rejection of tumours expressing only low levels of MHC class I molecules (108). The issue of stimulation of tumour specific CTL responses will be discussed in more detail in section 1.4.

The requirement of a second signal to activate T cells might also play an important role in the maintenance of T cell tolerance towards self proteins in normal non-cancerous cells *in vivo*, see also section 1.3.3. To mention an example, transgenic mice expressing high levels of class II molecules on islet cells in the pancreas are healthy, whereas mice expressing both high levels of class II and B7-1 develop autoimmune destruction of β–cells in the pancreas (109).

Adhesion mediated by several integrins on the T cells, such as LFA-1 or members of the VLA protein family, is also up-regulated upon TCR engagement (98). The interaction between ICAM-1 and LFA-1 has been found to deliver a costimulatory signal allowing for antigen induced proliferation of CD4 T lymphocytes. This signal is not linked to the induction of IL-2 secretion (106).

In addition to costimulatory signals provided by the interaction of surface molecules, a variety of different cytokines influence the generation of CTL. The number of known and unknown cytokines able to influence the CTL response either directly or indirectly (via the action on T helper cells) is huge. Only the major trends will be discussed here, for extensive reviews see (110; 111; 112; 113; 114).

IL-2 has been found to be both necessary and sufficient for the proliferation and differentiation of CTL into effector function (115). Exogenous IL-2 can be provided by T helper cells, but the mechanisms by which this help is provided *in vivo* are still not clear. In one *in vitro* model using cloned T helper cells, the T helper cell clones produced IL-2 and provided help to CTL only when the CTL and T helper clone interacted with peptides presented on the surface on the same antigen presenting cell, but the peptides did not have to be derived from the same molecule (116). However, not all CTL need help from T helper cells *in vivo* or *in vitro*. The use of transgenic mice has shown that T cells able to engage in high avidity interactions with antigen presenting cells are less dependent on help (99; 117). High avidity T cell interaction with an antigen presenting cell results in the production of IL-2 and IL-2 receptor by the CTL and proliferation of the cell. In contrast, low avidity T cell interactions with an antigen presenting cell results in no IL-2 production by the CTL and no proliferation although IL-2 receptor is synthesised (117). The nature of the antigen presenting cell interacting with the CTL plays an important role in determining the need for help. In that respect, dendritic cells have been found to stimulate primary CTL responses to some antigens in the absence of CD4 T helper cells (118; 119). Dendritic cells were used extensively as antigen presenting cells in the present work and will be described in more detail in chapter 4 and chapter 5.

During T cell culture *in vitro*, IL-2 is added exogenously to support proliferation and effector function of CTL irrespective of the avidity with which they are able to interact with the antigen presenting cells used in the culture. The situation is different *in vivo*. If *in vivo* immunisation is performed in a way which does not stimulate any T cell help or local production of IL-2, the proliferation of CTL able to engage in high avidity interactions with target antigen presenting cells will be favoured. NK cells also expand in response to IL-2, but higher concentration are required than for CTL, as NK cells predominantly express the p75 intermediate affinity IL-2 receptor (118).

In an *in vivo* setting, T helper cells will be a major source of cytokines influencing the CTL response in a positive or negative direction. T helper (Th) cells develop from a Th0 subset producing IL-2, IFN-γ and IL-4 into either Th1 producing IL-2, IFN-γ and tumour necrosis factor β (TNF-β) or Th2 producing IL-4, IL-5,IL-6,IL-10 and IL-13. Th1 cells stimulate cell mediated immunity, whereas Th2 cells primarily stimulate

humoral responses and some allergic type responses with the activation of eosinophils and basophils (120; 121). The two subsets are also involved in mutual regulation of their activities. This is important not only for mounting appropriate response to different pathogens, but also for regulation of autoimmune responses. Lymphokines produced by Th2 cells can regulate the activities of Th1 cells which in some cases of prolonged activation might be responsible for local inflammation and tissue damage (120). IL-12 and a newly identified cytokine, IFN-γ–inducing factor (IGIF), are known to stimulate CTL responses by stimulating production of IFN-γ by Th1 cells, whereas IL-4 can stimulate the development of Th2 responses (122; 123).

The source of the cytokines influencing T helper cell differentiation *in vivo* is unclear. Macrophages infected with intracellular organisms produce IL-12. IL-12 can rapidly stimulate NK cells to produce IFN-γ which, together with suitable APCs, can prime naive T cells to become IFN-γ producers. IL-4 could be derived from mast cells, basophils or both (113). It has been suggested that the interaction of B7-1 and B7-2 with CD28 on T helper cells could result in a Th2 and Th1 type response respectively (124). This finding conflicts with findings by other groups indicating that the effect is the opposite or that the two costimulatory molecules are involved in similar types of responses. These conflicting results could possibly be explained by variability of the strength of the TCR-mediated signals following exposure to antigen in the different systems (102).

The dose of antigen used and the affinity of the TCR for the MHC class II/peptide complexes have been found to influence the profile of cytokines secreted by T helper cells. Low and high antigen doses during immunisation of mice *in vivo* have been found to result in the development of primarily Th1 or Th2 responses respectively (113). In vitro experiments based on the analysis of a class II restricted T helper clone showed that if the T cell receptor bound with high affinity to a ligand, IFN-γ would primarily be produced, but if the T cell receptor bound with low affinity to a ligand, IL-4 would primarily be produced (125).

The molecular mechanism and cellular signalling events which follow engagement of the T cell receptor are complex. Binding of the TCR and CD8 to the MHC class I/peptide complex leads to the activation and effector function by at least two independent pathways, A and B. The two pathways may function in conjunction with each other. Binding of T cell CD28 to costimulatory molecules on APCs generates a third signal, but it is not clear how the signals derived from the TCR/CD8 and CD28 are linked (126).

Pathway A involves phosphoinositide (PI) turnover through the action of two different lipid kinases, the phosphoinositide kinases (PI kinases) PI 3 kinase and PI 4 kinase. The SH3 domain of the protein tyrosine kinase $p56^{lck}$ binds via hydrophobic interactions to

the regulatory p85 subunit of the PI 3 kinase. The PI 3 kinase then becomes activated and mediates the generation of inositides phosphorylated at the position 3 of the inositol ring. These phosphoinositides include phosphoinositide-3-phosphate (PI 3-P), phosphoinositide 3,4-diphosphate (PI 3,4-P_2) and phosphoinositide 3,4,5-triphosphate (PI 3,4,5-P_3). The function of the position 3 phosphorylated inositides is currently unclear. Unlike the phosphoinositides, they are not a substrate for phospholipase C-γ. p56 ^{lck} also binds to PI 4 kinase and activates it. PI 4 kinase phosphorylates phosphatidylinositol and eventually this results in the generation of phosphoinositide 4,5-diphosphate (PI 4,5-P2). This molecule is also generated from the second pathway, pathway B, activated by TCR ligation.

Pathway B involves the phosphorylation of the TCR ζ chains by the tyrosine kinase $p56^{lck}$. The phosphorylation of TCR ζ creates a binding site for the protein tyrosine kinase ZAP 70. Upon activation, ZAP 70 phosphorylates and activates phospholipase C-γ and a MAP-2 kinase. Phospholipase C-γ will cleave PI 4,5-P2 to Inositol 1,4,5-triphosphate (IP3) and diacylglycerol. These two compounds are involved in the mobilisation of intracellular Ca^{2+} and stimulation of a protein kinase C. MAP-2 kinase can also be activated by activated TCR ζ via the binding of phosphorylated TCR ζ to a small adaptor molecule, SHC. This adaptor molecule binds two other adaptor molecules Grb-2 and SOS. The latter binds to and activates p21ras. Activated p21ras then activates a series of kinases which finally activates MAP-2 kinase. It is thought that CD28 signalling also results in the activation of two pathways, one of which interacts with the pathways activated by TCR ligation, while the other involves activation of PI-3 kinase and finally IL-2 production. It is not well understood how signal transduction is mediated by CD28 or CTLA-4. It is thought that TCR and CD28 signals are both required for full activation of two mitogen activated protein kinases (MAPKs), JNK1 and JNK2, involved in phosphorylation of the c-Jun activation domain and the activation of transcription factor AP-1. This transcription factor plays a crucial role in T cell activation (127).

The presence of several pathways involved in T cell signalling could partly explain why different CTL functions such as effector function, proliferation and IL-2 production can be uncoupled, although the mechanism by which this occurs are not clear. Altered peptide ligands (APLs) are peptides which bind with similar affinity to a particular MHC class I molecule as a T cell agonist, but which do not activate or only partially activate the T cell. Some APLs have been shown to induce a different phosphorylation pattern on ZAP 70 compared with the pattern induced by a T cell agonist, but the significance of this finding in terms of the final outcome of the signalling cascade is unclear (128).

1.3.3. Mechanism of execution of effector function by CTL

Execution of effector function of CTL involves the following steps (98):

1) Initial contact between CTL and an antigen presenting cell via weak interaction between LFA-1 on the T cell and ICAM-1 on the antigen presenting cell.

2) T cell receptor binding to MHC class I/peptide complexes resulting in upregulation and activation of other molecules involved in adhesion such as CD8, LFA-1 and the VLA group of proteins.

3) Additional costimulation of the CTL.

4) Lysis of the target cell.

5) Recycling of the CTL to lyse additional targets.

CTL effector function is commonly evaluated by the ability to lyse target cells in a period of 4 to 6 hours. The duration of the assay will be sufficient to detect lysis of most target cells. Conjugate formation between T cells and target cells is rapid and usually takes place within a few minutes. Some target cells are lysed within 5 to 10 minutes whereas others require 2 to 3 hours. Only some tumour cells and fibroblast monolayers may require up to 24 hours to be lysed (129).

CTL predominantly use two pathways to lyse target cells directly. Both pathways induce apoptosis of the target cell as characterised by DNA fragmentation. One pathway involves the formation of pores in the target cell and the release of proteolytic enzymes from granules while the other pathway involves receptor mediated apoptosis of the target cell. Quiescent (G0) cells are refractory to CTL-induced DNA fragmentation whereas G1 stage targets are susceptible (130).

The first pathway CTL use to kill target cells involves the release of granules which contain perforin and different enzymes such as granzyme A and B. The release of granules is dependent on extracellular Ca^{2+} and an increase in intracellular Ca^{2+}. Perforin molecules insert themselves in the lipid bilayer of the target cell and create 15-20 nm holes in the cell membrane. As a result, granzyme A and B released from the CTL granules can enter the target cells (129). CTL are resistant to killing by their own secreted perforin due to presence of a membrane bound protein which interacts with perforin (131). The holes in the cell membrane can reseal, but the target cells will die because the action of granzymes A and B somehow mediates the DNA fragmentation characteristic of the apoptotic process (130).

CTL are serial killers in the sense that one T cell can kill several target cells. The serial killing occurs partly because granules fill up after lysis of a target cell and partly because CTL secrete approximately a third of newly synthesised lytic proteins instead of storing them in granules. The secretion of these lytic proteins might also lead to some bystander

lysis of target cells not expressing MHC class I/peptide complexes involved in the interaction with the T cell (132).
In vivo, the perforin pathway is crucial in the protection against cells infected with non-cytopathic viruses such as LCMV. The perforin pathway does not appear to important for the *in vivo* clearance of cells infected with cytopathic viruses such as vaccinia virus. This does not mean that CTL can not lyse target cells infected with vaccinia virus via the perforin pathway, but instead that the mechanism of recognition and lysis by CTL using this mechanism is too slow to significantly reduce the number of virus progeny produced by a cell infected with a cytopathic virus *in vivo* (133).

The second major pathway used by CTL to kill target cells is induction of apoptosis in the target cell by the interaction between Fas ligand (C95L) expressed on the CTL and Fas (CD95) expressed on the target cell. This pathway is independent of extracellular Ca^{2+} and observed with some target cells such as the Fas expressing thymoma cell line EL4 (130). Fas is a 48 kDa transmembrane glycoprotein homologous to the TNF-α receptor. Fas ligand is a 40 kDa transmembrane protein of the TNF family and it is expressed on activated T cells. CD4 positive cytolytic T cells do exist and current evidence indicates that the Fas-dependent pathway has a more important role in $CD4^+$ than $CD8^+$ mediated cytotoxicity and might even be the only lytic mechanism used by this T cell subset (133). It has been suggested that the signalling pathways for induction of perforin-and CD95-dependent cytolysis may be different. Alteration of one amino acid in a peptide otherwise known to induce CTL lysis of peptide coated target cells by both the Apo/Fas pathway and the perforin pathway was found to result in the same CTL being able to lyse peptide coated target cells only by the Apo/Fas pathway (134).

A third pathway consisting of lysis by surface bound TNF-α bound on the CTL is also known to exist and possibly be important in prolonged *in vivo* responses (135). The two receptors for TNF-α are elevated on many cells during inflammatory responses, but the pathway is much slower than the Apo/Fas and perforin pathways and would not be detected in a 4 hour ^{51}Cr release assay. All the lysis observed in a 4 to 6 hour ^{51}Cr release assay is therefore accounted for by the degranulation and Fas pathways (135). TNF-α was originally defined by its antitumour activity *in vivo* and *in vitro* but the cytokine has been found to exhibit cytotoxicity against only a limited number of tumour cells (136). Lately, it has been found that its cytotoxicity can be augmented by synergy with IFN-γ *in vitro* (137). This mechanism might play a role *in vivo* as non-cytolytic $CD8^+$ T lymphocytes secreting IFN-γ and TNF-α were found to mediate tumour rejection *in vivo* provided that the action of IFN-γ and to some extent TNF-α were not blocked (138). The tumour rejection might possibly have involved lysis by tumoricidal macrophages induced by IFN-γ produced by T cells as such IFN-γ activated macrophages have been found to lyse other tumour types (8).

As already mentioned, a 4 to 6 hour ^{51}Cr release assay is used to measure effector function of CTL in the present work. This means that the read out used is the ability of CTL to lyse target cells directly within a 4 to 6 hour assay via degranulation or the Apo/Fas pathway. Perforin knock-out mice and mice deficient in either Fas or Fas ligand have been used to clearly demonstrate the importance of the lytic pathway *in vivo* (139). Many experimentally induced murine tumours including fibrosarcomas and plasmacytomas are known to be lysed by direct lysis *in vivo* and *in vitro* (139; 140). The mechanism is also involved in the destruction of several human tumours. CTL activity against such tumours, and melanomas in particular, is commonly evaluated by using the ^{51}Cr release assay (20; 141; 142; 143; 144). The ^{51}Cr release assay is also considered to be extremely sensitve for detecting secondary CTL responses to viruses even when compared with *in vivo* assays (145).

Assays measuring the CTL responses to peptide coated target cells, have shown that the extent of degranulation is directly proportional to the concentration of peptide used to pulse class I, suggesting that activation as measured by degranulation is a direct function of the TCR occupancy level (146).

1.3.4. Mechanisms by which the T cell repertoire is influenced by processes taking place during T cell development in the thymus and in the periphery.

T lymphocytes are bone marrow derived cells which have separated into a thymus derived lineage of cells and an extra-thymically derived lineage of cells (120). The extra-thymically derived lineage of T lymphocytes consists of both αβ and γδ T lymphocytes which reside and mature in the gut epithelium. The function of these T cell subsets is still not clear. Intra-thymic development is by far the most important route for the production of mature T lymphocytes of both the αβ and γδ lineage. Development and export of T lymphocytes starts during the late phase of embryonic life and continues until puberty (120).

In the thymus, the immature T lymphocytes (thymocytes) undergo two major selection events, positive selection and negative selection. Positive selection involves the rescue of immature $CD4^+CD8^+$ TCR^+ thymocytes from apoptosis provided they carry T cell receptors which can bind with a certain strength to peptide/MHC class I complexes in the thymus. Positive selection takes place in the cortex of the thymus and can be mediated by several different antigen presenting cell types in addition to thymic epithelial cells. Thymocytes which are unable to be positively selected will undergo apoptosis, activation induced cell death or be functionally inactivated through anergy. $CD4^+CD8^+$ thymocytes develop into single positive $CD4^+CD8^-$ or $CD4^-CD8^+$ thymocytes. Down-regulation of either CD4 or CD8 appears to be a stochastic process occurring independent of the MHC specificity of the T cell receptor. During the final stages of positive selection, interaction of the transitional $CD4^+CD8^-$ or $CD4^-CD8^+$ thymocyte with APCs will result in maturation of the single positive thymocyte if the thymocyte express the appropriate coreceptor (147; 148).

The process of positive selection forms the basis of the MHC restriction of T lymphocytes. Peptides can influence the process of positive selection *in vitro* (149; 150), but it is not known whether the T cell receptor actually interacts with a peptide component *in vivo* or directly with the MHC class I molecule. Experiments have shown that the same peptide can induce positive selection, T cell activation and negative selection depending on the peptide concentration used (151). In other words, the avidity of the interaction between a T cell and an antigen presenting cell (as defined by the overall sum of the T cell receptor affinity, the density of costimulatory molecules and the density of peptide/MHC class I complexes on the cell surface) can determine the fate of the T cell (152).

The idea of low avidity interactions being responsible for positive selection is supported by the finding that selected peptides which are unable to stimulate T cells to effector

function *in vivo* (T cell antagonists) are able to induce positive selection of T cell *in vitro* (153). However, peptides which can stimulate T cells to effector functions (T cell agonists) have been found to be unable to induce positive selection (154). This suggests, that the fate of the thymocyte is not exclusively determined by the avidity of the interaction, but that different mechanisms might be involved in positive selection and T cell activation. T cells recognising viral (foreign) peptides presented by syngeneic class I molecules as well as T cells recognising peptides presented by allogeneic class I molecules, have been found also to recognise self peptides presented by syngeneic class I molecules *in vivo*. This degree of cross-reaction explains how T cells specific for peptides not found in the thymus are positively selected and supports the notion that the T cell receptor does not recognise a peptide in particular but rather a conformation/antigenic surface composed of the combination of peptide plus MHC (155).

The second major selection process taking place in the thymus is the process of negative selection. Transgenic mice expressing specific T cell receptors and/or the antigen recognised by an individual T cell receptor such as the male antigen HY or the murine class I molecule K^b have been fundamental for the understanding of this process (120; 156).

The process of negative selection results in the induction of apoptosis of T cells interacting with high avidity with cells in the thymus (120). As already mentioned, avidity is considered as the sum of the affinity of T cell receptors, the density of peptide/MHC class I complexes and density of other costimulatory molecules. This means that T cells recognising abundantly presented self peptides as well as T cells carrying high affinity or high density T cell receptors specific for particular self peptide/MHC complexes will be deleted. Deletion of T cells carrying specific receptors therefore depends on how well the antigen in question is presented on cells in the thymus and how well the T cell interact with peptide/MHC class I complexes via the T cell receptors. Negative selection is important to reduce the risk of CTL reacting to self proteins in the body. Negative selection can also influence the T cell response to foreign antigens. This is the case when T cells which can potentially recognise self MHC plus foreign peptide are eliminated because these T cells also react with high avidity with certain combinations of self MHC plus self peptide. As an example, recognition of HLA-B44 presented self peptide by CTL results in elimination of certain HLA-B8 restricted CTL responses to EBV proteins in HLA-B44, HLA-B8 positive humans (157).

Transgenic mice expressing exogenous proteins, such as LCMV nucleoprotein, hen's egg white lysozyme or the murine class I molecule K^b, as self proteins have been used to show that certain T cells specific for self proteins can avoid negative deletion and escape to the periphery. These T cells fall into two categories. The first category includes T cells

which are specific for epitopes known to be immunodominant and well presented in normal mice, but which can only engage in low avidity interactions with cells presenting these epitopes. The second category includes T cells which can interact with high avidity with antigen presenting cells, but where the epitopes are subdominant epitopes which are not efficiently presented and which do not stimulate CTL in normal mice (158; 159; 160; 161). Similar findings have been reached using specific T cell receptor transgenic mice and, either crossing them with mice expressing the antigen of interest such as a variant of haemoglobin (162), or by introducing the peptide recognised by the TCR intraperitoneally (163). In both cases, only T cells able to engage in low avidity interactions with cells presenting the antigen are found in the periphery.

The dose of self antigen can clearly influence the nature of the T cells found in the periphery, as shown in transgenic mice expressing different levels of hen's egg white lysozyme. At very high antigen doses, even T cells specific for subdominant epitopes are deleted, indicating that at these high protein concentrations even poorly presented peptides can be presented at sufficient high levels on the cell surface to allow high avidity interactions and subsequent deletion of autoreactive T cells (159).

Allo MHC molecules are not present in the thymus. This means that thymocytes with TCRs which can bind with high affinity to allo MHC or peptide/allo MHC would be able to escape negative selection. Indeed, a T cell clone specific for self peptide presented on a syngeneic MHC class I molecule was been shown to be of considerably lower affinity than a T cell clone specific for the same peptide but presented by an allogeneic MHC class I molecule (86). Although the generality of this finding can not automatically be assumed, higher avidity of allo CTL could explain the ability of an individual's T cells to react with high intensity against the disparate MHC proteins of other individuals of the same species (86) and the ability of some allo CTL to be activated by very low number of peptide/MHC class I complexes (164).

Activation of mature T cells to effector function requires higher avidity interactions with antigen presenting cells than negative selection of thymocytes (151; 165). This means that self peptide specific T cells which have escaped negative selection can exist in the periphery without doing any harm to normal tissue. It has been found that self specific T cells in the periphery can be activated if the avidity of the interaction between T cell and antigen presenting cells in the periphery is increased. This can be achieved by increasing the dose of antigen presented to self specific T cells, either by producing more protein or by increasing the level of MHC expression and/or by up-regulating the level of costimulatory molecules such as B7-1 (109; 160; 166). The addition of cytokines such as IL-2 (167; 168; 169) or viral, bacterial or parasitic infection (170; 171) can equally

contribute to the activation of autoreactive cells. Infection might contribute to breakage of tolerance by inducing the production of inflammatory cytokines such as IFN-γ resulting in the activation of T cells and/or up-regulation of antigen processing and MHC synthesis, but also by producing proteins which can act as superantigens (172) or generating peptides which mimic self peptides (91; 170).

When T cell specific for self peptides are exposed to additional levels of self antigen or activation signals in the periphery, they can not only become activated, but also deleted by activation induced apoptosis or become totally unresponsive to additional antigen by down-regulation of surface levels of T cell receptors (166; 173; 174; 175; 176). The outcome of T cell stimulation appears to depend on the type of antigen presenting cells used to stimulate the T cells as well as the dose of antigen. In mice carrying TCRs specific for the male antigen HY, a transient exposure of virgin T cells to the antigen leads to activation and development of memory T cells whereas an intermediate dose results in activation induced apoptosis of T cells and a high dose results in activation followed by anergy and down regulation of surface levels of CD8 (175).

1.4. Tumours as potential targets for CTL

As with conventional CTL target cells, tumour cells will only be able to be recognised and eliminated by CTL if the tumour cells produce suitable CTL epitopes and are able to serve as targets for CTL.

1.4.1. The contribution of altered protein expression in tumour cells to the generation of novel tumour antigens

Tumour cells or neoplastic cells are cells which differ from normal cells in that they grow abnormally and are no longer responsive to normal growth controlling mechanisms. Tumours can be benign or malignant. In the latter case, the tumours are referred to as carcinomas or sarcomas depending on whether they are epithelial or mesenchymal tumours. Tumours of the hematopoetic or lymphopoetic system are classified by the suffix "-emia". In the malignant cases, the tumour bearing state is referred to as cancer and the process which leads to the development of cancer is referred to as carcinogenesis (177).

Carcinogenesis is a multistep process. The first step is initiation, which is characterised by the induction of stable structural and/or functional alterations in the genes following (point)-mutations, deletions, substitutions, gene amplifications, chromosomal translocations or in some cases transductions and insertional mutagenesis. In most cases, the initiated cell will die or the altered genes will be repaired. However, if the initiated cell undergoes mitosis, the change is permanent and the cell is susceptible to tumour promotion. This does not include additional DNA changes but is rather due to altered expression of proteins in the initiated cells. The result is the generation of small nodules, polyps or papillomas. After this stage the benign tumour can progress to a malignant tumour through additional genetic alterations (177).

The genetic alterations which are important in carcinogenesis contribute to a loss of growth control of the cell. This happens either because cells divide all the time in an autonomous way or because the cells refuse to die, i.e. undergo apoptosis at stages where normal cells would do so. These cellular processes are the result of the actions of oncogenes or the inactivation of so-called tumour suppressor genes. Oncogenes are defined as genes which under certain conditions are able to induce cells to become neoplastic through mutation or change in the control of expression of normal genes, so-called proto-oncogenes. Retroviral oncogenes are derivatives of cellular genes linked to regulatory sequences elements and coding sequences of viral genes and carry the prefix "v-". Indeed, a retroviral oncogene, v-src, derived from Rous sarcoma virus was the first oncogene which was identified as being responsible for cell transformation (178).

Oncogenes generally fall into four categories (178; 179; 180): -
1) growth factors such as fibroblast growth factor and platelet derived growth factor ;
2) growth factor receptors and in particular the receptor tyrosine kinases such as her-2/neu (HER-2), the epidermal growth factor receptor or the fibroblast growth factor receptor;
3) cytoplasmic onco-proteins involved in signal transduction or regulation of the cell cycle such as ras and cyclins such as cyclin E and cyclin D1;
4) nuclear oncogenes involved in control of transcription such as c-myc, c-fos and c-jun or mdm2.

Finally mutations in tumour suppressor genes such as p53 are found to involved in 60% of human cancers (181).

Tumours have been known to be immunogenic for a very long time. Experimentally induced murine tumours were found to be rejected when transplanted into syngeneic mice and the rejection was found to be tumour specific. This led to the hypothesis that tumours expressed so called tumour specific transplantation antigens, TSTA. For a long time only surface bound tumour specific molecules recognised by antibodies were identified. These molecules were either differentiation antigens such as oncofoetal antigens α-feto-protein on human hepatomas and carcinoembryonic antigen on colon carcinomas or cell surface glycolipids such as gangliosides on melanomas and neuroblastomas (5). The work of Thierry Boon and co-workers in the late 1980's led to the identification of a series of tumour derived class I presented peptides which were recognised by tumour specific CTL. Peptides derived from proteins expressed in melanomas were the first naturally presented human CTL epitopes to be identified. Since then naturally presented CTL epitopes from other human and murine tumours have been identified as well as epitopes recognised by class II restricted tumour specific T helper cells, for detailed reviews see (5; 17; 182; 183; 184).

The antigens recognised on human tumours or murine experimental tumours by CTL or T helper cells can be divided into at least four categories.

The first category represents tumour antigens derived from viral proteins. This includes CTL epitopes derived from the Epstein-Barr virus (EBV) produced proteins EBNA2, 3, 4, 5 or 6 and LMP1 and LMP2 (185) or the CTL epitopes identified in human papillomavirus (HPV) type 16 E6 and E7 proteins (186). EBV and HPV are associated with Burkitt's lymphoma and cervical cancer respectively.

The second category of tumour antigens are derived from oncogenes activated by a point mutation, resulting in the generation of novel T cell epitope(s). Such epitopes include the $CD4^+$ T helper and $CD8^+$ CTL epitopes in p21 K-ras containing a glycine to aspartic acid substitution at amino acid 13 and recognised by T cells from a colon cancer patient (187). Another recent example is the HLA-A2.1 presented peptide derived from cyclin dependent kinase cdk4 with an arginine to cysteine substitution at amino acid 24 and recognised by CTL from a melanoma patient (188).

The third category of tumour antigens are derived from oncogenes which are activated as a result of a fusion of parts of genes encoding two different proteins. The most interesting example is the translocation of the human c-abl protooncogene to the specific breakpoint cluster region on chromosome 22 resulting in a bcr-abl fusion protein with abnormal tyrosine kinase activity. This translocation has been found in more than 95% of patients with chronic myeloid leukemia and preliminary experiments have shown that the protein can induce $CD4^+$T helper responses as well as $CD8^+$ CTL responses in mice (183).

The fourth category of tumour antigens are derived from proteins which do not carry any point mutation but are normal self proteins which are expressed at high levels in the tumour cell due to gene amplification or abnormal transcriptional or translational control. Most of the CTL epitopes and T helper epitopes which have been identified in cancer patients are derived from overexpressed self proteins. T helper responses and CTL responses to HER-2/neu have been detected in patients with breast cancer and ovarian cancer (189; 190; 191). The HER-2 protein is an example of a protein which is widely expressed in normal epithelial cells, but over expressed in a large percentage of breast -, lung-, colon- and ovarian cancers (between 20-80% depending on the cancer type) (183). In addition a wide variety of melanoma differentiation antigens including MART-1 (141; 142; 143; 192), gp100 (20; 144; 193) or the MAGE antigens (194; 195; 196; 197) stimulate human tumour specific $CD8^+$ CTL. Both $CD8^+$ as well as a $CD4^+$ T helper cell epitopes have been identified in the melanoma antigen tyrosinase (198; 199; 200).

1.4.2. Potential benefits and disadvantages of using normal self proteins as targets for tumour reactive CTL

Self proteins represent an attractive target for tumour specific CTL. Overexpression of a particular self protein is found in a large percentage of different cancers and this overexpression often appears to be linked to the role the overexpressed protein plays in maintaining the transformed phenotype of the tumour cell. In some respects, it is more advantageous to target normal self proteins than mutated proteins as CTL targets because the nature of mutations can vary from individual to individual and the number of mutations found within oncogenes or tumour suppressor genes have been found to be enormous. As an example more than 2000 different mutations have been found in p53 (181; 201). Due to the increased genomic instability of cancer cells, as many as one mutation in every 10-30 kb of the genome can be found in early tumour development (202).

CTL specific for proteins overexpressed in a tumour can mediate tumour rejection without causing damage to the surrounding tissue, as demonstrated in a murine model using the Friend Leukemia virus envelope (env) protein as a "self-protein" (203). CTL specific for the envelope protein and transferred to mice expressing low levels of the env proteins in normal tissue and high levels of the env protein in a transplanted tumour were found to mediate rejection of the tumour. The CTL did not damage the normal tissue expressing lower levels of the protein, thereby lending support to the hypothesis that tumour specific CTL would indeed be able to discriminate between cells expressing different levels of the same protein. In some cases, tumour specific CTL have been found to be able to distinguish between cells with only a two-fold difference in the level of expression of the protein giving rise to the antigen recognised by the CTL (204). The implications of these findings is that , as long as efficient priming of tumour specific CTL is provided either *in vitro* or *in vivo*, these CTL will potentially be able to selectively lyse tumour cells expressing as little as a few times more protein than normal tissue.

In several murine models, $CD8^+$ CTL have been shown to be directly responsible for the *in vivo* eradication of tumours expressing the antigen recognised by the CTL (6). The proof of a role of CTL in tumour rejection *in vivo* in humans is still indirect. Firstly, immunocompromised patients typically develop a variety of tumours of some viral origin. Secondly, tumour infiltrating CTL detected by immunohistochemistry in melanoma patients have in some cases been found to express a predominant TCR indicating clonality. These CTL have been found to cluster around melanoma cells *in vivo* and kill melanoma cells specifically in ^{51}Cr release assays *in vitro* (205). Thirdly, CD8 positive CTL isolated from tumour sites have been shown to carry the CD45RO isoform,

indicative of T cell memory and previous activation *in vivo* (206). Finally, transfer of tumour infiltrating lymphocytes specific for gp100 or tyrosinase to two different melanoma patients has been found to result in dramatic regression of cancer metastases (19; 211).

Many tumour antigens have been identified by culturing tumour infiltrating lymphocytes (TILs) or lymphocytes derived from peripheral blood (PBLs) with autologous tumours for an extendend period of time *in vitro* before specific cytotoxicity was detected. This could suggest that some CTL are anergic *in vivo* but activated by the exposure to cytokines and antigen *in vitro*. There is evidence of reversible T cell anergy towards some tumours *in vivo* based on the pattern of cytokine expression (207). However, there is also evidence of tumour specific T cells not being anergic but activated *in vivo*. Freshly isolated TILs derived from lung cancer patients and grown for only 6 days in culture (208) or TILs derived from melanoma patients and restimulated for a short time *in vitro* with IL-2 only and no antigen (209; 210) can specifically lyse tumour cells *in vitro*, suggesting that the T cells have indeed been activated *in vivo*. The precursor frequency of such activated CTL has been estimated to range from approximately 1/1000 to 1/30,000 as measured by limiting dilution of T cells derived from PBLs in melanoma patients (209).

In vivo, tumours can escape immune recognition by several mechanisms and this will obviously have to be taken into account when designing a strategy based on the use of overexpressed normal self proteins as targets for tumours specific CTL.

Firstly, tumour cells are constantly cycling. This means that the tumour burden can quickly overwhelm the host before the immune system has been sufficiently activated (7).

Secondly, tumours are heterogeneous due to genetic instability and possibly also due to pressure by the immune system (16; 202). Loss of antigens and development of antigen variants are known to occur in cancer patients *in vivo* as demonstrated by the loss of five different CTL epitopes and the gain of one completely new epitope by tumour cells in a melanoma patient in a period of five years (212). However, the chance of escape of antigen variants would be reduced by targeting normal overexpressed self proteins where a normal not mutated amino acid sequence is essential for the maintenance of the transformed phenotype.

Thirdly, tumours can escape immunosurveillance either by having low levels of class I molecules due to defects in the transcriptional regulation of the class I genes, deleted class I genes, deficiencies in the TAP transporters or lack of β_2m expression (213; 214; 215;

216). As mentioned in section 1.3.2., tumours can also escape immune recognition by providing insufficient levels of cell surface costimulatory molecules necessary to activate CTL. In murine tumour models, the problem of lack of costimulation has been overcome by immunising with cells expressing costimulatory molecules such as B7-1 or ICAM-1 alone or in combination with MHC molecules or cytokine molecules (106; 217; 218) or by transfecting tumour cells with cytokines enhancing the differentiation and activation of host APCs such as GM-CSF (106; 219). The tumour itself might not necessarily have to be involved in priming of the CTL. Bone marrow derived antigen presenting cells can present tumour derived antigens to class I restricted CTL (220). CTL could be primed by encountering tumour specific antigens on professional antigen presenting cells in the lymphoid organs. The tumour could then serve as targets for activated CTL as low levels of costimulatory molecules are not critical for execution of effector function by CTL (see section 1.3.2.).

Finally, the tumours are themselves able to inhibit the action of the immune system by the secretion of various inhibitory cytokines or interacting directly with cells of the immune system (5). This could result in defective signaling in CTL as most dramatically observed in the reduction in the level of $p56^{lck}$ and $p59^{fyn}$ and complete absence of CD3 ζ in mice carrying a murine colon carcinoma MC38 or in human colon cancer patients (221).

The potential problems mentioned above will need to be overcome if CTL based immunotherapy is going to be efficient. Nevertheless, the first problem to solve is to identify proteins which can serve as useful targets for tumour specific immunotherapy.

Cyclin D1, mdm2, fibroblast growth factor receptor I and p53 are all examples of oncogenes or mutated tumour suppressor proteins which are expressed at high levels in different human cancers. All four proteins have an important function in regulation of the growth and division of normal cells. The properties of the different proteins are listed in Table 1.4.1.

Cyclin D1 is a member of the cyclin family of proteins. These proteins are involved in the regulation of the cell cycle. Cyclin D1 is involved in the transition from the G1 phase to the S phase of the cell cycle. The protein associates with cyclin dependent kinase 4 (cdk4) and to some extent with cyclin dependent kinase 6 (cdk6). These complexes can associate with the tumour suppressor protein, the retinoblastoma protein, and this association somehow enables the cells to enter the S phase (222; 223). Cyclin D1 is present in the nucleus until the S-phase (224; 225). The intracellular level of the protein increases up to G1 but stays relatively constant during the rest of the cell cycle (223). The protein is expressed at low levels in most tissues and in the proliferative zones of epithelial tissues such as gastric and breast epithelial tissues in particular (225). In contrast to cyclin D2

and cyclin D3, cyclin D1 is not expressed in T cells (226) and can not be detected by immunocytochemistry in lymphoid tissues such as tonsil, spleen and lymph nodes (225). Despite being important for the regulation of the cell cycle, cyclin D1 knock-out mice are viable but exhibit some neurological defects, defects in the breast epithelial cells during pregnancy and reduced body size (227; 228). Cyclin D1 has been shown to contribute to cell transformation *in vitro* by substituting for other oncogenes (229) and *in vivo* where mice overexpressing the protein develop adenocarcinomas in the mammary glands (230). Paradoxically, it is difficult to create stable cell lines over expressing cyclin D1 and there seems to be some limit to how much of the protein a cell can tolerate (231; 232; 233). The protein was originally only associated with parathyroid adenomas and certain B cell neoplasms (234) but has since been found to be expressed at high levels in a large fraction of cancers in particular breast carcinomas, hepatocellular carcinomas, lymphomas, melanomas, head and neck squamous cell carcinomas and colorectal carcinomas (225; 235; 236; 237; 238; 239), see Table 1.4.1. As an example, 30% of breast cancer patients are reported to overexpress cyclin D1 moderately with levels being 2 to 3 times higher than in normal tissues, while 30% of patients overexpress the protein strongly with levels being 3.5 to 7 times higher than in normal tissue (236).

mdm2 and p53 are also involved in regulation of the cell cycle but in a different way than cyclin D1 is. Both proteins are expressed in all tissue including the spleen and the thymus (240; 241). The two proteins regulate each others activities both on the transcriptional and post translational level (242; 243). Both proteins are involved in the regulation of transcription, but in different ways. The activities of p53 are involved in negative regulation of the cell cycle by controlling the G1 to S transition. Mdm2 rather seems to have a positive role on G1 to S transition by its ability to bind to the transcription factors E2F1 and DP1 (244). Mdm2 is able to bind and inhibit the function of p53 (245; 246) and retinoblastoma protein (247). This means that the protein is able to block the action of proteins which inhibit cell cycle progression. This explains why mdm2 knock-out mice are not viable unless they are crossed with mice having a deleted p53 gene to create $mdm2^{--}p53^{--}$ mice (248; 249). On the other hand, p53 can influence the expression of mdm2 protein, as transcription of mdm2 can be regulated by p53 binding specifically to a region in intron 1 of the mdm2 gene (242).
Mdm2 has been found to be expressed at high levels in wide variety of cancers including sarcomas (250; 251; 252; 253; 254), leukemias (255; 256; 257), lymphomas (257), breast carcinomas (258) and pancreatic carcinomas (259), see table 1.4.1. To give a detailed example, 8 out of 12 patients (75%) with pancreatic carcinomas had more than 6 times higher levels of the mdm2 proteins in the tumour than in normal tissue (259).

P53 is a famous molecule, being elected molecule of the year in Science Magazine in 1992. The protein appears to negatively regulate the cell cycle and suppress tumour

formation. Two lines of evidence supports this hypothesis. Firstly, mutations in the protein have been identified in 60% of all cancer patients and 85.6% of these mutations are missense mutations resulting in a faulty gene (181). Secondly, p53 knock-out mice are viable but develop tumours at an early age (260).

Mutant p53 can form a complex with a potential normal version of the protein and inhibit its function. P53 in a mutant conformation has been found to be expressed at high levels in a wide variety of tumours. In addition, overexpression of p53 with apparently no mutations has also been observed in several tumours. In many cases, the wild type p53 has been found to be bound by other proteins such as overexpressed mdm2 and prevented from performing its function in the nucleus (261).
Mutated p53 would be an ideal target for tumour immunotherapy if it was not for the fact that so far more than 2000 different mutations have been identified in the protein (201). There are a number of mutational hot spots, but the mutations at these hot spots are not identical. As an example, at amino acid 175, 8 different mutations was found in 35 patients (262) Singling out individual mutations to stimulate T cell responses would be hopeless. Instead, one could exploit the fact that mutant p53 is commonly overexpressed and that it might be possible to induce CTL to normal epitopes found in common in most p53 proteins irrespective of the mutation found in the protein. Cancer patients have been found to have both p53 specific antibodies (263; 264; 265; 266; 267) and p53 specific T helper responses (265; 268). These responses are directed against non-mutated sequences in the protein, despite mutant versions of the protein being expressed in the individuals.

The fibroblast growth factor receptor (FR) represents the receptor tyrosine kinase category of oncogenes. In both mice and humans, the FR family of proteins is a multigene family and so far 5 distinct genes have been identified (269). The present work will involve a splice variant of the FRI gene. The cDNA encoding this form is 267 bp shorter than the longer splice variant of FRI (270; 271). The short form dealt with in the present work contains two extracellular immunoglobulin like domains (in contrast to three Ig domains in the long form), a transmembrane domain and a cytoplasmic tail involved in signaling. The homology between the murine and human proteins are 98% on the amino acid level (269).
The fibroblast growth factor receptor I has more limited tissue expression than cyclin D1, mdm2 and WT p53, but is expressed in a variety of tissues. The protein is expressed in endothelial and epithelial cells such as in neuroepithelial cells in the brain and epithelial cells of the cortex and the medulla of the thymus, the breast and the cervix. The protein has also been found in pancreatic acinar cells, keratinocytes, fibroblasts and on basophils and acidophils of the pituitary gland (270; 272; 273; 274). FRI is found to be over

expressed in cancers in the brain (malignant astrocytomas) (275; 276), breast carcinomas (273) and ovarian carcinomas (277) as well as pancreatic adenocarcinomas (274).

Altogether the four proteins cyclin D1, mdm2, p53 and fibroblast growth factor receptor I represent four proteins which are expressed in normal tissue at low levels but found to be expressed at high levels in several different human cancers. These proteins will be used in a murine system to investigate the extent of CTL tolerance to these proteins and whether it is possible to identify specific epitopes recognised by self protein specific CTL.
This would be the first step towards identification of CTL able to recognise tumours expressing high levels of the proteins.

Table 1.4.1.

Properties of the murine self proteins cyclin D1, mdm2, fibroblast growth factor receptor I (FRI) and wild type p53 (WT p53)			
Protein	**Function**	**Normal tissue distribution**	**Cancers characterised by high levels of expression of the protein**
Cyclin D1 (CD1) 34 kDa 295 aa	Cell cycle progression : G1 to S phase	Most cell types except T cells, B cells and fibroblast cell lines	Breast carcinomas Colorectal carcinomas Lymphomas Melanomas Cervical carcinomas
FRI 85 kDa 733 aa	Receptor for acidic and basic fibroblast growth factor, which are mitogens for a variety of cells and involved in angiogenesis	Epithelial cells Endothelial cells Keratinocytes Fibroblasts	Invasive bladder carcinomas Ovarian carcinomas Hepatocellular carcinomas Pancreatic adenocarcinoma Malignant astrocytomas
mdm2 95 kDa 489 aa	Negative regulator of p53 Transcription factor	Ubiquitious:kidney, brain, lung, testis, thymus etc.	Sarcomas Breast carcinomas. Leukemias Lymphomas
p53 53 kDa 393 aa	Transcription factor Negative regulator of cell growth Induces apoptosis in response to DNA damage Blocks cells in G1 phase.	Ubiquitious: but highest in spleen and thymus	Colorectal carcinomas Breast carcinomas Soft tissue sarcoma Astrocytomas Lymphomas

1.5. Properties of selected recombinant vaccinia virus and adenovirus expression systems

In the present work, the experimental approach used to investigate the state of tolerance to selected murine self proteins is based on :

1) the use of recombinant viruses expressing the proteins for *in vivo* immunisation and stimulation of CTL *in vitro* and

2) peptides for *in vivo* and *in vitro* immunisations.

The two viruses used in the present work are vaccinia virus and adenovirus. The properties of the two viruses and the reasons underlying the choice of these viral expression systems will be outlined below.

1.5.1. Biology of vaccinia virus

Vaccinia virus is a cytopathic DNA virus. The virus belongs to the genus of Orthopoxviruses which is a part of the Poxviridae Family. Orthopox viruses comprise at least 10 species including cowpox virus and variola virus. The latter is the cause of human smallpox (278). Vaccinia virus has been used to immunise against smallpox at least since 1967 where the WHO Intensified Smallpox eradication programme began. The origin of vaccinia virus and its relation to variola virus and cowpox virus remains obscure. Originally, cowpox virus was used to immunise humans against smallpox, but no strain of virus used for vaccine production since 1967, and probably very much earlier, has been characterised as cowpox. Instead, all strains have had the biological characteristics of vaccinia virus (278).

Vaccinia virus has a broad host range but so far no natural animal reservoir of vaccinia virus has been discovered apart from some observations with buffalo pox in India. In humans, infection with vaccinia virus is unlikely to occur except by deliberate immunisation or during experimental work with the virus. Work with the virus requires certain precautions and is usually carried out in a class II containment laboratory. Infection can cause skin complications such as eczema or generalised vaccinia, eye infections or in severe cases encephalitis. However, due to strong immune response to the virus, fatal complications mostly occur in immunocompromised individuals and the virus is relatively safe to work with (278).

Vaccinia virions appear as oval-or brick shaped bodies about 200-400 nm long (279). The virion is composed of a lipoprotein bilayer surrounding a biconcave core (279).

The vaccinia virus genome is now well characterised and consists of a linear duplex 191 kb long DNA molecule. Each terminus is composed of an incompletely base-paired hairpin of 101 nucleotides, so one may consider the genome as self complementary circular strand. One strain, the Copenhagen Strain, has been completely sequenced and

found to encode as many as 263 potential proteins, of which most have a role in nucleic acid metabolism or biosynthesis or in evading the host immune response (280).

The life cycle of the virus takes 20 to 24 hours and is divided into virus entry, early transcription, DNA replication and late gene expression followed by virion assembly and virus dissemination, Figure 1.5.1. As many as 10,000 infectious virus particles can be generated per cell (279). Often the vaccinia virus will remain in the infected cell until it is harvested. This intracellular form is referred to as intracellular naked virus (INV) but does contain an envelope. Extracellular enveloped virus acquires a second membrane upon exiting the cell and is important in virus dissemination in an infected host animal (281).

It takes approximately 30 minutes for the virus to enter the cell. Once the virus has entered the cell, early transcription begins. Vaccinia virus and members of the pox virus family are the only known DNA viruses (with the exception of African swine fever virus) that synthesise their mRNA in the cytoplasm of the infected cell, Figure 1.5.1. The virus core contains all the enzymes necessary for the transcription of the early genes including a RNA polymerase, transcription and termination factors, RNA capping and methylating enzymes and a poly (A) polymerase (279). Replication starts within the first 2 hours of infection and goes on for up to 12 hours post infection. Host protein synthesis is shut off. Vaccinia virus has also been reported to interfere negatively with class I surface molecule expression. In one case, infection with vaccinia virus was shown to reduce the surface levels of some class I molecules 2 to 5 fold upon 24 hours post infection (282).

Vaccinia virus is useful for expressing foreign genes for several reasons. Apart from the broad host range of the virus, at least 25 kb of foreign DNA can be inserted into the genome without any deleterious effect on virus yield. Post translational modification including glycosylation, phosphorylation, ADP-ribosylation and acylation of eukaryotic proteins seem to occur faithfully (281; 283). The virus has been used to express a variety of different eukaryotic, viral and bacterial proteins including Factor IX, Influenza A viral proteins and *E. coli* proteins (283).

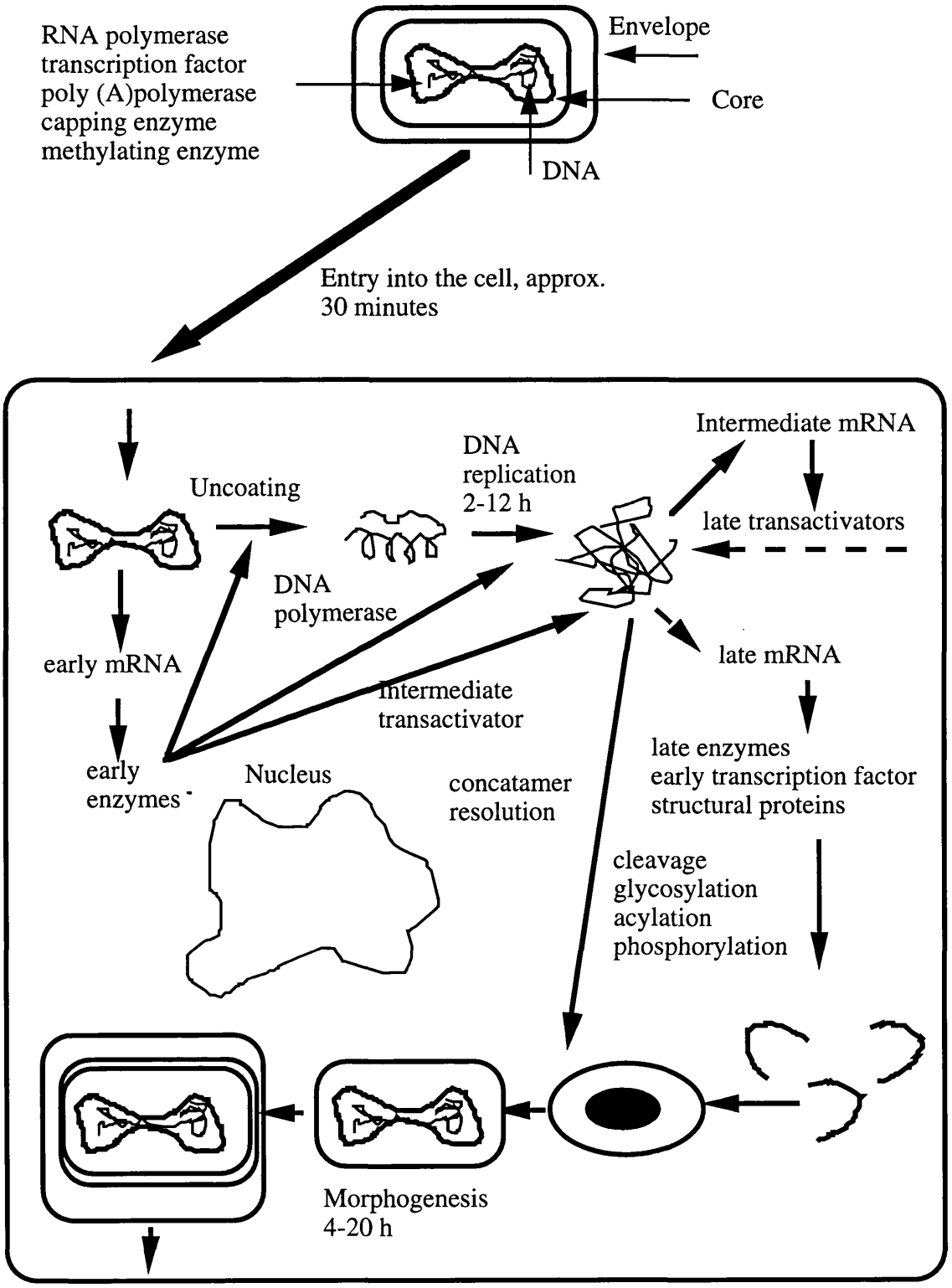


Figure 1.5.1
Outline of the replication cycle of vaccinia virus.
See text for details.

1.5.2. The immune response to recombinant vaccinia virus

Expression of recombinant proteins by vaccinia virus can induce a strong humoral as well as a cellular response to the recombinant protein.

There are examples of the generation of neutralising and protective antibodies to proteins expressed in vaccinia virus in many different species including mice, rabbits, chicken, cattle, monkeys and humans. Some examples include the generation of protective antibodies to rabies virus in foxes by immunising the animals with recombinant vaccinia expressing rabies virus glycoprotein and the generation of protective antibodies to influenza virus with recombinant vaccinia virus expressing influenza hemagglutinin (284).

Vaccinia virus is also very efficient in delivering exogenous recombinant proteins to the class I pathway because the entire life cycle of the virus takes place in the cytosol. CTL have clearly been shown to be essential for the immuneresponse to many recombinant proteins expressed by vaccinia virus. This has been demonstrated by CTL assays *in vitro* and by passive transfer experiments of primed CTL as well as by blocking the function of CTL *in vivo* (284; 285). CTL have been induced to numerous recombinant proteins expressed by vaccinia virus in a variety of hosts. In mice, several protocols exist for inducing CTL *in vivo* (for one review see (285)). When mice are immunised with recombinant vaccinia virus, intraperitoneal or intravenous administration is commonly used and both methods work equally well (285). When spleen cells from immunised mice are put into culture, the CTL activity will peak after 6 to 7 days in culture (285). In mice, CTL responses have been generated to viral proteins such as influenza virus nucleoprotein or human papillomavirus proteins (285; 286) or various oncogenes such as mutant ras (287). In humans, recombinant vaccinia virus expressing a tumour antigen, carcinoembryonic antigen (CEA), has recently been used to elicit specific CTL responses in cancer patients suffering from metastatic breast carcinoma (288).

It seems that the natural protection against vaccinia virus *in vivo* is mediated by antibodies as well as by $CD4^+$ and $CD8^+$ T lymphocytes (133; 278), although experiments in $CD8^+$ T cell deficient mice have shown that the virus itself can be cleared in mice deficient of $CD8^+$ CTL (289). Secretion of IFN-γ and TNF-α by $CD4^+$ and $CD8^+$ T cells is involved in the immunity against poxviruses. Experiments with knock-out mice have shown that Fas and perforin do not seem to be necessary for clearance of the virus. The number of CTL precursors specific for vaccinia virus components can be as much as 10 to 100 times higher than for other viruses also known to be potent inducers of CTL such as influenza virus (285). Nevertheless, it has been suggested that recognition and

lysis of an infected cell by CTL is too slow to significantly reduce the number of virus produced by a cell that is infected with a cytopathic virus such as vaccinia virus (133).

Vaccinia virus can enter the body through abrasions in the skin or oral mucosa membrane, through inoculation or through infection via the respiratory and sometimes the intestinal tract. In natural infections, respiratory infection was the principal mode of infection in smallpox (278). The virus first infects cells at the port of entry and causes lytic death of infected cells. When lytic cell death occurs as a consequence of viral infection this leads to a local inflammatory response. Local dendritic cells subsequently become activated and migrate to draining lymph nodes, where they present exogenous viral antigens (290). The virus itself moves from the port of entry to the lymph nodes where it replicates (278). Once the virus has entered the lymph nodes it can again be presented by professional antigen presenting cells to CTL.

Spleen cells from vaccinia virus infected mice have been shown to produce various cytokines such as IFN-γ, IL-6 and TNF-α within 12 hours of restimulation with virus-infected UV-irradiated syngenic cells *in vitro* with $CD4^+$ T cells and adherent cells being responsible for the IFN-γ production and adherent cells producing IL-6 and TNF-α (291).

The frequency of CTL precursors specific for vaccinia virus itself is high. This means that the CTL response to the virus itself will be very strong and might dominate the immune response if the conditions for restimulation of T cells are not carefully chosen. In addition, vaccinia virus is also able to induce *in vitro* primary responses. If mice have been immunised *in vivo* with vaccinia virus, it will be advantageous to stimulate the responder T cell *in vitro* by other means than by using vaccinia virus because otherwise the vaccinia response tends to overwhelm all but the strongest of responses to extrinsic antigens (285).

1.5.3. Vectors used to construct recombinant vaccinia virus

pSC11 (292) is a 7.88 kb vector widely used to clone a variety of eukaryotic, prokaryotic and viral cDNAs aimed at being expressed in vaccinia virus, Figure 1.5.2. and for review see (283; 293; 294). The vector contains the same features as the vaccinia virus vector pGS20 (295). This means that pSC11 is pUC9 based and that the foreign cDNA is cloned under the P7.5 promoter (see below) between the left and the right end of the vaccinia virus thymidine kinase gene. The thymidine kinase sequences mediate homologous recombination with wild type vaccinia virus. Recombinant virus will then be thymidine kinase negative (tk^-). This provides an opportunity to select recombinant tk^- virus with bromodeoxyuridine (BrdU) (292; 295). BrdU is a thymidine analogue. Wild type virus harbouring an intact thymidine kinase gene will be able to phosphorylate BrdU and incorporate it into their DNA with fatal consequences. In contrast tk^- recombinant virus will not be able to phosphorylate the BrdU and it will not be incorporated into the DNA (296).

In addition to the features in common with pGS20, pSC11 contains the β-galactosidase gene (lacZ) under the control of the vaccinia virus late promoter P11. β-galactosidase is able to convert the colourless compound 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) to its indoxyl derivative which in turn is oxidised to the blue dye 5,5'-dibromo-4,4'cloroindigo (296). This means that cells containing recombinant virus and thus β-galactosidase can be identified by their blue colour.

Vaccinia virus contains and encodes its own RNA polymerase which directs transcription from vaccinia virus promoters but not eukaryotic gene promoters (294). Transcribed mRNAs are not spliced. This means that expression of eukaryotic genes is only achieved if the cDNA is inserted under control of vaccinia promoter. In pSC11, the vaccinia early/late promoter of the 7.5 gene (297) is used to direct expression of foreign cDNAs. The cDNA is cloned in an unique SmaI site immediately downstream of the promoter (292; 295). The vector does not have an ATG codon positioned downstream of the promoter so foreign sequences need to contain their own ATG initiation codon. Expression can be detected within one hour of infection and continues throughout the whole infective cycle of the virus (295; 298). The level of protein expressed using the P7.5 promoter has been reported to be as much as 1-2 mg protein per litre cell culture (283)

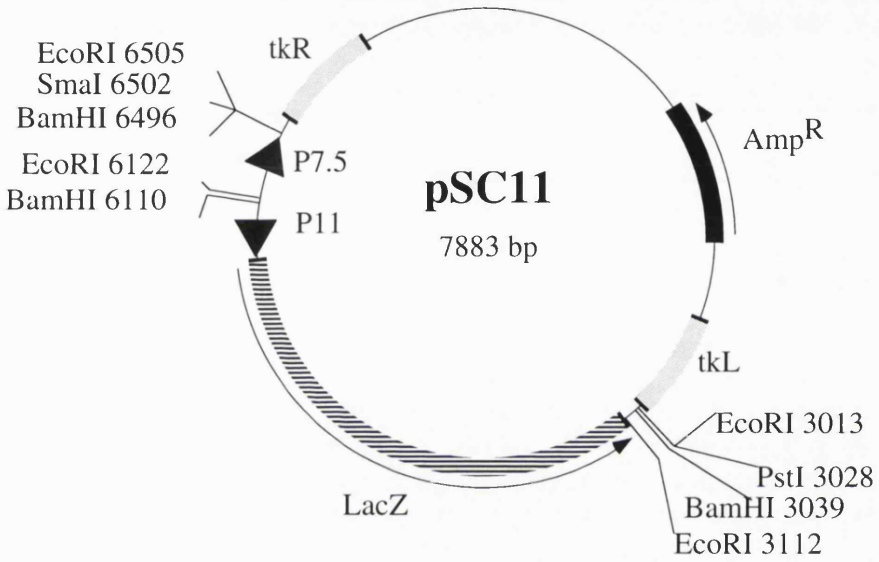


Figure 1.5.2.
Schematic representation of the vaccinia virus expression vector pSC11.
The foreign cDNA is inserted in the SmaI site under control of the vaccinia early/late promoter P7.5.
Recombinant virus is generated by homologous recombination with wild type vaccinia virus via the flanking thymidine kinase sequences, tkR and tkL.
Expression of β-galactosidase under the late promoter P11 allows identification of recombinant virus.

1.5.4. Biology of human adenovirus type 5 (Ad5)

Adenoviruses are intermediate-size lytic DNA viruses. The virus is an icosahedron of 70 to 90 nm in diameter with 252 capsomeres, 12 of these at the vertices carrying filamentous projections. The virion consists exclusively of protein and DNA (299; 300).

Adenoviruses have been isolated from a large number of mammalian and fowl species and to date more than 100 different serotypes have been isolated. The human serotypes have been grouped into six subgenera (A-F) whose members have close sequence similarity and share a variety of other properties. The most well characterised are Adenovirus type 2 and 5 (subgenus C), type 7 (Subgenus B) and 12 (Subgenus A) (299). The present work will deal with Adenovirus type 5 (Ad5).

Human Ad5 is not generally associated with serious illness. In most cases, infection is limited to the upper respiratory tract with or without fever, but some variants might be more pathogenic than others. Retrospective antibody sampling studies of antibody titres to Ad5 show that, depending on the geographic region, from 50% to almost 100% of the population over the age of 5 years have been infected with Ad5.

Adenoviruses including Ad5 can cause latent infections and are isolated with high frequency from tonsils of asymptomatic patients. The genome consists of 36,000 bp of linear double stranded DNA. The virus can transform several different cell types, resulting in integration of the viral DNA into the host chromosome (301).
Several human adenovirus serotypes including Ad5 can cause tumours in animals such as hamsters but there is no evidence that the virus plays any role in human cancer except in certain rare instances (299; 301). The region of the adenovirus DNA which are important in oncogenic transformation of cells in culture and in tumour induction *in vivo* are contained within the left approximately 12% of the viral genome. This region corresponds to the so-called early region 1 (E1). This is one of four regions which are expressed prior to DNA replication (301). Although human cells transformed by human adenoviruses exist, they have been generated with great difficulty using non-infectious viral DNA fragments. This was the case with the 293 cell line (see below). No human cell transformed by infectious adenovirus has ever been established as a permanent cell line (299). In Britain, work with adenoviruses has to be carried out in Containment level II laboratories.

The human adenovirus has a very restricted host range compared to vaccinia virus. Human epithelial cells are most efficiently infected although the viruses grow reasonably well in some human fibroblast cell lines (299). The virus is also able to infect a variety of different human brain cells including neurons, oligodendrocytes and myelinated axons as

well as muscle cells (302). Only a small fraction of human lymphocytes are infected by and capable of replicating human adenoviruses. In general, infection of cultured cells of other species results in much less or no virus production when compared with human epithelial cells. Mouse cells in culture are semipermissive for Ad5 replication (299). The virus has also been reported to infect and express recombinant proteins in mouse spleen cells and liver cells (299; 303).

Adenovirus is attractive as mammalian expression vector because the genome is relatively stable and inserts of foreign genes are generally maintained without change through successive rounds of replication (299). Adenovirus 5 is being used in vaccine research, but the virus is currently also being intensively investigated for use in human gene therapy (302).

The virus cycle takes 32 to 36 hours and differs from the cycle of the vaccinia virus in several ways. In particular, many parts of the adenovirus life cycle are carried out in the nucleus where the vaccinia virus life cycle is entirely cytoplasmic. Adenovirus replication starts at 6 to 9 hours post infection and takes place in the nucleus. During replication, host DNA synthesis is reduced by up to 90%. Virus assembly also takes place in the nucleus after single polypeptides have been assembled into capsomeres in the cytoplasm (304).

The virus replication cycle contains 2 major phases. The early phase precedes DNA replication. During this phase, four non-contiguous regions are expressed namely early region E1 consisting of E1A and E1B and early regions E2, E3 and E4. E1A activates transcription of the other early regions. DNA replication requires both virally encoded and cellular functions. After the onset of DNA replication, the major late promoter (MLP) drives much of the viral transcription. The late transcripts are processed into a complex array of different mRNAs that encode most of the structural virion proteins. Production of late structural proteins is extremely efficient because of selective shut-off of host-cell protein synthesis (299).

Infected cells produce 1000 to 10,000 plaque forming units of virus and virions remain concentrated within the cell long after yields have reached maximum levels. This clearly facilitates collection of virus and virus produced protein (299).

Adenovirus type 5 vectors with inserts resulting in viral DNA close to or less than a net genome size of 105% of the wild type genome grow well and are relatively stable, but if the size of the DNA exceeds this, the resulting vector will grow poorly and undergo rapid rearrangement (305). This means that there is a relatively tight constraint on the amount of DNA which can be packaged into virions compared to vaccinia virus.

About 2 kb of DNA in addition to the wild type adenovirus genome can be packaged in virions. Part of the adenovirus genome can be deleted to accommodate more DNA provided that the modifications are not essential for viral replication and packaging. Foreign DNA can be inserted into essential regions such as E1A provided that the defect is compensated for by suitable host cells such as 293 cells. These cells are human embryonic kidney cells which contain the extreme left of the adenovirus genome including the E1A gene (306), see also section 2.2.1. Adenovirus vectors with insertions in the E1A gene can consequently be used to generate infectious viruses in the 293 cell line. Deletions of up to 3.2 kb can be made in E1 without compromising the ability of the virus to grow in 293 cells.

Alternatively, substitutions can be made in the E3 region or in a region just before the start of E4 transcription (299).

1.5.5. The immune response to recombinant adenovirus

Recombinant adenovirus are being increasingly used to induce humoral and cellular immune responses. Despite the inefficient replication of human adenoviruses in cultured cells of most animal species, Ad5 recombinants produced either by insertion in the E1A gene or the E3 gene have successfully been used to induce antibodies to a variety of proteins. As an example, the pMV60/pJM17 system, which uses insertion in the E1A gene and will be described below, has recently been used to generate protective humoral immunity to tick-borne encephalitis virus in mice (307). In addition, Ad5 recombinants carrying inserts of herpes simplex virus, vesicular stomatitis virus or rabies glycoproteins in the E3 region have been shown to stimulate neutralising antibodies in rhesus monkeys, cows, dogs, foxes, striped skunks, racoons and mice (299).

The CTL response to Ad5 itself is well characterised in several strains of mice. In H-2^b mice, the CTL response is directed exclusively against the products of the E1A region (308). Adenovirus has not been used to generate CTL responses to recombinant proteins as much as vaccinia virus has, but there are several reports in the literature. As an example, CTL responses have been generated in H-2^k mice to human cytomegalovirus glycoprotein B expressed under the E3 promoter. Immunisation with recombinant adenovirus was found to be superior to immunisation with a recombinant vaccinia virus expressing the protein under the vaccinia virus H6 early/late promoter (303).

The biggest problem encountered when using adenovirus to induce or stimulate CTL responses is the ability of the E3/19 K protein to interfere with the transcription of class I molecules or the export of class I molecules from the endoplasmic reticulum. The ability to do so depends on the individual strain of virus. Ad5 E3/19 K does not interfere with transcription of class I molecules but it does bind various class I molecules (309). However, the affinity of E3/gp19K for mouse K^b and D^b molecules is reportedly low. To illustrate this point, expression of Ad5 E3/19K by vaccinia virus has been found not to alter CTL responses (310). The recombinant adenovirus used in the present work contains a deletion in the E3 gene, see section 1.5.6.

1.5.6. Vectors used to generate recombinant adenovirus.

The pMV60/pJM17 cloning system used to generate recombinant adenovirus involves the use of three different vectors, pMV100, pMV60 and pJM17.

The system is based on homologous recombination between a plasmid (pJM17) which contains most of the Ad5 genome and a plasmid which contains the foreign DNA flanked by a fraction of the Ad5 E1 gene in a pUC based vector (pMV60). Neither plasmid can give rise to infectious virus on its own. The foreign cDNA is cloned into pMV60 after first having been cloned behind the cytomegalovirus immediate early promoter (CMV-IE promoter) in the intermediate vector pMV100. The expression cassette containing the CMV-IE promoter plus foreign cDNA is cut out from pMV100 with HindIIII and cloned into the pMV60 vector, Figure 1.5.3. (311). Vector pMV100 is used as an intermediate because it is convenient to have a smaller vector for the intermediate manipulation steps.

pMV60 is derived from the vector pXCX2 (312). pXCX2 contains the left 16% of the adenovirus genome, minus a 2.7 kb deletion in the Ad5 E1 region, cloned into BamHI/SalI of pBR322. The vector still contains bp 20-458 and bp 3318-5578 of the E1 region. Regions which are necessary for cis viability including part of the left inverted terminal repeat (ITR) (1-103 bp) and packaging signals (194-300 bp) or for the structure of the virus as (the protein IX gene, bp 3500 to the end of the E1 gene) (313) have been left intact. pMV60 is identical to pXCX2 except that the XbaI cloning site has been substituted with a HindIII cloning site (311).

The big plasmid, pJM17, contains the entire Ad5dl309 genome (314; 315) plus a 4.3 kb insertion of the pBRx plasmid in the E1A gene (316). The pBRx fragment contains the ampicillin and the tetracyclin resistance genes. The Ad5dl309 is a mutant of Ad5 with a deletion in the E3 region. On its own, pJM17 is 40.3 kb in size and thus approximately 2 kb to big to be packaged into infectious virions. However, the E1A sequences can be used to mediate homologous recombination with the smaller pMV60 plasmid.

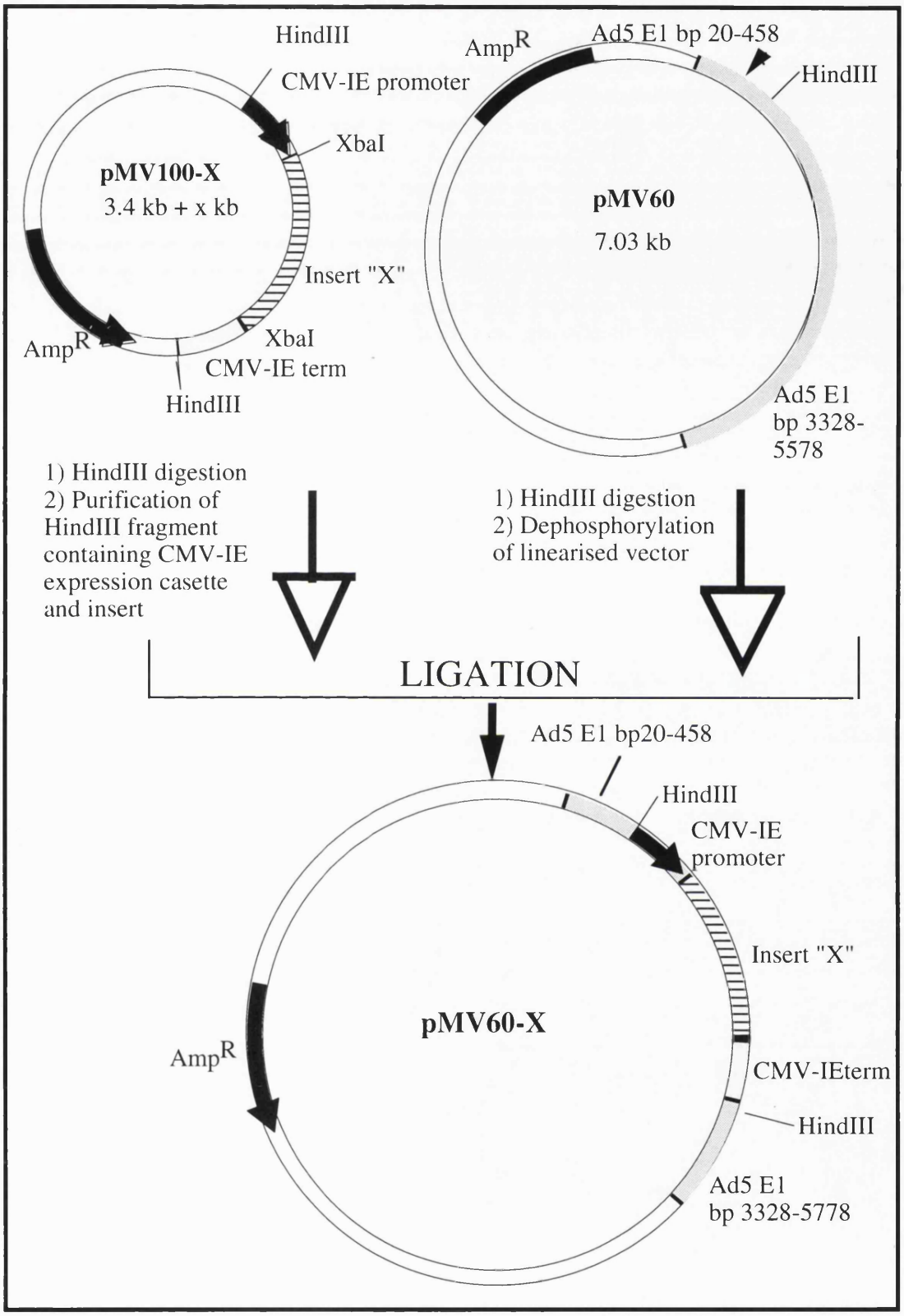


Figure 1.5.3.
Schematic representation of the cloning strategy used to generate the recombinant adenovirus plasmid pMV60 -X.
Recombinant live adenovirus is generated by cotransfecting this vector together with vector pJM17 into 293 cells, see text for details.

During homologous recombination between pJM17 and pMV60, the E1A region with the pBRx plasmid will be excised from pJM17 and replaced with the E1A region from pMV60 containing the CMV-IE expression cassette. The resulting plasmid will therefore contain major deletions in E1 and be smaller than pJM17 provided that the cDNA cloned into pMV60 is not bigger than 5 kb. The plasmid will be able to give rise to infectious virus, as long as the recombination takes place in a cell line providing the amputated E1A function such as the 293 cell line. The pMV60 vector cannot give rise to any virus on its own due to the lack of the necessary adenovirus genes.

The CMV-IE promoter is derived from the immediate early promoter from human cytomegalovirus (HCMV) strain AD169 and is strong due to an efficient enhancer element (317). The promoter can be used for both transient and constitutive expression and is functional in a wide range of different cell types. A construct containing a truncated form of the promoter from bp -299 to + 69 and the CMV-IE polyA signal (also derived from HCMV strain AD169) has been shown to give high levels of protein expression up to 144 hours after infection in a fibroblast cell line. In other cell lines maximum levels of protein expression are reached after 48 hours (311). A map of the CMV-IE expression cassette is shown in Figure 1.5.4.

There are several advantages of an adenovirus system based on insertion into E1A and with the foreign gene driven by the CMV-IE promoter. Infection of cells lacking the E1 helper function results in constitutive expression from the CMV-IE promoter with simultaneous activation of the adenovirus immediate early-phase gene expression. Secondly, inserted genes are generally maintained without change through successive rounds of replication (313).

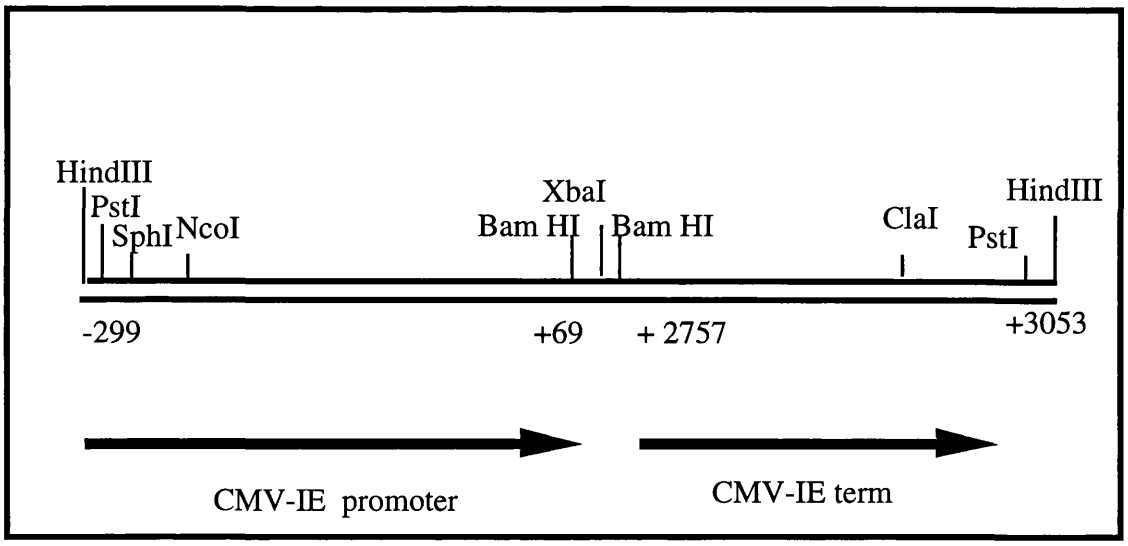


Figure 1.5.4.
The eukaryotic CMV-IE expression cassette present in vector pMV100.
The expression cassette contains the CMV-IE promoter (-299 to +69) upstream of a XbaI cloning site and a poly A signal also derived from the major CMV-IE gene (+2757 to + 3053). The whole fragment between the two HindIII sites is 735 bp.

2. Materials and methods

2.1. Molecular biology and protein chemistry techniques

2.1.1. Growth and handling of bacteria

Bacterial strains

E. coli XL1-Blue (Stratagene) was used for transformation and amplification of recombinant plasmids containing the ampicillin resistance gene. The genotype is recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, [F'proAB, $lacI^q$ ΔM15,Tn10 (tet^r)].

Bacterial medium and antibiotics

LB medium (Luria-Bertani medium) and SOB medium were supplied by ICRF Central Media Department, Clare Hall.

LB-medium contains 1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl. LB agar plates were made from LB medium solidified with 1.5% (w/v) agar.

SOB medium contains 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.06% (w/v) NaCl, 0.02% (w/v) KCl, 0.2% (w/v) $MgCl_2$ and 0.25% (w/v) $MgSO_4$.

Tetracycline was made up in ethanol as a 5 mg/ml stock solution and used at a concentration of 12.5 μg/ml.

Ampicillin was made up in H_2O as a 50 mg/ml stock solution and used at concentration of 50 μg/ml.

Cryopreservation of bacterial stocks

Bacterial stocks were stored in 10% (v/v) sterile glycerol in 1.5 ml Eppendorf tubes at -70°C. To resuscitate the bacteria, some frozen cells were streaked out an LB agar plate with appropriate antibiotics and grown overnight at 37°C.

Preparation of a small bacterial stocks (5 ml culture)

A single colony from a LB agar plate with appropriate antibiotics was used to inoculate 5 ml of selective LB medium. The culture was grown to saturation (12-16 hours).

Large scale bacterial stock (200-400 ml culture)

A freshly prepared small scale bacterial stock was diluted 1:100 into a larger volume of selective LB medium and regrown to saturation (16-18 hours).

2.1.2. General procedures for handling of DNA

Phenol equilibration

Phenol was equilibrated to pH above 7.5 as described by (318). One volume of liquid phenol with added 0.1% (w/v) hydroxyquinoline was mixed with one volume 0.5 M Tris-HCl, pH 8.0. The mixture was stirred for 15 minutes protected from light. The phases were allowed to separate and the top aqueous phase was removed. 1 volume of 0.1 M Tris-HCl, pH 8.0. was added to the bottom phenol layer. The mixture was again stirred for 15 minutes protected from light. The phases were allowed to separate and the top aqueous phase was removed. pH was checked and 1/10 volume 0.1 M Tris-HCl, pH 8.0 containing 0.2% (v/v) β-mercaptoethanol was added to the phenol for storage. The equilibrated phenol was stored for up to one month at 4°C in a glass container protected from light.

Phenol/chloroform extraction

Proteins were removed from plasmid and DNA fragment preparations by phenol chloroform extraction unless something else is indicated. Immediately prior to use, 1 volume equilibrated phenol was mixed with 1 volume chloroform. 1 volume of the phenol/chloroform mixture was added to 1 volume DNA solution. The emulsion was mixed on a whirli mixer for 60 seconds and centrifuged for 2 minutes in a microcentrifuge at 12000 rpm (6000 x g). The aqueous top layer was transferred to a fresh tube and mixed with 1 volume chloroform using a whirli mixer for 60 seconds. After centrifugation, the top aqueous DNA containing layer was transferred to a new tube.

Agarose gel electrophoresis of DNA

DNA fragments were separated by electrophoresis in agarose gels in 45 mM Tris-borate, 1 mM EDTA buffer (0.5 x TBE buffer). The agarose content of the gel depended on the size of the DNA fragments to be separated in the gel. 0.7-0.8% gels or 1% gels were used for most purposes and would separate DNA fragments between 0.8 to 10 kb (318). 1.5% gels are efficient for separating fragments between 0.2-3 kb (318), so these gels were always used to separate PCR products.

A 10 x TBE buffer stock solution was made from 108 g/l Tris base, 55 g/l boric acid and 40 ml/l 0.5 M EDTA, pH 8.0 (318). Gels were made by melting agarose in 0.5 x TBE buffer in a microwave oven, cooling the solution to 60°C and adding ethidium bromide (EtBr) to give a final concentration of 0.5 µg/ml. After having cast the gel, one volume DNA sample was mixed with 1/5 volume DNA loading buffer and loaded on the gel. The 6 x concentrated DNA loading buffer contained 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose in water. Electrophoresis was performed in 0.5 x TBE under constant voltage. Under these conditions, the bromophenol blue migrates at approximately the same rate as linear double-stranded DNA 300 bp in length. After electrophoresis, the binding of EtBr to DNA allowed visualisation of the DNA fragments under UV light. EtBr binds to DNA by intercalating between the bases and causing the double helix to unwind.

Precipitation of DNA

DNA was precipitated with 1/10 volume 3 M NaAc, pH 5.3 and 2 volumes of ethanol. Samples estimated to contain at least 10 µg/ml DNA were precipitated for 10 minutes at -70°C whereas samples with lower DNA concentrations were left overnight at -20°C. The latter procedure will precipitate DNA at concentrations as low as 10 ng/ml. Following precipitation, the DNA was peleted by centrifugation for 15 minutes at 4°C at 12,000 x g (15,000 rpm in a Sigma 2K15 centrifuge), washed once in 70% (v/v) ethanol, dried under vacuum and resuspended in H_2O or TE, pH 8.0

Small scale plasmid preparations

Small scale plasmid preparations were obtained by the alkaline lysis method essentially as described by (318).

1.5 ml of bacterial culture from a fresh small scale bacterial stock was transferred to a 1.5 ml Eppendorf tube. The cells were pelleted in a microcentrifuge, washed once in 1 ml of STE buffer (100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)) and resuspended in 100 µl 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0). The cells were lysed by adding 200 µl of freshly made 0.2 M NaOH, 1% (w/v) SDS. Proteins, cellular debris and chromosomal DNA were precipitated by adding 150 µl

ice-cold 3.0 M KAc, pH 5.3, incubating for 5 minutes on ice and centrifuging at 12,000 x g for 10 minutes. Remaining proteins were removed by one phenol/chloroform extraction. The DNA was precipitated with 1/10 volume 3 M NaAc, pH 5.3 and 2 volumes of EtOH. After precipitation, the DNA was resuspended in 50 µl TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)) containing 20 µg/ml RNAse. The yield from high copy number plasmids such as the pUC based pSC11 or pMV100 (see section 1.5.) was about 3-5 µg DNA per millilitre original culture. For lower copy number plasmids such as the pBR322 based pMV60 (see section 1.5.), the yield was roughly 1-2 µg DNA per millilitre original culture.

Large scale plasmid preparations

Large scale plasmid preparations were obtained with the Qiagen Plasmid Maxi Kit (Qiagen GmbH). The method is based on the use of Qiagen-tip 500 columns to selectively bind DNA. The column consists of a resin with a hydrophilic surface containing diethylaminoethyl (DEAE) groups. The resin functions as an anion-exchanger that selectively separates nucleic acids from other substances, such as proteins and carbohydrates. Six different solutions are used in the Qiagen kit. Solution P1 is 50 mM Tris-HCl (pH 8.0), 10 mM EDTA with added RNAse to give a final concentration of 100 µg/ml. Solution P2 is 200 mM NaOH, 1% (w/v) SDS and solution P3 is 3.0 M KAc, pH 5.5. Buffer QBT is 750 mM NaCl, 50 mM MOPS (pH 7.0), 15% (v/v) EtOH, 0.15% (v/v) Triton X-100. Buffer QC is 1.0 M NaCl, 50 mM MOPS (pH 7.0), 15% (v/v) EtOH and buffer QBF is 1.25 M NaCl, 50 mM Tris-HCl (pH 8.5) and 15% (v/v) EtOH.

First, a large scale bacterial stock was made. 100 ml of culture was used to prepare high copy number plasmids such as pSC11 and pMV100 and derivatives of these vectors whereas 500 ml of culture was used to prepare low copy number plasmids such as pMV60 and derivatives of pMV60. The culture was harvested and the bacteria was centrifuged 15 minutes at 2500 x g (3750 rpm in a centrifuge with a rotor radius of 16 cm i.e. Beckman GPR centrifuge). The bacterial pellet was resuspended in 10 ml of buffer P1. 10 ml of buffer P2 was added, the sample was mixed and incubated at room temperature for 5 minutes exactly. 10 ml of chilled buffer P3 was added, the sample was mixed and incubated on ice for 20 minutes. The samples were centrifuged at 4°C for 45 minutes at 2,500 x g (3750 rpm). The supernatant was removed and immediately applied to a Quiagen-tip 500 column which had been equilibrated with 10 ml of buffer QBT. The column was washed 2 times with 30 ml of buffer QC, and the DNA was subsequently eluted with 15 ml of buffer QF. The DNA was precipitated with 0.7 volumes of isopropanol (room temperature) and centrifuged for 30 minutes at 4°C at 15,000 x g (11,000 rpm in a Beckman J2-21 with a JA-20 rotor, radius 10.7 cm). The DNA was washed with 15 ml of ice-cold 70% (v/v) ethanol, air dried for 5 minutes and redissolved

in 1 ml of TE buffer. The DNA concentration and quality were evaluated by agarose gel electrophoresis and by measuring A_{260} and A_{280}. An A_{260}/A_{280} ratio of 1.75 indicating a high DNA/protein ratio was found to be acceptable on the basis of the recommendations of (318). Under these circumstances, the DNA concentration was calculated on the basis of the assumption that an A_{260} value of 1 corresponds to 50 µg/ml DNA (318).

Restriction digests

Most restriction digests were done in One-Phor-All Buffer Plus (Pharmacia). The 10 x stock solution contains 100 mM Tris-acetate (pH 7.5), 100 mM magnesium acetate and 500 mM potassium acetate. The stock solution was diluted according to instructions given by the manufacturer.

Restrictions digests using NotI were done in 1 x Gibco BRL Basic REACT buffer 3 (50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 100 mM NaCl) and Eco72I and SphI digests were done in 1 x Promega buffer K (10 mM Tris-HCl (pH 7.1), 10 mM $MgCl_2$, 150 mM KCl). SphI digests were done in Pharmacia One-Phor-All Buffer Plus, if the enzyme was used in conjunction with other restriction enzymes which did not work well in Promega buffer K.

Purification of small DNA fragments

DNA fragments used as inserts in ligation reactions or as probes in blots were resin purified with the Geneclean method (Stratech Scientific). This method is based on the binding of DNA to an insoluble silica matrix "Glassmilk". The DNA fragments were separated by agarose gel electrophoresis and the relevant band was excised under UV light. The agarose fragment(s) containing the DNA was weighed and the volume of the slice was determined on the assumption that 1 g equals 1 ml of gel. 3 volumes NaI solution was added per volume gel. The tube was kept for 5 minutes at 55°C in a waterbath to dissolve the agarose. Glassmilk was added to the tube. Approximately 5 µl Glassmilk was used per 5 µg DNA to be bound. The suspension was incubated for 5 minutes on ice. The DNA/Glassmilk complexes were pelleted by brief centrifugation and the pellet was washed three times with the "New Wash" washing solution. The washing solution contains so-called New Formula (supplied with the kit by Stratech Scientific), H_2O and EtOH in a ratio (vol: vol: vol) of 14: 280: 310. Great care was taken after the last wash to make sure that as much of the washing solution had been removed as possible. The DNA was finally eluted using the same volume of TE as the volume of Glassmilk used to bind the DNA. The samples were incubated at 55°C for 3 minutes and centrifuged. The supernatant containing the DNA was kept. The DNA content of the supernatant was checked by agarose gel electrophoresis.

Filling in of recessed 3' termini on inserts

The Klenow fragment of DNA polymerase I was used to fill in recessed 3' termini to create blunt ends. The Klenow fragment retains the complete polymerase activity and 3' to 5' exonuclease activity of DNA polymerase I, but lacks the 5' to 3' exonuclease activity (296). Up to 5 µg digested DNA was reacted in 50 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 100 µM DTT and 50 µg/ml BSA (fraction V) using 80 µM of each dNTP and 5 units Klenow fragment in a total volume of 25 µl for 30 minutes at room temperature (318). The reaction was stopped with the addition of 1 µl 0.5 M EDTA, pH 8.0. The fragments were phenol/chloroform extracted and precipitated. After resuspension in a small volume of TE, pH 8.0 (usually 10 µl), the inserts were ready for ligation.

Dephosphorylation of vectors

5' terminal phosphate residues were removed on vectors prior to ligation by treatment with calf intestinal alkaline phosphatase (Pharmacia) to prevent vectors from religating. Only foreign DNA inserts providing phosphate groups will be ligated efficiently to the dephosphorylated vector DNA to give an open circular DNA containing two nicks. This circular DNA will transform more efficiently than the linear vector and the two remaining open phosphodiester bonds are closed *in vivo* after transformation into the bacterial cell (296; 318). Between 1 and 5 µg vector were dephosphorylated in 1 x One-Phor-All buffer (Pharmacia) in a volume of 50 µl with 0.1 unit calf intestinal phosphatase (Pharmacia) for 30 minutes at 37°C. Following dephosphorylation, the alkaline phosphatase was inactivated by heating the sample for 15 minutes at 85°C. The dephosphorylated vector was used directly with no change of buffer in subsequent ligation reactions.

Ligation of vector and insert

Inserts were ligated to dephosphorylated vectors with T4 ligase for 16 hours at 16°C in 1 x One-Phor-All buffer (Pharmacia) and 0.5 mM ATP in a total volume of 10 µl. For cohesive end ligations, the molar ratio of vector to insert was approximately 0.5, and 0.5 -5 units T4 ligase was used. For blunt end ligations the molar ratio of vector to insert was approximately 3, and 1 µl of 8 units/ml T4 ligase was used. For blunt end ligations, ligation for 4 hours at room temperature worked as well as 16 hours at 16°C overnight.

Following ligation, 4 µl of the ligation mixture were used to transform 100 µl of competent XL1 Blue *E. coli* cells.

Transformation of *E. coli*

Competent cells:

The composition of the solutions used to make competent cells is given in Table 2.1.1. A single colony *E. coli* XL1 BLue was used to inoculate 7 ml of SOB medium overnight. The following morning 4 ml of the primary culture were transferred to 400 ml SOB and grown for between 2 to 4 hours to A_{550} 0.45-0.55. The cell suspension was aliquoted into 8 ice-cold 50 ml Falcon Tubes and kept on ice for 3 minutes. The cells were pelleted by centrifugation at 4°C for 5 minutes at 1130 x g (2500 rpm, 16 cm rotor radius). The cells were resuspended in 5 ml ice-cold solution I after which additional 15 ml solution I were added. The cells were kept on ice for 30 minutes and centrifuged for 5 minutes at 4°C at 1130 x g (2500 rpm). The cells were resuspended in 4 ml ice-cold solution II and aliquoted into pre-cooled Eppendorf tubes. The cells were kept at 4°C overnight prior to freezing them at -70°C.

Table 2.1.1.

Solutions used for making competent *E. coli* cells
Solution I
30 mM KAc
50 mM $MnCl_2$
100 mM KCl
10 mM $CaCl_2$
15% (w/v) Glycerol
pH was adjusted to 5.8 with 0.2 M Acetic Acid
Solution II
10 mM MOPS
75 mM $CaCl_2$
10 mM KCl
15% (w/v) Glycerol

For transformation, 100 µl competent cells were thawed on ice. Between 4 and 40 ng DNA in a volume of no more than 4 µl were added to the cells. The cells were incubated with the DNA for 40 minutes on ice, heat shocked for 90 seconds at 42°C and then again transferred to ice for one minute. 400 µl LB medium were added to the cells, followed by a one hour incubation at 37°C. Finally, 100 µl cells were plated out on selective LB agar plates and incubated at 37°C overnight.

2.1.3. Transfection of mammalian cell lines.

Transfection by $CaPO_4$ precipitation

Recombinant DNA was introduced into adherent cells by a modification of the $CaPO_4$ precipitation method (319). The buffers used during the procedure are listed in Table 2.1.2.

One day in advance of the transfection, adherent cells were seeded in T75 flasks in order to reach 70% confluency the following day.

On the day of transfection 10-20 µg DNA was mixed with 200 µl transfection buffer 2 in a 20 ml universal tube. Water was added to give a final volume of 500 µl. 500 µl of transfection buffer 1 was added. After 30 minutes at room temperature, the medium was removed from the T75 flask containing the cells to be transfected. The DNA mixture was added to the cells. Following a 25 minutes incubation at 37°C, 5 ml of E4, 5% FCS were added. The cells were incubated for 4 hours at 37°C. 1 ml of a mixture of 56% (v/v) DMSO and 44% (v/v) RPMI, 5% FCS was added and left on the cell layer for 25 minutes at 37°C. The medium and DMSO were removed and replaced with 15 ml of E4, 5% FCS. The cells were placed in the incubator. The following morning, the medium was replaced with fresh E4, 5% FCS.

Table 2.1.2.

Buffers used for transfection of DNA via calcium precipitation
Transfection buffer 1
274 mM NaCl
10 mM KCl
1.4 mM Na_2HPO_4
12 mM glucose
42 mM HEPES, pH 7.1
Transfection buffer 2 = 5 x $CaCl_2$
625 mM $CaCl_2$
pH of both buffers were checked and adjusted to 7.1 if necessary

Transfection by electroporation

Mammalian cells were transfected with linearised DNA by electroporation using a Biorad Gene Pulser linked to a Biorad Capacitance Extender. 5 x 10^6 cells were mixed with between 10 to 20 µg recombinant DNA and 20 µg sonicated and boiled salmon sperm carrier DNA in a volume of 1 ml IMDM without any FCS. The sample was transferred to a cuvette and kept on ice for 5 minutes to avoid cell damage during local heating during the following pulsing. The cells were electroporated using the conditions listed in Table 2.1.3. After electroporation, the cells were left on ice for 5 minutes to prolong the period where the pores would be open. The electroporated cells were finally plated out in a 24 well plate at a concentration of 2 x 10^5 cells per well on top of a layer of 1 x 10^6 syngeneic irradiated (3000 rads) splenocytes per well. In the present work, all transfected plasmids contained the neo gene, so transfected cells could be selected on Geniticin (G418). G418 is an aminoglycoside antibiotic which inhibits protein synthesis. Resistance to G418 is conferred by the neo gene which encodes an aminoglycoside phosphotransferase which phosphorylates G418, thereby rendering it inactive. The frequency of spontaneous resistance is lower than 10^{-7} (296). Selection was initiated at 48-96 hours after electroporation, depending of the apparent health of the cells. The concentration of G418 used for selection would depend on the transfected cell line, see Table 2.1.3. The medium would be changed every 2 to 4 days for 2 to 3 weeks to get rid of debris of dead cells and to allow colonies of resistant cells to grow out.

Table 2.1.3.

Conditions used for transfection of mammalian cells by electroporation			
Cell line	**Voltage (V)**	**Capacitance (µF)**	**Active concentration of G418 used for selection (µg/ml)**
EL4	450	125	450
RMA	200	960	600
P1HTR	800	25	350
A20	450	250	450

2.1.4. Isolation and analysis of genomic DNA from mammalian cells

Isolation of genomic DNA

Genomic DNA was isolated by lysing cells with SDS followed by proteinase K digestion and several rounds of phenol and chloroform extractions. Briefly, 10^7 mammalian cells were harvested, centrifuged for 5 minutes at 300 x g, washed once in PBSA and resuspended in 100 µl PBSA. The cells were lysed by adding 1 ml of 50 mM Tris (pH 7.5), 100 mM EDTA, 0.5% (w/v) SDS. Proteins were digested by adding 80 µl of a 5 mg/ml stock solution of proteinase K to give a final concentration of 300 µg/ml enzyme. Proteinase K is a serine protease with broad specificity and suitable for non-specific elimination of cellular proteins. The samples were incubated overnight at 55°C. Enzyme as well as digested proteins and lipid components were removed the following day by first extracting twice with phenol for at least 2 hours on a rotor and then extracting twice with chloroform for 10-20 minutes on a rotor. The DNA was precipitated with 0.1 volume 3 M NaAc, pH 5.3 and 2 volumes ice-cold 100% ethanol and centrifuged immediately for 15 minutes at 12,000 x g at 4°C. If no firm precipitate was formed after the centrifugation, the sample was kept for one hour at -70°C before centrifugation. The precipitate was washed in 80% ethanol and resuspended in 50 µl TE, pH 8.0 containing 10 µg/ml RNAse.

The quality of the DNA was checked by agarose gel electrophoresis. An estimate of remaining protein which could interfere with subsequent treatment and analysis of the DNA was obtained by measuring the optical absorbance of the sample at 260 nm and 280 nm (A_{260} and A_{280}). An A_{260} to A_{280} ratio greater than 1.75 was considered satisfactory. Under these conditions, the concentration of the DNA would be calculated from the assumption that an A_{260} value of 1 corresponds to 50 µg/ml double stranded DNA (318).

Analysis of genomic DNA with Southern blots

Between 10 and 15 µg genomic DNA was digested in a volume of 60 µl. The fragments were separated by electrophoresis in a 0.7% agarose gel overnight at 0.7 V/cm. Following electrophoresis, the gel was soaked for 1 hour in 0.5 M NaOH, 1.5 M NaCl to denature the DNA. The gel was rinsed briefly in distilled H_2O and neutralised by soaking it two times for 30 minutes each time in 0.5 M Tris (pH 7.4), 1.5 M NaCl. The DNA was transferred to a nylon membrane (Hybond N) by capillary transfer under neutral conditions in 20 x SSC (318).

Hybridisation of radiolabelled probe to the immobilised DNA

All incubations including pre-hybridisation and washes of the filter were carried out in hybridisation bottles at 68°C using a Hybaid Maxi hybridisation oven.

The filter was pre-washed in 0.1 x SSC, 0.5% (w/v) SDS for 1 hour and pre-hybridised for 1 hour in a solution made up from 1 volume 7% (w/v) SDS, 1% (w/v) BSA (fraction V), 1 mM EDTA and 1 volume 1 M Na_2HPO_4 pH 7-7.5 ("hybridisation solution"). The hybridisation was carried out by removing the pre-hybridisation solution and adding 15 ml fresh hybridisation solution and labelled probe.

The DNA probe was labelled in advance by resuspending approximately 100 ng DNA in 29.5 µl H_2O. The DNA was made single stranded by boiling it for 5 minutes and transferred to ice. 10 µl OLB buffer (see Table 2.1.4.), 2 µl BSA (fraction V) from a 10 µg/ml stock solution and 5 µl ^{32}P-dCTP (>3000 Ci/mmole,10 mCi/ml, Amersham) and 2 units *E. coli* DNA polymerase I (Klenow fragment) were added sequentially. The labelling reaction was allowed to proceed for 2.5 hours at 37°C and stopped by adding 1 µl 0.5 M EDTA, pH 8.0. Un-incorporated nucleotides were removed by gel filtration with TE buffer, pH 8.0 on a Sephadex G50 column (Pharmacia). The probe was eluted in 500 µl TE, pH 8.0. 1 µl of the probe was diluted in 10 ml scintillation liquid and counted on Beckmann LS1800 Scintillation counter. The labelled probe was kept on ice and boiled for 5 minutes just prior to addition to the hybridisation solution. 10^6 cpm of probe was added per ml hybridisation solution.

The DNA probes used in the present work are listed in Table 2.1.5.

Table 2.1.4.

Solutions used for labelling of DNA probes for DNA-DNA hybridisation on nylon membranes		
Solution O 1.45 M Tris-HCl, pH 8.0 0.145 M $MgCl_2$	**Solution A** 865 µl Solution O 18 µl β-mercaptoethanol 50 µl 10 mM dATP 50 µl 10 mM dGTP 50 µl 10 mM dTTP	**Solution B** 2 M Hepes, pH 6.6
Solution C 90 Unit/ml Hexanucleotide (Pharmacia) in 3 mM Tris-HCl (pH 7.5), 0.2 mM EDTA (pH 8.0)	**OLB Buffer** 100 µl Solution A 250 µl Solution B 150 µl Solution C	

After overnight hybridisation, the filter was washed once in 5 x SSC, 1% (w/v) SDS for 30 minutes, once in 2 x SSC, 0.1% (w/v) SDS for 30 minutes and finally once in 0.2 x SSC, 0.1% (w/v) SDS for 30 minutes. The filter was exposed to a Fuji autoradiography film at -70°C for varying lengths of time.

Table 2.1.5.

DNA probes used in hybridisations		
Probe	**Obtained from**	**Length**
Murine cyclin D1 (CD1) cDNA	CD1 in pJ7-omega vector (V. Fantl, ICRF)	1.3 kb EcoRI fragment
Murine fibroblast growth factor receptor I (FRI) cDNA	FRI in pDO-R vector (N. Lemoine, ICRF)	2.2 kb EcoRI/SalI fragment
Murine mdm2 cDNA	mdm2 in Bluescript KS vector (A. Levine, Princeton)	1.7 kb EcoRI fragment
Murine wild type p53 (WT p53) cDNA	p53 in pT7-7 vector (L. Crawford, ICRF)	2.0 kb XbaI-HindIII fragment
Murine β-actin cDNA	β-actin in pUC8 vector (Ludwig Institute for Cancer Research, London)	1.8 kb PstI fragment

2.1.5. Isolation and analysis of mRNA from mammalian cells

Isolation and analysis of RNA was carried out taking special precautions recommended by (318) to avoid degradation of RNA by RNAse. H_20 was treated for 2 hours with diethylpyrocarbonate (DEPC) at 37°C to give a final concentration of 0.1% (v/v) DEPC and subsequently autoclaved. Most solutions were treated similarly, except solutions containing amines such as Tris buffers or solutions which could not be autoclaved. All glassware was baked for 7-8 hours at 180°C. Gel tanks and trays which could not be baked was cleaned with detergent, rinsed with water, dried with ethanol and treated with 3% (v/v) H_20_2. After 10 minutes at room temperature, the equipment was rinsed with DEPC treated H_20.

Isolation of total RNA from mammalian cells

RNA was purified from mammalian cells with a mixture of guanidiumthiocyanate salt, urea and phenol using the total RNA isolation reagent from Advanced Biotechnologies based on the method of (320).

$5x10^6$ cells were suspended in 1 ml of the guanidiumthiocyanate and phenol containing RNA isolation reagent. This procedure will lyse cells, denature proteins and release proteins bound to RNA and DNA. Subsequent physical separation of the RNA in an aqueous top phase from protein, lipid and DNA in the interface and the organic bottom phase was achieved by adding 200 µl chloroform to the sample followed by centrifugation at 12,000 x g for 15 minutes at 4°C. The RNA was precipitated by adding an equal volume of isopropanol to the sample, leaving it on ice for 15 minutes and centrifuging it at 12,000 x g for 15 minutes at 4°C. The precipitate was washed once in 80% (v/v) ethanol and resuspended in 30 µl of DEPC treated H_20. The purity of the final RNA preparation with respect to contaminating protein was estimated by measuring the ratio of A_{260} to A_{280}. A ratio of 1.8 or more was considered satisfactory. The concentration of RNA in the sample was estimated assuming that an A_{260} value of 1 corresponds to 40 µg/ml of single stranded RNA (318). A measure of the quality of the RNA was obtained by checking for the presence of abundant 18S and 28S Ribosomal RNA bands upon gel electrophoresis.

Reverse transcription of RNA

First strand cDNA was synthesised from mRNA with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) as follows: 5 µg RNA in 10 µl DEPC treated H_2O was heated to 94°C for 1 minute and put on ice. Reverse transcription was performed in 1 x Reverse Transcriptase buffer from Gibco BRL (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, 40 units RNAse inhibitor, 1 mM of each dNTP, 0.036 units random hexamer (Pharmacia) and 400 units Murine Moloney Leukemia virus Reverse Transcriptase. The reverse transcription reaction was allowed to proceed for 1 hour at 37°C. Samples were kept at 4°C until further use. 5 µl of the cDNA mixture was routinely used per PCR reaction.

Polymerase Chain Reaction (PCR)

The polymerase chain reaction was carried out with Thermus species thermostable DNA polymerase (Promega). The buffer used was PCR reaction buffer IV from Advanced Biotechnologies. The 10 x concentrated buffer contains 200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl, pH 9.0 (at 25°C), 0.1% (w/v) Tween and 15 mM $MgCl_2$.

The PCR reaction mixture consisted of 1 x Buffer IV, 125 µM of each dNTP, 0.5 µg (approximately 50 picomoles) of each oligonucleotide primer and finally target DNA as appropriate. Water was added to give a volume of 90 µl.

The PCR reaction was carried out in a Perkin Elmer Cetus DNA Thermal cycler as follows: The samples were heated for 5 minutes at 94°C and then cooled to 57°C. The temperature was kept at 57°C for 5 minutes during which time 10 µl of a 0.25 units/ µl Taq polymerase were added to each sample.

After addition of the Taq enzyme, the programme consisted of 30 cycles of each 1 minute at 94°C for strand separation, 1 minute at 57°C for annealing of the primers and 1 minute extension at 72°C followed by a final extension step for 5 minutes at 72°C. The samples were kept at 4°C until analysed in a 1.5% agarose gel. The oligonucleotides used for PCR amplification of cDNA products are listed in Table 2.1.6.

Table 2.1.6.

Oligonucleotides used as primers in PCR			
cDNA and EMBL accession number	**1. primer (sense)**	**2. primer (antisense) written as reverse complement sequence**	**Amplified fragment**
Murine cyclin D1 M64403	CTC AAG ACG GAG GAG ACC TG	ATG GCG GCG AGG TTC CAC TT	Bp 281-651 = 371 bp
Murine fibroblast growth factor receptor I (FRI) M28998	GAT AAG GAC AAA CCC AAC	TCA GCA GCT TGA AAA GTT	Bp 1291-1930 = 640 bp
Murine mdm2 X58876	ACA CAG ATG AGC TAC CTG GG	CTT CTT CTG CCT GAG CTG AG	Bp 704-1261 = 558 bp
Murine wild type p53 (WT p53) X01237 Set 1	TAT TCT GCC AGC TGG TGA AGA CGT G	AAC TCT AAG GCC TCA TTC AGC TCC C	Bp 546-1197 =652 bp
Murine wild type p53 (WT p53) X01237 Set 2 ("Donehower")	GGG ACA GCC AAG TCT GTT ATG TGC	CTC CCG GAA CAT CTC GAA GC	Bp 497-1195 = 699 bp
Murine hypoxanthine phosphoribosyl transferase (HPRT) (positive control) J00423	GTT TGT TGT TGG ATA TGC CCT TGA C	GGG GAC GCA GCA ACT GAC ATT TCT A	Bp 645-895 = 251 bp (mouse) Bp 643 -909 = 267 bp (human) NB: the murine sequence covered by the antisense primer differs at two nucleotides from the corresponding human sequence

Analysis of total RNA with Northern blots

Total RNA was isolated by the guanidinium thiocyanate method as described above. The RNA was separated in gels containing denaturing formaldehyde and using the buffers and reagents listed below (318). All solutions were DEPC treated unless otherwise specified.

SOLUTIONS PREPARED FOR ELECTROPHORESIS OF RNA IN FORMALDEHYDE GELS

1) 5 x concentrated formaldehyde gel running buffer

The buffer contained 0.1 M MOPS (pH 7.0), 40 mM sodium acetate and 5 mM EDTA (pH 8.0) and was prepared as follows: 20.6 g MOPS was dissolved in 800 ml DEPC treated 50 mM NaAc. pH was adjusted to 7.0 with 2 M NaOH and 10 ml DEPC treated 0.5 M EDTA was added. The volume was adjusted to 1 litre. The solution was filtered through a 0.2 µm filter and kept at room temperature protected from light.

2) Deionised Formamide

Formamide was deionised my mixing 50 ml formamide with 5 g Dowex G50 mixed bead resin for 30 minutes at room temperature in the dark. The solution was filtered twice through Whatman n°1 filters.

3) Gel loading buffer

50% (v/v) glycerol
1 mM EDTA, pH 8.0
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF
made up in DEPC treated H_2O

The electrophoresis gel was made up by mixing
1) 3.5 part 1.6% (w/v) melted agarose in DEPC treated water and cooled to 60°C
2) 1 part 12.3 M formaldehyde (pH>4.0) and
3) 1.1 part 5 x formaldehyde gel running buffer

The RNA samples were prepared by mixing approximately 20 µg RNA in 4.5 µl DEPC treated H_2O with 2.0 µl formaldehyde gel running buffer, 3.5 µl formaldehyde and 10 µl deionised formamide. A sample containing 1 µg 0.24 kb-9.5 kb RNA ladder was also prepared. The samples were heated at 65°C for 15 minutes, put on ice for a few minutes,

centrifuged briefly and put back on ice. 2 µl sample loading buffer were then added to each sample.

The formaldehyde gel was pre-run for 5 minutes at 3-4 V/cm after which the samples were loaded immediately on the gel. The gel was usually run at 2 V/cm.

After electrophoresis, the lane containing the RNA ladder was cut off the gel. The ladder was stained for 45 minutes in 0.1 M NH_4Ac containing 0.5 µg/ml EtBr and photographed under UV light, juxtaposed to a ruler. The rest of the gel was rinsed several times in DEPC treated water to remove formaldehyde, soaked in 0.05 M NaOH for 20 minutes to subject the RNA to partial hydrolysis, rinsed in DEPC treated water and soaked in DEPC treated 20 x SSC for 45 minutes.

Transfer of RNA to Hybond N filters was exactly as described for Southern blots, except that all solutions used were DEPC treated. After transfer, the filter was washed for 5 minutes in 6 x SSC and dried on a paper towel. The RNA was immobilised by baking the filter for 2 hours at 80°C.

The probes used in hybridisation were always DNA probes labelled with ^{32}dCTP as described for Southern hybridisations.

The pre-hybridisation and hybridisation buffer were identical and contained 1 M NaCl, 10% dextran sulphate (w/v), 50% deionized formamide (v/v) and 1% (w/v) SDS. The pre-hybridisation solution was made up fresh, heated to 42°C for 30 minutes and added to the filter. After 10 minutes, the pre-hybridisation solution was removed and replaced with fresh pre-heated hybridisation solution containing no more than 1 x 10^5-4 x 10^5 cpm/ml of labelled and boiled probe and 100 µg denatured salmon sperm DNA. The hybridisation reaction was carried out for 15-18 hours at 42°C.

The filter was washed twice with 2 x SSC for 5 minutes at room temperature, twice with 2 x SSC, 1% (w/v) SDS at 60°C for 30 minutes at constant agitation and finally twice with 0.1 x SSC for 30 minutes at room temperature with constant agitation. The filter was exposed to autoradiography with a Fuji film for varying lengths of time at -70°C.

2.1.6. Isolation and analysis of cellular proteins

Immunoprecipitation of cellular proteins

The solutions and reagents used for immunoprecipitations are listed below.

SOLUTIONS AND REAGENTS USED FOR IMMUNOPRECIPITATION

1) **RIPA buffer:**

0.05 M Tris-HCl, pH 7.2
0.15 M NaCl
0.1% (w/v) SDS
1% (w/v) sodium deoxycholate
1% (v/v) Triton X-100.

2) **Protein A beads**:

500 mg of beads were swelled in 25 ml of RIPA buffer on a roller wheel at 4°C. The beads were washed three times by centrifugation at 400 x g (1500 rpm) for 5 minutes and resuspended in RIPA buffer. The final pellet was resuspended in 3.5 ml of RIPA buffer and stored at 4°C.

3) **Protein Loading buffer**

5% (w/v) SDS
12.5% (v/v) β-mercaptoethanol
25% (v/v) glycerol
0.1% (w/v) bromophenol blue

5 x 10^6 cells were washed in methionine free E4 medium. 500 µCi of S^{35} methionine (50 µl of a 1 mCi/100 µl solution (Amersham)) were added to the cells in a volume of no more than 5 ml methionine free E4 medium. The medium contained 0.5% (v/v) heat inactivated FCS which had been dialysed to remove free amino acids. The cells were harvested 10 -12 hours later after the addition of the S^{35} methionine. Adherent cells were

washed 3 times in PBSA and then scraped off using a rubber police man into PBS. Non-adherent cells were harvested, washed 3 times in PBSA and finally once in PBS.

The labelled cells were resuspended and lysed in 250 µl RIPA buffer containing 2 µg/ ml leupeptin, 10 µg/ml soybean trypsin inhibitor and 1 mg/ml aprotinin and left for 10 minutes on ice. Leupeptin inhibits serine and thiol proteases, soybean trypsin inhibitor inhibits serine proteases and aprotinin inhibits serine proteases. The cell lysate was centrifuged for 15 minutes at 12,000 x g to pellet the nuclei.

The supernatant was transferred to a fresh tube and pre-cleared with 25 µl of rabbit pre-immune serum (DAKO X901) at 4°C for 15 hours on a rotor. 50 µl of Protein A Sepharose in RIPA were added and the sample was mixed for 30 minutes on a rotor at 4°C. The beads were pelleted by centrifugation at 12,000 x g for 5 minutes and specific antibody was added to the supernatant. The volume of antibody added was 5-12 µl if it was a polyclonal antiserum and 50-100 µl if it was a supernatant from a hybridoma producing monoclonal antibodies. The antibodies were allowed to bind for 4 hours at 4°C on a rotor. 50 µl of Protein A beads were added and following a one hour incubation at 4°C on a rotor, the beads were pelleted by centrifugation for 5 minutes at 12,000 x g. The beads were washed at least 5 times in RIPA buffer and resuspended in 100 µl of protein gel loading buffer. The samples were left for 15 minutes at 25°C and then clarified by brief centrifugation. The supernatant was heated for 2 minutes at 100°C and then loaded on a polyacrylamide gel.

The antibodies used for detection of recombinant proteins are listed in Table 2.1.7.

Table 2.1.7.

Antibodies used for immunoprecipitations			
Antibody	**Specificity**	**Type**	**Source**
αD1 (321)	Human and murine cyclin D1 C-terminus aa 281-295 LACTPTDVRDVDI	Rabbit polyclonal	G. Peters & S.Bates ICRF, UK.
2A10, clone 11. (322)	Human mdm2 Zn finger domain aa 294-339 Cross reacts with murine mdm2	Mouse monoclonal	A. Levine, Princeton University, NJ, USA.
BG90	Human fibroblast growth factor receptor I (flg) C terminus RHPAQLANGGLKRR	Rabbit polyclonal	Hing Leung & N. Lemoine ICRF, UK.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

SDS-PAGE was carried out using the discontinuous buffer system originally described by Laemmli (324). The separating gel was 12% T (total cross linker) and the stacking gel was 5% (T). The gels were 0.75 mm thick. The reagents used for electrophoresis are listed below and are adapted from (325).

Preparation of separation and stacking gels for SDS-PAGE of proteins

30 ml Separating gel (12% T)

The gel was prepared by mixing:
12.0 ml Protogel (30% Acrylamide)
7.5 ml 1.5 M Tris-HCl, pH 8.8
300 ml 10% (w/v) SDS and
9.9 ml H_2O
To this mixture was added:
300 µl 10% APS (freshly made in H_2O) and
12 µl TEMED

The gel was poured immediately after adding APS and TEMED. After pouring the gel, it was overlaid with water saturated isobutanol to prevent oxygen from interfering with the polymerisation. The gel was allowed to set for 30 minutes. The isobutanol was removed with a filter paper.

10 ml Stacking gel (5% T)

The stacking gel was prepared by mixing:
1.7 ml Protogel
1.25 ml Tris-HCl, pH 6.8
100 ml 10% (w/v) SDS and
6.8 ml H_2O
To this mixture was added:
100 µl 10% APS (freshly made in H_2O) and
10 µl TEMED

The stacking gel was poured immediately after adding the latter two components. The gel was allowed to polymerise for 30 minutes.

Running buffer

The running buffer was 0.192 M Glycine, 25 mM Tris and 0.1% (w/v) SDS.

The protein samples were boiled for 5 minutes before loading them onto the gel. A ^{14}C high molecular weight protein marker from Gibco-BRL was used as protein standard. Electrophoresis was performed in Hoefer Scientific Instruments Vertical Slab Gel Unit, model SE600 attached to a Hoefer PS500X DC Power Supply and a Hoefer RCB refrigerated Circulating Waterbath. The gel was run at 40 mA constant current. The temperature of the waterbath was kept at 16°C during electrophoresis to prevent over heating of the gel.

Following electrophoresis, the gel was treated for 15 minutes in 1 M Sodiumsalicylate to enhance the signal. The gel was dried in a Biorad Model 583 gel dryer for 1 hour at 80°C and exposed to autoradiography with a Fuji film for 1-3 weeks at -70°C.

2.2. Tissue culture

2.2.1. Chemicals, animals and cell lines

Buffers and stock solutions

All media and solutions used for tissue culture were made with water prepared at ICRF, Clare Hall. The water was double distilled, purified by a Millipore Reverse Osmosis system and finally organic filtered to give water at < / 10MΩ.cm.

All buffers and solutions with the exception of PBSA, PBSB and PBSC were made in the lab. PBSA was supplied from ICRF Clare Hall together with PBSB and PBSC. The recipes for making the most commonly used buffers and stock solutions are in Appendix I.

Animals

BALB/c ($K^dD^dL^d$), C57BL/10ScSn (K^bD^b) and C57BL/6 (K^bD^b) mice were supplied by ICRF breeding colony. Mice were used at the age of 6 to 12 weeks.

Media

All media used except IMDM were obtained ready made and filter sterilised from the central cell services at ICRF Clare Hall.

RPMI, 2% bicarbonate was used as growth medium for all cells in suspension except T cells. For simplicity, the medium will be referred to as RPMI. RPMI medium from Clare Hall was made up with 10.392 g/litre RPMI powder (Gibco BRL), 2g/litre $NaHCO_3$, 2 mM L-Glutamine, 100 units/ml penicillin and streptomycin and with pH adjusted to 7.0 with CO_2.

E4 (Dulbecco's modified Eagle's medium) was used as growth medium for all adherent cells. The E4 made up by ICRF Clare Hall was identical to the E4 medium manufactured by Gibco BRL.

MEM (Minimum Essential Medium) was used when infecting cells with vaccinia virus or adenovirus. MEM was made up with 9.68 g/l MEM powder (ICN) with added 4.36 g/l HEPES and 100 units/ml penicillin and streptomycin and with pH adjusted to 7.0 with 10 M NaOH.

The medium used was always less than 6 weeks old. Additional glutamine was not added to the medium which at the time of preparation contained 4 mM glutamine.

IMDM (Iscove´s modified Dulbeccos medium) was used for T cell culture and for transfections.
IMDM medium was made up in this laboratory. It was prepared with 17.66 g/l IMDM powder (Gibco BRL), 5 µg/ml transferrin, 100 units/ml penicillin and streptomycin and 3.02 g/l (36 mM) $NaHCO_3$. The medium was filter sterilised and used within two weeks of the preparation date.

Details of the individual components of the media such as mineral, vitamin, amino acid and antibiotic content can be obtained from ICRF Clare Hall (E4, RPMI), from Gibco BRL (E4, RPMI, IMDM) or ICN (MEM).

For most purposes, except T cell culture, heat inactivated foetal calf serum was used. The foetal calf serum was thawed on water bath at 37°C and complement was inactivated by incubating the serum for 30 minutes at 56°C in a waterbath. The heat inactivated serum was aliquoted and kept at - 20°C.
T cell culture foetal calf serum was taken from special T cell culture tested FCS batches and used directly without heat inactivation.

Cell lines

The origin and haplotype of the cell lines used are summarised in Table 2.2.1. The table also includes transfectants which have been generated as part of the present project.

Murine cell lines

RMA cells originate from a Rauscher virus-induced C57BL/6N T cell lymphoma RBL-5 which was mutagenised with ethyl methane sulfonate (326).

RMA-S cells are RMA cells which were exposed to five rounds of anti-H-2 alloantisera and rabbit complement treatment to select for low H-2 expressing cells (326).
The cell line expresses 5% of the level of assembled H-2 D^b, K^b and β_2m expressed by RMA cells (327). Incubation of the cells at 26°C induces up-regulation of expression of empty class I molecules (35; 36; 326). The defect in surface class I expression is due to the lack of a functional TAP 2 protein. The cell line contains only one copy of the TAP 2 gene. The gene has a point mutation at nucleotide 97 which generates a premature stop codon (328).

EL4 cells were established in tissue culture from a murine 9:10- dimethyl-benzan-thracene induced thymoma from C57BL/6N mice (329).

Tp53 is a murine thymoma cell line established from a p53 negative mouse (260) and L. Dianda, ICRF, unpublished results. Class I expression is low on this cell line, but can be up-regulated by adding 10 -100 u/ml IFN-γ to the culture (own observation).

C57 is a murine epithelial cell line derived from a C57BL/6 mouse and immortalised by HPV (H. Stauss, personal communication).

EL4 FR1 ("EF") are EL4 cells transfected with the pDO-R vector (330) containing the cDNA encoding murine fibroblast growth factor receptor I.

EL4 mdm2 ("EM") are EL4 cells transfected with the pDO-R vector (330) containing the cDNA encoding murine mdm2.

P1.HTR is derived from the murine DBA/2 methylcholantrene induced mastocytoma P815. The cell line has a high efficiency of transfection for exogenous DNA (184).

P1.HTR FR1 ("PF") are P1.HTR cells transfected with the pDO-R vector (330) containing the cDNA encoding murine fibroblast growth factor receptor I.

P1.HTR mdm2 ("PM") are P1.HTR cells transfected with the pDO-R vector (330) containing the cDNA encoding murine mdm2.

Human cell lines

293 cells are human embryonic kidney cells transformed with DNA fragments of Adenovirus type 5. The cells contains 4-5 copies of the left hand 12% of the Ad5 genome and 1 copy of the right hand 10% of the Ad5 genome. This has resulted in a cell line producing the Ad5 E1A and E1B proteins (306).

Tk^-143 (143B) is a human osteogenic sarcoma cell line transformed with Kirsten mouse sarcoma virus (Ki-MSV). The cell line does not express thymidine kinase (332).

Hela K^b cells are Hela cells which have been transfected with the murine class I K^b molecule. The cell line is an epithelial cell line derived from a cervical carcinoma (333).

Hamster cell lines

CHO cells are derived from the ovaries of an adult chinese hamster (334).

Table 2.2.1

Murine cell lines				
Cell line	**Origin**	**MHC class I expression**	**Growth medium**	**Source**
RMA	Murine T cell lymphoma	K^b, D^b	RPMI, 8% FCS	K. Kärre, Stockholm, Sweden
RMA-S	Murine T cell lymphoma	K^b, D^b	RPMI, 8% FCS	K. Kärre, Stockholm, Sweden
EL4	Murine thymoma	K^b, D^b	RPMI, 8% FCS	ATCC
EL4FRI ("EF")	Murine thymoma	K^b, D^b	RPMI, 10% FCS 450 µg/ml G418	Produced at the TIU
EL4mdm2 ("EM")	Murine thymoma	K^b, D^b	RPMI, 10% FCS 450 µg/ml G418	Produced at the TIU
Tp53	Murine thymoma	K^b, D^b	RPMI, 10% FCS	L. Dianda, ICRF
C57	Murine epithelial cell line	K^b, D^b	RPMI, 10% FCS	L. Crawford, ICRF
P1.HTR	Murine mastocytoma	K^d, D^d, L^d	RPMI, 8% FCS	ECACC
P1.HTR-FRI("PF")	Murine mastocytoma	K^d, D^d, L^d	RPMI, 10% FCS 350 µg/ml G418	Produced at the TIU
P1.HTR-mdm2 ("PM")	Murine mastocytoma	K^d, D^d, L^d	RPMI, 10% FCS 350 µg/ml G418	Produced at the TIU
P1.HTRK^b ("PK^b")	Murine mastocytoma	K^d, D^d, L^d, K^b	RPMI, 10% FCS 350 µg/ml G418	Produced at the TIU
P1.HTRD^b ("PD^b")	Murine mastocytoma	K^d,D^d, L^d, D^b	RPMI, 10% FCS 350 µg/ml G418	Produced at the TIU

Table 2.2.2.

Human cell lines			
Cell line	**Origin**	**Growth medium**	**Source**
$Tk^{-}143$ (143B)	Human osteogenic sarcoma	E4, 8% FCS 25 µg/ml BrdU	ATCC
Hela K^{b}	Human epithelial cells	E4, 8% FCS	Produced at the TIU
293	Human embryonic kidney cells	E4, 8% FCS	ATCC

Table 2.2.3.

Hamster cell lines			
Cell line	**Origin**	**Growth medium**	**Source**
CHO	Chinese hamster ovary cells	E4, 8% FCS	ATCC

Preparation of Con A supernatants

Spleens from normal rats were teased into single cell suspension. The cells were pelleted at 250 x g for 10 minutes and washed once in MEM. The cells were resuspended at 5 x 10^6 nucleated cells/ml in IMDM, 5% FCS and 3 μg/ml Concanavelin A in a T125 flask. After 48 hours culture at 37°C, the cells were spun at 800 x g for 20 minutes. The supernatant was collected, filtered through a 0.2 μm filter and stored at -20°C until use.

Peptides

Peptides were synthesised by the Peptide Synthesis Laboratory at ICRF, Lincoln's Inn Fields by standard fmoc chemistry. The quality of the peptide was checked by mass spectrometry and HPLC.

The peptides derived from cyclin D1, mdm2 and p53 were given the prefix cd, md and p53 respectively followed by the number of the first amino acid according to published protein sequences. As an example, a peptide derived from cyclin D1 starting at position 20 in the protein sequence would be abbreviated "cdp20".

8 and 9 mer peptide stock solutions were made up as a 1 mg/ml (roughly 1 mM) solutions in PBSA except for the peptides cdp41, cdp84, p53p127 and p53p320. These peptides were dissolved in DMSO and PBSA was then added to give a final DMSO concentration of 10% (v/v). The peptide solutions were filtered through 0.2 μm and stored at -20°C.

2.2.2. Culture of immortalised tumour cell lines and transfectants

Handling of tumour cell lines and transfectants

Tumour cell lines and transfectants were washed or transferred to fresh medium by centrifugation of the cells for 5 minutes at 300 x g (1300 rpm in a Beckman GPR with rotor radius of 16 cm) followed by resuspension of the cells in the appropriate medium.

Culture of cells growing in suspension

Cells grown in suspension were usually seeded at 2 x 10^5 cells/ml and repassaged at a concentration around 1 x 10^6 cells/ml. The medium used for culturing individual cell lines is listed in Table 2.2.1., 2.2.2. and 2.2.3. Transfectants were grown in the same medium as the parental cell line but with a selecting antibiotic such as Geneticin, G418. The concentration of G418 in the medium depended of the cell line used, see Table 2.2.1.

Culture of adherent cells

Adherent cell lines such as Tk^-143, CHO and Hela K^b were subcultured by detachment of the cells with trypsin as follows:

4 ml of 0.25% (w/v) trypsin in Tris Saline was mixed with 16 ml of 0.02% (w/v) versene (EDTA) in PBSA and kept at 37°C.

The medium was removed from the adherent cell layer, the cells were washed once with PBSA and the pre-heated trypsin/versene was added to the cells. The volume of trypsin/versene added depended on the size of the flask containing the cells. 1 ml was used to a T25 flask, 2.5 ml was used to a T75 flask and 5 ml were used to a T125 flask. The cells were incubated for a few minutes at 37°C until the cells had detached from the tissue culture flask. The cells were centrifuged and washed 3 times with E4, 2% FCS.

Semiadherent cell lines such as the 293 cells were not trypsinised. The cells were washed once with PBSA and detached by gently tapping the flask.

2.2.3. General methods for analysis of cells

Viability

Cell viability was estimated by trypan blue exclusion. 1 volume of cells was mixed with one volume of trypan blue. The cells were counted using an improved Neubauer standard haemocytometer.

FACS Analysis

Cells were analysed by flow cytometry by direct or indirect immunofluorescence staining.

The cells were washed once in PBSA, 2% FCS, 0.01% (w/v) sodiumazide and distributed at 1 x 10^5 cells per well in 96 well round bottom non-tissue culture treated Falcon plates (referred to as assay plates). Cells were incubated with a saturating concentration of first layer antibody. The first layer antibody was either unconjugated (indirect staining) or conjugated to a fluorescent dye (direct staining). The cells were left for 30 minutes on ice and washed 2 times with PBSA, 2% FCS, 0.01% (w/v) sodiumazide. For indirect immunofluorescence staining, a second layer antibody conjugated to a fluorescent dye was then added. If the first layer consisted of a biotinylated antibody, then the second layer would be a streptavidin conjugate. The cells were incubated for 30 minutes on ice protected from light. Cells were washed twice with PBSA, 2% FCS, 0.01% (w/v) sodiumazide. Stained cells were analysed on a Becton Dickinson FACSscan flow cytometer. The mean fluorescence channel FL1 was determined on a 4- logarithmic scale containing a total of 1023 fluorescence channels. The values were converted to linear values by using the formula:
mean FL1 = $10^{(\text{mean FL1 channel}/1023)}$ x 4 (335).

The monoclonal and polyclonal antibodies used for cell staining are listed in Table 2.2.4. and Table 2.2.5.

The conjugated antibodies or other molecules used in the present work are listed in Table 2.2.6.

Table 2.2.4.

Antibodies directed against murine determinants			
Antibody	**Specificity**	**Type**	**Source**
Hb 27 (28-14-8S) (336)	D^b, D^q, L^d α^3 domain	Mouse cytotoxic mAb IgG_{2a}, κ	ATCC
Y3 (337; 338; 339)	K^b α1 + α2 domains	Mouse mAb IgG_{2b}, κ	ATCC
Hb31	L^d	Mouse mAb IgG_{2a}, κ	ATCC
Hb75 (34-4-20S) (340)	D^d	Mouse cytotoxic mAb IgG_{2a}, κ	ATCC
Tib 120 (M5/114.5.2) (341)	I-A $^{(b,d,q)}$ I-E $^{(d,k)}$	Rat mAb IgG_{2b}, κ	ATCC
N418 (342)	α-chain of a 150,90 kDa integrin (CD11c) on DCs. Not on splenic lymphocytes and macrophages.	Hamster mAb	ATCC
RA3-6B2 (343; 344; 345)	B220: 220 kDa cell surface protein on B lymphocytes and NK cells.	Rat mAb $IgG_{2a,\kappa}$	PharMingen
16-10A1 (346)	B7-1	Hamster mAb	D.Harlan Wisconsin
M1/70.15 (347; 348)	Mac1 (CD11b): macrophages, monocytes, granulo-cytes and NK cells	Rat mAb IgG_{2b}	Sera-lab

Table 2.2.5.

Antibodies directed against human determinants			
Antibody	**Specificity**	**Type**	**Source**
Hb95 (Hw6/32) (349)	HLA-A, B, C (heavy chain, not with β_2-micro-globulin)	Mouse mAb $IgG_{2b,\kappa}$	ATCC

Table 2.2.6.

Conjugated antibodies or other molecules used for FACS analysis			
Specificity	**Type of antibody**	**Molecule(s) conjugated to the antibody/ molecule**	**Supplier**
Mouse CD8α (Ly-2)	Rat IgG_{2a}, κ mAb Clone 53-6.7	FITC	PharMingen
Mouse CD4 (L3T4)	Rat IgG_{2a}, κ mAb Clone RM4-5	R-PE	PharMingen
Mouse polyvalent immunoglobulins (IgG, IgA, IgM)	Affinity purified goat polyclonal antibodies	FITC	Sigma
Hamster polyvalent immunoglobulins	Goat polyclonal antibodies	FITC	Nordic Immunological Laboratories
Rat polyvalent immunoglobulins	Goat polyclonal antibodies	FITC	Nordic Immunological Laboratories
B220	Mouse mAb IgG_{2a}, κ (Table 2.2.4)	Biotin	PharMingen
Biotin	-	Streptavidin-cytochrome C	PharMingen

Peptide binding assays

The ability of peptides to bind to murine class I K^b or D^b molecules was determined using a whole cell binding assay. The assay measures the ability of peptides to stabilise class I molecules on the cell surface of temperature-induced RMA-S cells.

The day before the assay, RMA-S cells were seeded at 1 x 10^6 cells/ml in RPMI, 10% FCS in a T25 flask. The cells were temperature induced for 24 hours at 26°C in a 5% CO_2 incubator to increase expression of class I molecules on the cell surface (35).

The medium used for the binding assay consisted of RPMI, 2% FCS. The medium had been heated for 10 minutes at 100°C in a waterbath to inactivate proteases (350). Serial dilutions of peptides were prepared in assay medium in 96 well, U-bottom, non-tissue culture treated assay plates (referred to as assay plates). Two rows with medium but no peptide were also prepared. The temperature-induced RMA-S cells were centrifuged and resuspended at 1 x 10^6 cells/ml in assay medium. A sample of these cells was subsequently withheld and kept on ice as positive control. The remaining RMA-S cells were added to the assay plate. 1 x 10^5 cells were added to each well in each row in the assay plate, except one of the rows without peptide. This row would be used later for the positive control cells. The other row without peptide and to which RMA-S cells were added would serve as negative control.

Binding of the RMA-S cells to the peptides in the assay plate was allowed to proceed for 2 hours at 37°C. After this incubation period, the RMA-S cells serving as positive control were added to the assay plate to the row which did not contain peptide and which had not received any RMA-S cells previously. All cells were subsequently washed once with PBSA, 2% FCS, 0.01% (w/v) sodiumazide. The cells were stained with monoclonal antibodies specific for murine K^b or D^b molecules and analysed by FACS analysis as described above. Half maximal binding for an individual peptide was determined as the concentration of peptide needed to obtain 50% of the maximum mean FL1 signal obtained for the peptide when using the mean FL1 obtained with RMA-S cells incubated without any peptide (negative control cells) as baseline.

CTL assays

Cytotoxic T cell activity was determined in a ^{51}Cr release assay. The assay uses the release of chromium from pre-labelled target cells to measure lysis of target cells after contact with effector T cells.

The design of the assay depended of the purpose of the assay. Three different assay designs were used. The overall principle of mixing effector and labelled target cells for 4 hours at 37°C was always the same, but the details of the assay set-up varied. For reasons of clarity, the three main assay types will be explained separately.

Design and set up of assays

All CTL assays were carried out in 96 well U-bottom non-tissue culture treated assay plates from Falcon. In the following, these plates will simply be referred to as assay plates.

CTL Assay type I:

Standard assay using a fixed concentration of target antigen but varying concentrations of effector cells.

This assay was the most widely used. The release of chromium from the target cells is measured as a function of an increasing effector to target cell ratio, where the T cell effectors is the variable parameter whereas the target cell concentration remains constant.

The assay medium used was RPMI, 5% FCS, 10 mM HEPES, pH 7.0.

Step 1: Pre-treatment of target cells

The target cells used in the chromium release assay would either be tumour cell lines, virus infected or peptide pulsed tumour cell lines.

a) Virus infected tumour cell lines : 1 x 10^6 cells were infected overnight with 10 pfu/cell virus in a volume of 2 ml RPMI, 2% FCS in a 24 well tissue culture plate. The following morning, the infected cells were harvested, spun down and resuspended in 50 µl assay medium and transferred to an assay plate.

b) Uninfected tumour cell lines except RMA-S cells: The cells were seeded at a concentration of 5 x 10^5 cells/ml in RPMI, 10% FCS the day before the assay. On the day of the assay 1 x 10^6 target cells were spun down, resuspended in 50 µl RPMI, 10% FCS and transferred to an assay plate

c) RMA-S cells: The cells were seeded at 1 x 10^6 cells/ml in RPMI, 10% FCS the day before the assay. The cells were temperature induced for 24 hours in a 26°C incubator. On the day of the assay, 1 x 10^6 target cells were spun down, resuspended in 50 µl RPMI, 10% FCS and transferred to an assay plate.

Step 2: Labelling of target cells.

The cells were labelled for 1 hour with 50 µCi $Na^{51}CrO_4$ by adding 10 µl of 5 mCi $Na^{51}CrO_4$ to the target cells in the assay plate.

The labelling was done in a 26°C incubator for RMA-S cells and a 37°C incubator for all other cell lines. The cells were washed three times and resuspended in a 20ml tube at 5 x 10^4 cell/ml in RPMI, 5% FCS, 10 mM HEPES, pH 7.5. If peptide loaded RMA-S cells were used as targets, RMA-S cells would be incubated at this stage with 100 µM peptide for 1 hour, washed once and resuspended again at 5 x 10^4 cells/ml. During the incubation of the RMA-S cells with peptide, the other target cells would be kept in an incubator at 37°C.

Step 3: Preparation of effector cells

A serial dilution of effector cells was done in assay medium in an assay plate. The volume in each well was 100 µl prior to the addition of target cells. Rows with 100 µl medium or 100 µl 0.5% (w/v) SDS representing spontaneous and maximum release respectively were prepared on separate plates.

Step 4: Incubation of target cells with the effector cells

5 x 10^3 ^{51}Cr labelled target cells i.e. 100 µl were added to the wells containing the effector cells and the wells representing spontaneous and maximum release. The plates were centrifuged for 2 minutes at 450 x g and incubated for 4 hours at 37°C.

CTL Assay type II

Assay using different concentrations of blocking antibodies and a fixed effector to target cell ratio

This type of assay was based on the use of fixed effector to target cell ratios and variable concentrations of antibody targeted at CD4 or CD8.

The assay medium used was RPMI, 5% FCS, 10 mM HEPES, pH 7.0.

Step 1 and 2: Pre-treatment and labelling of target cells

The pre-treatment and labelling of target cells were as for the standard assay using fixed ratio of effector cells and target antigen (type I, see above) except that the target cells were resuspended at a concentration of 1 x 10^5 cells/ml after the labelling.

Step 3: Preparation of effector cells and incubation with anti CD4 or anti CD8 antibodies

Serial dilutions of blocking antibody were done in duplicate in wells containing 100 µl assay medium in an assay plate. The highest concentration of antibody used was 100 µg/ml. (This would give a final concentration of 50 µg/ml once the effector cells and target cells were added to the well). Rows containing 100 µl medium but no antibody were also prepared.

Effector T cells were harvested, spun down and resuspended at the appropriate concentration. 50 µl of T cells were added to the wells containing the antibody dilutions or the medium alone. The plates were incubated for 30 minutes in a 37°C incubator.

Step 4: Incubation of target cells with the effector cells.

50 µl ^{51}Cr labelled target cells (5 x 10^3 cells) were added to each well containing T cells and antibody or T cells and medium. 50 µl target cells was also added to wells containing 150 µl medium with no T cells and no antibody representing spontaneous release as well as to wells containing 150 µl 0.5% (w/v) SDS representing maximum release. The plates were centrifuged for 2 minutes at 450 x g and incubated for 4 hours in a 37°C incubator.

CTL Assay type III:

Assay using a fixed concentration of effector cells and varying concentrations of target antigen (peptide titration)

This type of assay was used to measure the effect of decreasing peptide concentration on the lysis of peptide coated target cells by effector T cells.

The assay medium used was RPMI, 5% FCS, 10 mM HEPES, pH 7.0 which had been heated for 10 minutes at 100°C to inactivate proteases (350).

Step 1: Pre-treatment of target cells.

RMA-S cells were used as target cells. The labelling of the RMA-S cells were as described for the standard assay (type I).

Step 2 : Labelling and preparation of target cells

The cells were labelled for 1 hour with 50 µCi $Na^{51}CrO_4$ by adding 10 µl of 5 mCi $Na^{51}CrO_4$ to the target cells in the assay plates.

The labelling was done in a 26°C incubator. After 1 hour, the cells were washed three times and resuspended to give a concentration of 1 x 10^5 cell/ml.

During labelling of the target cells, serial dilutions of peptides were set up in duplicate in assay medium. The volume in each well was 100 µl. The concentrations of peptide covered the range from 2 µM to 200 fM with 10 fold dilution steps. This would give a final concentration range from 1 µM to 100 fM when the RMA-S cells and effector T cells were added.

For spontaneous release, a row was prepared with no peptide, but 150 µl assay medium only. For maximum release, a row was prepared with no peptide but 150 µl 0.5% (w/v) SDS only. Peptide toxicity was checked by setting up wells containing 100 µM of each peptide in 150 µl assay medium.

5 x 10^3 labelled and washed RMA-S cells in a volume of 50 µl were added to all the wells in the assay plate, including wells containing peptide and wells with no peptide representing spontaneous and maximum release. Binding of peptide to the RMA-S cells was allowed to proceed for 1 hour at 37°C.

Step 3: Preparation of effector cells

Meanwhile effector T cells were counted. The T cells were spun down and resuspended in assay medium at the concentration needed to give the E/T ratio wanted in the assay.

Step 4: Incubation of effector T cells with the peptide coated target cells.
50 µl of T cells were added to the wells containing the RMA-S cells and the peptide. No T cells were added to the wells set up to check the toxicity. These wells received 50 µl medium instead.
The plates were centrifuged for 2 minutes at 450 x g and incubated for 4 hours in a 37°C incubator.

Harvest of assay and analysis of results

Independent of the assay type used (CTL assay type I, II or III), the harvest and analysis of results were identical.

100 µl supernatant was harvested from each well in the assay plates and counted in a LKB Wallac 1272 Clinigamma γ-counter linked to an Olivetti PCS 286 computer.

The "% specific release" was calculated as:

$$\frac{\text{(experimental release-spontaneous release)}}{\text{(maximum release - spontaneous release)}} \times 100\%.$$

Cell separation using magnetic beads

Subpopulations of cells could be obtained by using the MACS magnetic cell separation system (Miltenyi Biotec) by following the instructions given by the manufacturer:
The cells were resuspended at concentration of 10^7 cells per 90 µl in PBS, 0.5% (w/v) BSA. The cells were incubated with 10 µl monoclonal antibody coated magnetic beads per 10^7 cells for 15 minutes at 4°C. The cells were washed once with PBS, 0.5% (w/v) BSA, resuspended in 500 µl PBS, 0.5% (w/v) BSA and applied to the top of a MACS separation column attached to a MACS separator providing a magnetic field. The column had been equilibrated with PBS, 0.5% (w/v) BSA. A 23 G needle was attached to the MACS column. After having added the cells, the column was washed with 3 ml of PBS, 0.5% (w/v) BSA. To elute the cells, the column was removed from the separator and the cells were collected in 500 µl PBS, 0.5% (w/v) BSA.

Preparation of peptides from whole cell lysates

Peptides were prepared from cell lysates as described (351), with some minor modifications. The preparation of peptides included the following steps:

1) Preparation of a whole cell lysate

Peptides from virus infected cells were obtained from cells which had been infected overnight at a density of 1 x 10^6 cells/ml in RPMI, 2% FCS with 10 pfu /cell recombinant virus.

The following day, 2 x 10^8 infected or uninfected cells were washed three times in PBSA. The cells were centrifuged at 350 x g (1400 rpm) for 5 minutes. All supernatant was removed. The cells were lysed by resuspending them with a glass pipette in 3 ml 0.7% TFA. The cell lysate was kept at -70°C until further use.

Peptides from spleen cells were obtained from whole spleens which had been depleted of red blood cells with Gey's solution. Briefly, the spleens were passed through a mesh and the cell suspension was spun down at 350 x g for 5 minutes. The cells were washed once in PBSA and separated into universals so that each universal would contain cells from 6-7 spleens or less. Red blood cells were lysed by resuspending the cells in 1 ml Gey's solution per spleen and incubating for 3 minutes. 10 ml PBSA were added per universal, the cells were spun down and washed 3 times with PBSA. Finally, the cells were pooled and resuspended with a glass pipette in 3 ml 0.7% TFA per 5 spleens. The cell lysate was kept at -70°C until further use.

Gey's solution for lysis of red blood cells

Solution A	Solution B
35 g NH_4Cl	2.1 g $MgCl_2 \cdot 6H_2O$
0.56 g Na_2HPO_4	0.7 g $MgSO_4 \cdot 7H_2O$
1.85 g KCl	1.7 g $CaCl_2$
0.12 g KH_2PO_4	H_2O up to 500 ml
5 g Glucose	
H_2O up to 1000 ml	Solution C
	11.25 g $NaHCO_3$
	H_2O up to 500 ml

Complete Gey's solution is made up by mixing Solution A, B and C to give 20% (v/v) Solution A, 5% (v/v) Solution B and 5% (v/v) Solution C.

2) Preparation of peptides from whole cell lysates

The cell lysate was transferred to a 20 ml universal tube, put on ice and sonicated with a Branson sonicator on output 2, duty cycle at 50% and timer on hold. 3 x 10 bursts were delivered. The sample was allowed to cool and transferred with a glass pipette into two 2 ml Eppendorf tubes. The tubes were vortexed for 3 minutes and centrifuged for 60 minutes at 12,000 x g (15,000 rpm in a Sigma 2 K15 centrifuge).

The supernatant was transferred into a centricon 10 filter unit and centrifuged at 4°C for 2.5 hours at 5000 x g (6500 rpm in a Beckmann J2-21 centrifuge using a 45 angle JA20 rotor). The material which had passed through the filter and which contained small proteins and peptides was either used directly for HPLC separation or stored at -70°C.

3) Separation of peptides by HPLC

Peptides were separated by reverse phase HPLC using a Pharmacia HPLC system according to the protocol described by (351). The equipment used consisted of a Pharmacia LKB HPLC pump 2248, a Pharmacia LKB UV monitor VWM 2141, a Pharmacia LKB fraction collector FRAC-100, a Pharmacia LKB reverse phase column Superpac Pep-S No 80-1266-36.

Buffer A and B consisted of 0.1% (v/v) TFA in H_2O and 0.1% (v/v) TFA in acetonitrile. The peptide retention standard was from Pierce Rockford. It was resuspended in 0.1% (v/v) TFA to make a 0.1 mg/ml stock solution.

The program used for separating the peptides consisted of

1) 0-40 min : increase in buffer B from 0% to 40% at a rate of 1% per minute
2) 40-46 min: increase in buffer B to 90%
3) 46-51 min: buffer B constant at 90%
4) 51-52 min: decrease of buffer B from 90% to 0%

2.2.4. Induction of cytotoxic T lymphocytes

In vivo immunisations

a) Immunisation with recombinant vaccinia virus

The route of immunisation was intraperitoneal injection.

The recombinant virus was diluted in PBSA and no adjuvant was used. The immunisation schedules used in individual experiments will be specified and discussed in more detail in chapter 4.

Mice were either immunised once with 2 x 10^7 recombinant vaccinia virus and spleens were taken either one week or three weeks later. Alternatively, the mice were immunised twice or more, first time with 2 x 10^7 pfu recombinant virus and second time two weeks later with 1 x 10^7 pfu recombinant vaccinia virus. The boost was occasionally repeated one week later. Spleens were taken one week after the last boost.

b) Immunisation with synthetic peptide.

Synthetic peptides were administered by subcutaneous injection. 50 µg peptide was given per injection.

Three types of adjuvant were used:

The peptide was given as a 1:1 water in oil emulsion with Incomplete Freund's Adjuvant. Alternatively 50 µg peptide was mixed with 5 µg of a muramyl peptide derivative and mixed 1:1 (vol: vol) with adjuvant STP ("aqueous adjuvant": 0.2% Tween-80 (Sigma), 5% squalane (BASF), 2.5% L121 polymer (Aldrich)) or with adjuvant STP_m ("lipid adjuvant": 0.6% Tween-80, 15% squalane and 0.015% L121 polymer). The muramyl dipeptide used was N-acetyl-D-glucosaminyl-(beta-1-4)-N-acetylmuramyl-alanyl-D-isoglutamine (GMPD) (352). The muramyl dipeptide and the ready made STP and STP_m adjuvants were kindly provided by Dr. R. Hornung, National Cancer Institute-Frederick Cancer Research and Development Center, Maryland.

Mice were mostly either immunised once and the spleens were taken one or three weeks later or the mice were immunised once followed by a boost ten days later and removal of the spleen after seven days. The immunisation schedule used in individual cases will be discussed in chapter 5.

In vitro restimulation of T cells

T cells were restimulated *in vitro* with uninfected or vaccinia infected tumour cell lines, peptide pulse or vaccinia infected dendritic cells or adenovirus infected spleen cells or tumour cell lines. The conditions used will be described below and in chapter 4.
Irrespective of the antigen presenting cells used to stimulated the T cells, all T cells were grown in the same type of medium and on a layer of feeder cells after 6 days in culture.

T cell medium

The T cell medium was IMDM, 10% FCS, 10 u/ml rIL-2 and 5 x 10^{-5} 2-ME. For the first 6 days of culture, the IL-2 was omitted.

General handling of splenocytes and T cell cultures

T cells or splenocytes were washed or transferred to fresh medium by centrifugation of the cells for 10 minutes at 180 x g (1000 rpm in a Beckman GPR centrifuge with a rotor radius of 16 cm) unless anything else is specified.

Effector cells

Effector T cells were obtained from spleens of immunised or naive mice. Spleens from at least two animals were pooled and were used per experiment. Spleens were removed from the dead animals, transferred to a universal with RPMI, 10% FCS and teased through a mesh to make a single cell suspension. The spleen cells were centrifuged, leaving most cell debris, connective tissue fragments and dead cells in the supernatant. The cells were resuspended in a small volume of T cell medium, counted and diluted to give the appropriate concentration.

Feeders

Freshly prepared spleen cells from a syngeneic mouse were used as feeders. A single cell suspension of 5-10 x 10^6 cells/ml in RPMI, 10% FCS was irradiated with 3000 rads from a ^{137}Cs γ-irradiator to prevent DNA replication. The cells were centrifuged. The cells were resuspended in a small volume of fresh T cell medium, counted and diluted to give the appropriate concentration.

Antigen presenting cells and culture conditions

The different types of antigen presenting cells used and the set up and expansion of lines will be described below. The combinations used for individual T cell lines and clones will be discussed in more detail in chapter 4 and 5.

I) Cultures using syngeneic untransfected, transfected or vaccinia infected tumour cells as antigen presenting cells

Step 1 : Pre-treatment of stimulator cells

If vaccinia infected tumour cell lines were used as APCs, then 5 x 10^6 tumour cells were infected with 10 pfu/cell vaccinia virus for 4 hours in 5 ml RPMI, 2% FCS.

Infected or uninfected tumour cell lines were treated with mitomycin C to prevent DNA and RNA synthesis. The mitomycin C treatment was done by resuspending the cells at 2.5 x 10^6 cells/ml in serum free medium in an universal wrapped in aluminium foil followed by the addition of mitomycin C to give a concentration of 50 µg/ml. Cells were incubated for 60 minutes at 37°C and washed three times with medium containing 5% FCS.

Step 2: Set up and expansion of cultures.

Bulk cultures were set up in 96 well plates using 4 x 10^5 responder T cells and 4 x 10^4 stimulators in a total volume of 200 µl per well. On day 6, the cells were harvested and tested for cytotoxicity. The T cells were passed on to new 96 well plates with fresh APCs and feeders. Each well contained 5 x 10^4 T cells, 5 x 10^3 stimulator cells and 3 x 10^5 syngeneic spleen cells as feeders. The cells were passaged weekly. Cells aimed for cloning were passaged with two weeks intervals. The T cells were assayed for cytotoxic activity in ^{51}Cr release assays on day 6 and/or day 7 after restimulation.

II) Cultures using cell preparations enriched for dendritic cells as APCs

Dendritic cells were enriched from spleens and then incubated with peptide or infected with recombinant vaccinia virus or adenovirus and used as APCs.

Step 1: Enrichment of dendritic cells from mouse spleens

Dendritic cells were enriched from mouse spleens by taking advantage of two properties of dendritic cells: adherence and low buoyant density. Macrophages and other strongly adherent cells adhere to tissue culture flasks and stay adherent after an over night incubation whereas dendritic cells do adhere to tissue culture flasks initially, but start detaching after 1-2 hours and will be non-adherent after an overnight incubation. These non-adherent and low density dendritic cells can subsequently be separated from other non-adherent but high-density cells such as lymphocytes by centrifugation on a layer of metrizamide (353).

Spleens were removed from syngeneic mice and teased through a mesh to make a single cell suspension. Cells were centrifuged and resuspended at 5 x 10^6 cells/ml in RPMI, 10% FCS. 1 x 10^8 spleen cells were seeded per T75 flask and allowed to adhere over night. The following day, non-adherent cells were taken from the flasks. The non-adherent cells were centrifuged and resuspended at 10^7 cells per ml in RPMI, 10% FCS. 8 x 10^7 cells in a volume of 8 ml were layered on top of 2 ml 14.5% (w/v) metrizamide in RPMI, 10% FCS in a 10 ml universal tube. The cells were centrifuged for 10 minutes at 600 x g (1800 rpm). The low density cells in the interface were collected and washed. For reasons of simplicity, the cell preparation will be referred to as DCs.

Step 2: Use of cell preparations enriched for dendritic cells as APCs.

The DCs were used as APCs either after having been pulsed with peptide or after having been infected with recombinant vaccinia virus or adenovirus.

1 x 10^6 DCs in a volume of 1 ml RPMI, 2% FCS were either incubated for 4 hours with 100 μM peptide or infected for 4 hours with 10 pfu per cell vaccinia virus or adenovirus. The cells were washed once and used as APCs.

Step 3: Initiation and expansion of T cell cultures

Bulk T cell cultures were set up in 96 well plates using an effector : DC ratio of 50. Each well contained 4 x 10^5 responder T cells and 8 x 10^3 peptide pulsed or virus infected DCs. 100 nM peptide were added to cultures containing peptide pulsed DCs. The cells were tested for cytotoxicity and restimulated on day 6. From this day, the T cells were passed every two weeks. Each well contained 5 x 10^4 responder T cells, 5 x 10^3 peptide pulsed or vaccinia infected DCs and 3 x 10^5 syngeneic mouse spleen cells as feeders in a volume of 200 μl. This gives an effector: DC ratio of 10. Again, additional 100 nM peptide were added to the cultures containing peptide pulsed DCs. From day 20 i.e. the

second restimulation, the dendritic cells were omitted from the cultures which had been set up originally with peptide pulsed DCs. Instead, the T cells were restimulated with 100 nM peptide only. The T cells were assayed for cytotoxic activity in ^{51}Cr release assays on day 6 and day 7 after restimulation and were restimulated every two weeks.

III) Cultures using adherent spleen cells or unfractionated spleen cells as APCs

On a few occasions, adherent spleen cells or unfractionated spleen cells were used as APCs. Splenocytes were obtained as described above and allowed to adhere overnight. The following morning, the non-adherent cells would be discarded and the adherent cells would be infected with recombinant adenovirus in a volume of 5 ml E4, 0.5% FCS for 8-12 hours. Alternatively, recombinant adenovirus would be added to the whole flask without removing the non-adherent cells. Following infection, the cells were harvested and washed once. T cell cultures were set up exactly as when using peptide pulsed or virus infected DCs, see above.

Sometimes T cell cultures were set up using a tumour cell line for the first 6 days of *in vitro* restimulation followed by peptide pulsed or vaccinia infected DCs as antigen presenting cells. The conditions will be described for individual lines in chapter 4 and 5.

T cell cloning.

T cells were cloned after having been 34-35 days in culture. The cells were cloned 14 days after the previous restimulation. 96 well plates containing 1 T cell, 10 T cells or 100 T cells per well were set up. In addition to T cells, each well contained 3 x 10^5 irradiated syngeneic spleen cells as feeders and 10 u/ml IL-2. Peptide specific T cell lines were cloned using 100 nM peptide per well.

2.3. Generation of recombinant vaccinia virus and adenovirus

2.3.1. Construction, amplification and analysis of recombinant vaccinia virus

Maps of all vectors used are in section 1.5.

Generation of pSC11 recombinant plasmids

For details of the cloning techniques described below, see section 2.1.2.

The vector, pSC11, was digested with SmaI and dephosphorylated. Inserts were prepared as follows: All cDNAs used in the present work were cut out of their original plasmids by using restriction enzymes generating 5´ overhanging ends. The inserts were resin purified using the Geneclean method and the recessed 3´ends were filled in with the Klenow fragment of DNA polymerase. The blunt ended insert was subsequently ligated to the dephosphorylated pSC11. After transformation and identification of recombinant constructs containing the insert in the correct orientation, a large stock of recombinant DNA was prepared by making maxi preps with the Qiagen method.

Generation of recombinant virus *in vivo*

One day prior to transfection, 2 x 10^6 Tk^-143 cells were seeded in 15 ml RPMI, 10% FCS in a T75 tissue culture flask. The following day, the semiconfluent cell layer was infected with 0.05 pfu per cell wild type vaccinia virus in 2 ml MEM, 2% FCS. The tissue culture flask was gently moved every 30 minutes (virus absorption time) to optimise the number of cells infected. After 2 hours, the inoculum was removed. The infected cells were transfected by the $CaPO_4$ method (section 2.1.3.) using between 10 and 20 μg non-linearised pSC11 vector containing the cDNA of interest. No additional carrier DNA was used. After 48 hours without any selection, the cells were harvested, centrifuged at 300 x g for 5 minutes and resuspended in 1 ml MEM, 2% FCS. Virus was liberated by subjecting the cells to three rounds of freeze/thawing and sonicated briefly. Recombinant virus were identified by titration and selection with BrdU and X-Gal as described below.

Titration of recombinant vaccinia virus

Recombinant vaccinia virus were thymidine kinase negative and were selected by adding BrdU to cultures of virus infected cells. The pSC11 vector contains the β-galactosidase gene, see section 1.5. The presence of this gene allows recombinants to be identified by the formation of blue plaques generated by the conversion of the substrate X-Gal to the blue coloured dye 5,5' -dibromo-4,4'cloroindigo using the procedure described by (354) with some minor modifications.

One day in advance, 6 x 10^5 Tk^-143 cells in E4, 10% FCS were seeded per well in 6 well plates. The following day, 6 dilutions of virus were made in MEM in universals.

The medium was removed from the adherent cells and the cells were washed with 1 ml of MEM per well. 0.25 ml of each virus dilution was added per well and the plate was put in a 37°C incubator. The plates were gently moved every 30 minutes. 2 hours later, the virus inoculum was removed. The inoculum was replaced with 3 ml of a mixture containing equal volumes of : 1) E4, 4% FCS, 50 µg/ml BrdU and 2) E4, 2% low melting point agarose. After 36-48 hours, plaques were visible at some of the virus dilutions depending on the concentration of the virus. At this stage, a second agarose layer consisting of E4, 1% low melting point agarose and 300 µg/ml X-Gal was added. Recombinant virus were identified by blue colour development. To prevent virus inactivation, plaques were picked from the plate with a plastic Pasteur pipette no more than 6 hours after the addition of the X-gal layer. If the colour development was weak, the plate was left for 1/2 hour at room temperature to intensify the colour (354).

For estimation of the entire virus concentration including the concentration of non-recombinant virus, a dilution series of virus was made and added to semiconfluent Tk^- 143 cells as described above. The infected cells were incubated in E4, 2% FCS, 25 µg/ml BrdU with no agarose. The medium was removed after approximately 48 hours. The cells were washed once with PBSA and stained for 1 minute with 1 ml of 1% (w/v) crystal violet in 70% (v/v) ethanol. After removal of the dye, the plates were left to dry in the tissue culture hood. Plaques were identified and counted on the basis of the appearance of holes in the blue background cell layer.

Preparation of a vaccinia virus stock

Virus stocks were generated and maintained by following the procedure recommended by (293) with some minor modifications.

About half the virus recovered from one plaque in a volume of 0.5 ml was used to infect a 25 cm^2 monolayer of Tk^-143 cells which had just reached confluence. After 2 hours the inoculum was removed and replaced with 5 ml E4, 4% FCS, 25 µg/ ml BrdU. After 36-48 hours, most of the cells looked rounded up and plaques were visible. The cells were harvested by scraping with a rubber policeman, resuspended in 2 ml 10 mM Tris-HCl, pH 9, 1 mM EDTA and freeze/thawed 3 times. This small scale stock ("T25 stock") was kept at -70°C.

The T25 stock was used for DNA, mRNA and protein analysis and to generate larger stocks. The stock was thawed and briefly sonicated prior to use. To make a large stock, half the virus from the small stock was incubated with 0.1 volume 2.5 mg/ml trypsin for 30 minutes at 37°C. The virus stock was diluted to 16 ml with MEM, 2% FCS. 2 ml were used to inoculate each of 8 T125 flasks containing a just confluent layer of Tk^-143 cells. After 2 hours the inoculum was removed and 40 ml E4, 4% FCS were added. The cells were incubated for 48 hours, harvested by cell scraping, resuspended in 2 ml MEM per monolayer and freeze/ thawed 3 times.

As an alternative to this, larger stocks could also be generated via an intermediate step. 100 µl virus from a T25 stock was used to infected 5 T75 flasks containing semiconfluent layers of Tk^-143 cells. The cells were harvested after 36-48 hours, lysed, resuspended in 1 ml MEM per T75 flask and freeze/ thawed 3 times. This stock was called "The master stock". 100 µl of master stock was used to infect 10 T125 flasks containing semiconfluent layers of Tk^-143 cells. The cells were harvested after 36-48 hours, lysed, resuspended in 1 ml per T125 flask and freeze/ thawed 3 times. This stock was called the "final stock".

The lysed cells and the supernatant were usually frozen directly at this stage at -70°C. For some later stocks, the cell debris were removed by centrifugation and the supernatant was kept at -70°C.

2.3.2. Construction, amplification and analysis of recombinant adenovirus

Generation of recombinant adenovirus.

Recombinant adenovirus was generated by co-transfecting vector pJM17 with vector pMV60 containing the cDNA of interest into 293 cells by following the guidelines outlined by (316).

The plasmids used were transfected as circular DNA and an equimolar amount of the two plasmids were used. The plasmids were transfected into the 293 cells by using the $CaPO_4$ method described in section 2.1.3. The medium used was MEM, 5% FCS. After transfection, the medium was changed approximately every 2 or 3 day due to the rapid metabolism of the cells. When plaques were visible 7- 8 days after the transfection, the cells were harvested by scraping of the cells with a rubber policeman.

Extraction of adenovirus from infected cells.

Adenovirus were isolated from infected cell by extracting the cells once with Arklone (300). The cells were spun down for 5 minutes at 500 x g. The supernatant was removed as completely as possible and the cells were resuspended in 1 ml PBS per 2 x 10^7 cells or less. The cells were put on ice and one volume Arklone was added. The cells were mixed thoroughly for 4 x 15 seconds on a whirli mixer while being kept on ice in between the mixing. The cells were centrifuged for 5 minutes at 500 x g. The upper aqueous phase containing the virus was carefully removed and stored at -20°C. The aqueous phase was opalescent if the virus had expanded well in the cells. The bottom fluorocarbon phase and the interface were occasionally extracted once more with an equal volume of PBS, but the second extraction rarely increased the virus yield considerably.

Titration and growing of a large stock of adenovirus.

The adenovirus which had been harvested and extracted after the $CaPO_4$ transfection was titrated on layers of 293 cells. 1 x 10^6 293 cells were seeded per well in a 6 well plate. The following day, dilutions of newly generated recombinant adenovirus or a control recombinant adenovirus, Rad35, were done in MEM. 1 ml of each virus dilution was added per well containing 293 cells. 2 hours later, 3 ml E4, 5% FCS was added to each well. The wells were observed for 6-8 days. Wells containing cells which had similar morphology as cells infected with the control Rad35 virus were harvested.

A master stock was produced by using 100 µl of virus extract from one 6 well to infect a semiconfluent layer of 293 cells in a T75 flask. The infected cells were harvested after 5-6 days, spun down and resuspended in 1 ml per T75 flask. The timing of the harvest of the cells depended on the appearance of the cell layer. The virus was extracted from the cells with Arklone. 100 µl of the master stock was used to infect a semiconfluent layer of 293

cells in a T125 flask. The infected cells were harvested after 5-6 days, the virus was extracted with Arklone and stored at -20°C.

To get an estimate of the virus concentration, triplicate dilutions of virus were done in E4, 0.5% FCS in a 96 well flat bottom tissue culture treated plate to give a volume of 100 µl per well. Subsequently 100 µl of 293 cells in E4, 0.5% FCS at a concentration of 3×10^5 cells/ml were added to each well. The plates were put in a 37°C incubator and left for 6 days. The lowest concentration of virus which was seen to give partial destruction of the 293 cell layer was used to calculate the titer of the virus.

3. Generation of expression systems for the production of mdm2, cyclin D1, fibroblast growth factor receptor I and wild type p53 in mammalian cells.

Introduction

The ability of normal self proteins to serve as targets for tumour specific cytotoxic T lymphocytes (CTL) would first be investigated by establishing whether it would be possible to stimulate CTL to recognise and eliminate cells overexpressing selected self proteins. Priming, restimulation and analysis of CTL specific for peptides derived from normal self proteins required the generation of suitable expression systems which would allow the selected self proteins to get access to antigen presenting cells and be presented to CTL *in vivo* and *in vitro*.

The choice of expression systems had to meet several requirements.

1) Firstly, the expression systems should allow the expressed proteins to gain access to antigen presenting cells, be processed endogenously and presented via the MHC class I pathway *in vivo* and *in vitro*. Viral expression systems based on vaccinia virus and adenovirus involve delivery of the relevant cDNA by infection of the appropriate cells. Recombinant protein is produced transiently within the cytosol of infected cells and gain access to the class I pathway. In contrast to the transient viral expression systems, transfection of cells with the cDNA encoding the protein of interest can ensure stable production of protein.

2) Secondly, expression systems producing high levels of protein as well as expression systems producing only moderate levels of protein, relative to the levels found in normal cells, should be available.
Viral expression system such as the vaccinia virus and adenovirus systems produce high levels of protein compared to the levels found in normal cells and should therefore be useful for T cell priming, stimulation and analysis.
Mammalian cells stably transfected with exogenous cDNA express lower levels of protein than the levels which can be achieved by viral infection and this would be useful for restimulation and analysis of already activated T cells. Stably transfected tumour cell lines would also be useful to study the ability of T cells to recognise and eliminate tumour cells overexpressing the protein *in vitro* and *in vivo*.

3) Thirdly, the expression system should allow proteins to be expressed in cells of different haplotypes.
Viral expression systems such as vaccinia virus and adenovirus infect a broad range of cell types independent of haplotype. This makes stimulation and analysis of CTL restricted by different MHC class I alleles easy. In order to get stable transfectants of different haplotypes, it is necessary to transfect different cell lines, a process which is more time consuming than viral infection of different cells with a single stock of virus.

4) It should be possible to achieve protein expression in primary cells *in vitro* and *in vivo.* Viruses such as vaccinia virus and to a less extent adenovirus infect a wide range of cell types including professional antigen presenting cells.

5) The expression system used for *in vivo* immunisation of mice should have an adjuvant effect. Immunisation with recombinant virus such as vaccinia virus and adenovirus induce a local inflammatory response which might contribute to the breakage of tolerance to self proteins.

6) It would be desirable to have more than one expression system for each individual protein to avoid CTL induction and stimulation to components of the vector used to deliver the proteins.

Finally, the choice of expression systems would also depend on properties of the individual proteins under investigation (mdm2, cyclin D1, the fibroblast growth factor receptor I and wild type p53). The expression of each of the four proteins is tightly regulated in the normal cell and overexpression of the proteins could potentially interfere with the growth of the cells expressing the proteins.

mdm2 does not seem to possess any properties which would disfavour the generation of stable cell lines expressing high levels of the protein. It has been possible to achieve stable overexpression of the protein *in vitro* under the control of its own promoter or exogenous stronger promoters in several different cell lines such as Rat2 cells or NIH3T3 cells (241; 355). No detrimental effect on cell growth due to transfection of mdm2 cDNA has been reported. Instead transfection of cells have resulted in immortalisation of primary cells and tumorgenicity of previously non-tumorgenic immortalised cell lines (241). On basis of this information, it was decided to express mdm2 using recombinant vaccinia virus, adenovirus as well as transfectants.

In contrast to mdm2, high levels of exogenously derived cyclin D1 are reported to mostly have a negative effect on cell growth. Despite the protein being expressed at high levels in several tumours, attempts to create stable cells lines which overexpress cyclin D1 has

either been fruitless or resulted in cells with markedly inhibited growth (231; 232; 233; 356). In rodent fibroblasts, cyclin D1 could not be stably expressed under a strong promoter such as the Moloney Murine Leukemia virus promoter or the cytomegalovirus promoter but only under a less strong promoter such as the avian Rous sarcoma virus promoter. On average, a three fold overexpression of the protein could be achieved but as much as five to ten fold overexpression of the protein was possible in NIH 3T3 cells (233). There appears to be an upper limit to the amount of cyclin D1 a cell can tolerate (357). It was therefore decided not to try to make stable transfectants overexpressing cyclin D1 but instead use EL4 thymoma cells for restimulation of T cells. EL4 cells overexpress cyclin D1 presumably due to proviral insertion by Friend murine leukemia virus at the Fis-1 locus upstream of the murine cyclin D1 gene on chromosome 7 (358) and G. Peters, ICRF, personal communication. The EL4 thymoma cell line would therefore provide stable expression of cyclin D1 whereas transient production of the protein would be achieved by using recombinant vaccinia virus and adenovirus.

The murine fibroblast growth factor receptor I (FRI) has been stably expressed in a functional form in a variety of cell lines including chinese hamster ovary cells (CHO), Rat-2 fibroblasts and FDC-P1 myeloid cells and overexpression is not known to have any negative impact on cell proliferation (359). For this reason, it was decided that FRI should be transiently expressed by recombinant vaccinia virus and stably expressed by cell lines of two different haplotypes transfected with the FRI cDNA.

Finally, stable overexpression of wild type p53 protein has been found to have a profound negative effect on cell proliferation in a variety of different cell types. Introduction of WT p53 was found to be incompatible with growth in Saos-2 osteosarcoma cells (360; 361) and S6 myeloid leukemia cells (362). In the latter case, the effect was found to be dose dependent and most likely due to p53 induced apoptosis. In GM47.23 glioblastoma cells (363; 364; 365) or the pre-B cell line L12 (366) overexpression of WT p53 cells did not affect viability but resulted in pronounced slow growth. On the basis of this information, only transient viral expression systems would be generated to deliver WT p53 to antigen presenting cells *in vitro* and *in vivo*.

To summarise, it was decided to generate recombinant vaccinia virus expressing four selected self proteins, cyclin D1, mdm2, wild type p53 (WT p53) and fibroblast growth factor receptor I (FRI) for priming of CTL *in vivo* and restimulation and analysis of CTL *in vitro*. Alternative expression systems based on adenovirus and transfectants would be produced to the widest extent possible. The expression systems which would be generated to prime, stimulate and analyse CTL specific for the four selected self proteins cyclin D1,mdm2, WT p53 and FRI are summarised in Table 3.1. Details of the cloning techniques used to generate the constructs are in chapter 2.

Table 3.1.

Expression systems for priming, stimulation and analysis of CTL *in vivo* and *in vitro*	
Protein	**Expression system**
mdm2	Recombinant vaccinia virus Recombinant adenovirus EL4 cells transfected with mdm2 cDNA P1.HTR cells transfected with mdm2 cDNA
Cyclin D1 (CD1)	Recombinant vaccinia virus Recombinant adenovirus EL4 cells, untransfected
Fibroblast growth factor receptor I (FRI)	Recombinant vaccinia virus Recombinant adenovirus EL4 cells transfected with FRI cDNA P1.HTR cells transfected with FRI cDNA
Wild type p53 (WT p53)	Recombinant vaccinia virus Recombinant adenovirus

3.1. Generation of mdm2 expression systems

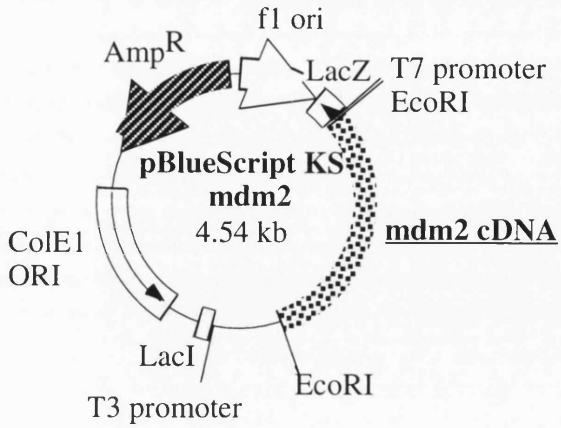
The mdm2 cDNA in the vector pBluescript KS^- was a generous gift from Dr. A. Levine, Princeton University. The sequence of the cDNA was retrieved from the EMBL data base, accession number X58876.

3.1.1. Generation of recombinant vaccinia virus expressing mdm2

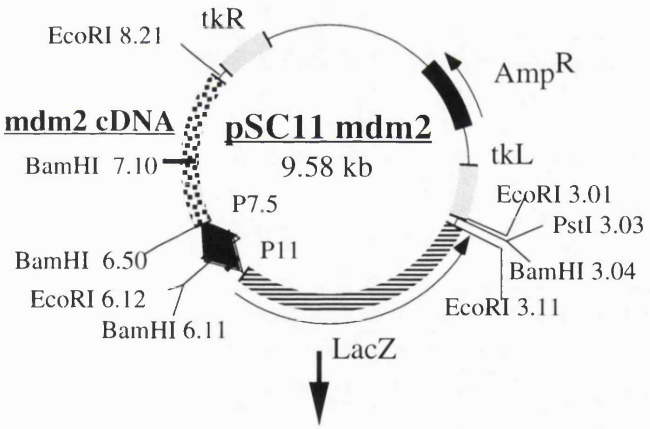
The procedure used to generate recombinant vaccinia virus expressing mdm2 is outlined in figure 3.1.1.

The entire murine 1.7 kb mdm2 cDNA was cloned into the SmaI site of pSC11 by blunt end ligation and used to transform *E. coli* XL1 Blue cells. Plasmid DNA from transformed cells was analysed for the presence and orientation of the mdm2 cDNA by restriction digestion with BamHI which cuts pSC11 a few nucleotides upstream of the SmaI cloning site in pSC11 and murine mdm2 cDNA at position 601 (see Figure 3.1.1.). Plasmid DNA producing a novel 0.6 kb BamHI fragment upon digestion was subjected to PCR analysis using primers spanning nucleotide 704 to 1261 of the mdm2 cDNA to confirm the identity of the insert (data not shown). Plasmid pSC11 mdm2 was selected and amplified.

Recombinant vaccinia virus expressing mdm2 was obtained by homologous recombination of pSC11 mdm2 with wild type vaccinia virus in the thymidine kinase negative human cell line Tk^-143. The recombinant virus was subjected to four rounds of plaque purification. Two plaques ,VV mdm2 3.1.2. and mdm2 3.2.1., were picked from the final round of purification and analysed. Virus stocks prepared from the plaques appeared to contain more than 95% recombinant virus as determined by comparing the ability of the stocks to produce plaques expressing β-galactosidase (blue plaques) with the overall ability to produce plaques as determined by staining of infected cell layers with crystal violet. Due to the number of plaque purifications used and the purity of the viral stocks, the preparations were considered to be derived from single virus clones and will be referred to as clonal.



1) EcoRI digestion of pBlueScript KS⁻ mdm2.
2) Purification of 1.7 kb EcoRI fragment containing mdm2 cDNA.
3) Fill in of 3' recessed ends with Klenow fragment of DNA polymerase I.
4) Digestion of pSC11 with SmaI.
5) Dephosphorylation of pSC11.
6) Ligation of 1.7 kb mdm2 fragment to pSC11.



1) Generation of recombinant vaccinia virus by homologous recombination of pSC11 mdm2 with wild type vaccinia virus VV WT in Tk⁻143 cells.
2) Plaque purification.
3) Analysis of clone VV mdm2 3.1.2. and clone VV mdm2 3.2.1.

Figure 3.1.1.
Outline of the procedure used to generate recombinant vaccinia virus expressing murine mdm2.

Integration of the entire mdm2 cDNA in the vaccinia virus genome was confirmed by Southern blot analysis of DNA from cells infected with the recombinant virus (Figure 3.1.2.)

10^7 Tk^-143 cells were either not infected or infected with wild type vaccinia virus or one of the two recombinant vaccinia virus mdm2 stocks at 1 pfu per cell. Cellular DNA was isolated 15 hours post infection. 15 μg DNA from each sample was digested with EcoRI and BamHI, (Figure 3.1.1.). The combined action of these two enzymes would cut out any fragment cloned into the SmaI site and additionally cut mdm2 at the internal BamHI site at nucleotide 601 in the mdm2 cDNA. The DNA fragments were separated by agarose gel electrophoresis, transferred to a nylon membrane and probed with the entire mdm2 cDNA.

Figure 3.1.2. shows that EcoRI and BamHI digested DNA derived from cells infected with the recombinant vaccinia virus mdm2 stocks, VV mdm2 3.1.2. and VV mdm2 3.2.1., contained 0.6 kb and 1.1 kb fragments hybridising to the mdm2 cDNA. The size of these bands corresponded to the expected size based on the nucleotide sequence of the mdm2 cDNA and pSC11. The two bands were also present in the positive control, pSC11 mdm2 DNA digested with EcoRI and BamHI. No corresponding bands were found in uninfected Tk^-143 cells or Tk^-143 cells infected with wild type vaccinia virus. Clone VV mdm2 3.2.1. was used for further analysis.

The murine and human mdm2 coding regions are 80.3% homologous on the nucleotide level (254). Stringent washing conditions including a final wash at 68°C in 0.2 x SSC was used for the blot shown in Figure 3.1.2. The T_m of a DNA-DNA hybrid is expected to decrease by 1-1.5°C with every 1% decrease in homology (318) so the washing conditions most likely accounts for the insignificant background of endogenous mdm2 bands seen in the human Tk^-143 cells.

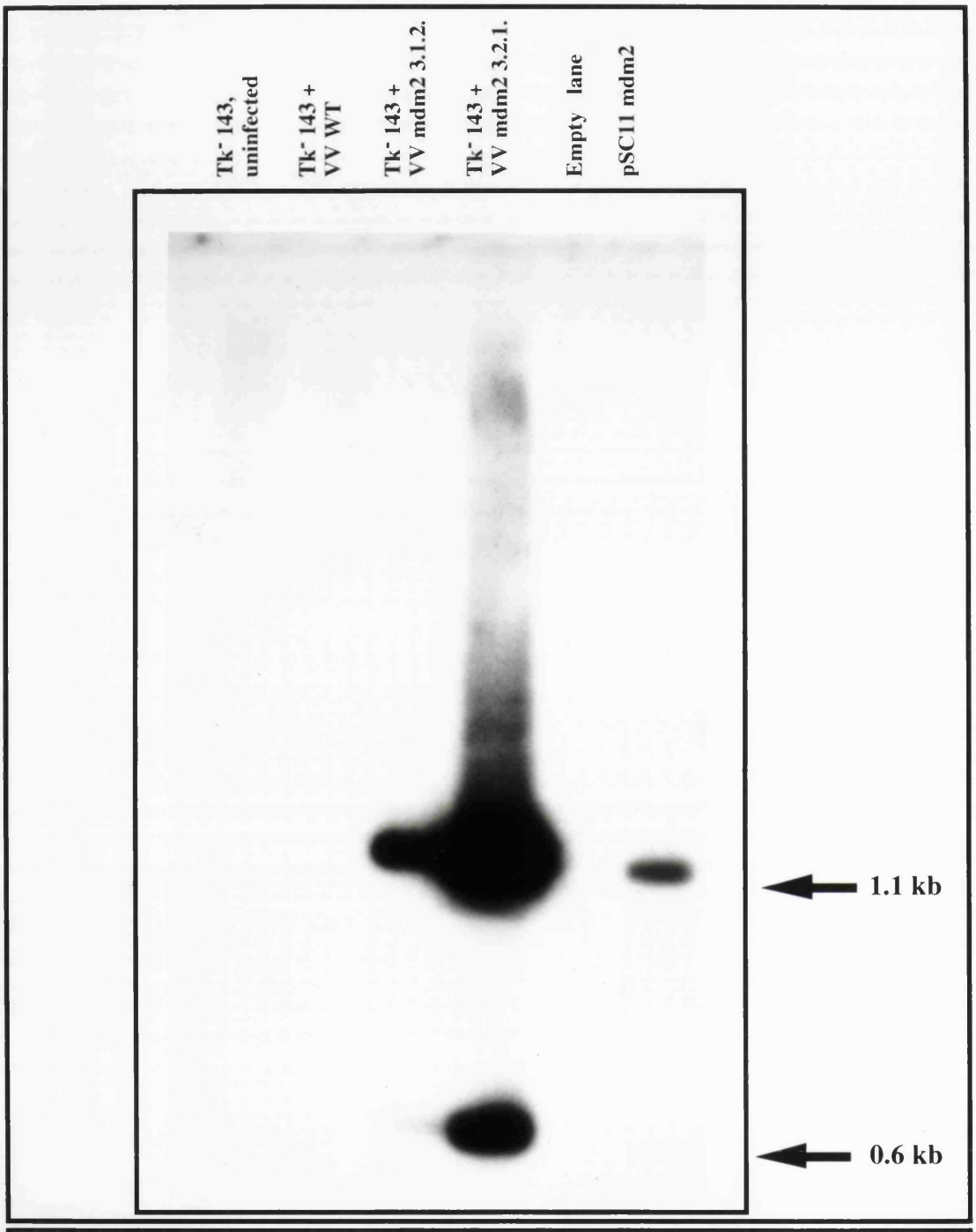


Figure 3.1.2.

Presence of mdm2 cDNA in Tk$^-$143 cells infected with recombinant vaccinia virus mdm2.

Southern blot of DNA from uninfected Tk$^-$143 cells, Tk$^-$143 cells infected with wild type vaccinia virus (VV WT) or recombinant vaccinia virus mdm2 (VV mdm2 3.1.2. or VV mdm2 3.2.1.). Tk$^-$143 cells were infected with 1 pfu virus per cell over night. Cellular DNA was isolated, digested with EcoRI and BamHI, separated in a 1 % agarose gel, transferred to a nylon membrane and probed with a 1.7 kb fragment containing the entire mdm2 cDNA. pSC11 mdm2 DNA digested with EcoRI and BamHI was used as positive control.

mRNA production in cells infected with recombinant VV mdm2 3.2.1. was analysed by RT-PCR, Figure 3.1.3.

Tk^-143 cells were either not infected with virus or infected for 12 hours with 5 pfu recombinant or wild type vaccinia virus per cell. The cells were harvested at 12 hours post infection as there are reportedly two peaks in the transcription activity of the P7.5 promoter which drives the transcription of the mdm2 insert in pSC11. These peaks are about 2 hours post infection and at 12 hours post infection (298). Following isolation, total RNA was reverse transcribed and analysed by PCR by using the mdm2 primers spanning bp 704 to bp 1261 which selectively amplify a 558 bp fragment in the murine mdm2 cDNA sequence (254). Since the sequence homology between mouse and human mdm2 correspond to 70% identity for the sense primer and 75% identity for the antisense primer it should be possible to selectively amplify mdm2 cDNA.

Figure 3.1.3. shows that reverse transcribed RNA derived from Tk^-143 cells infected with VV mdm2 3.2.1 generated the correct 558 bp fragment in the PCR reaction whereas this fragment was not seen in samples from uninfected Tk^-143 cells or Tk^-143 cells infected with wild type vaccinia virus.

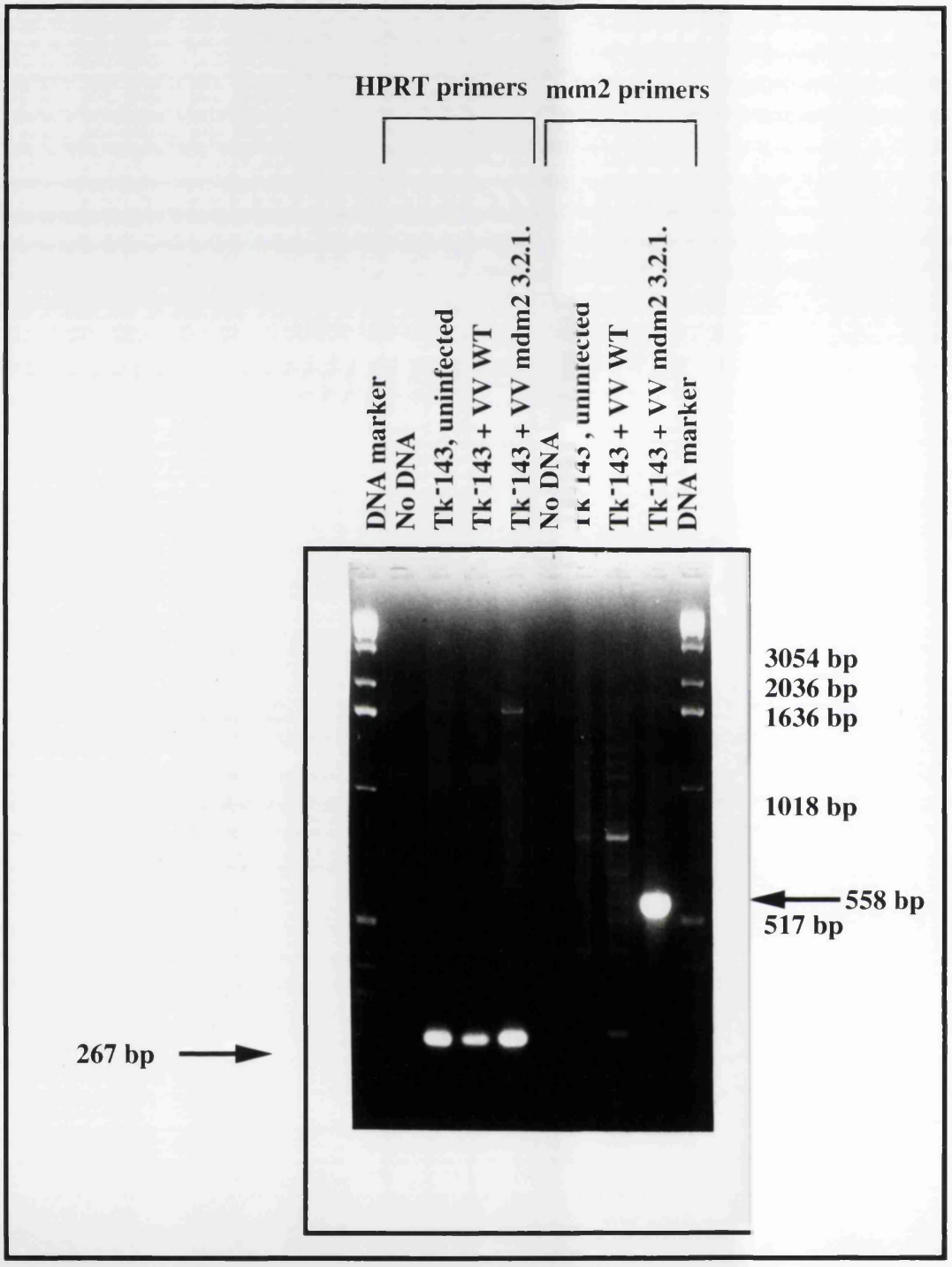


Figure 3.1.3.

Production of murine mdm2 mRNA in Tk⁻143 cells infected with recombinant vaccinia virus mdm2.

RT-PCR of total RNA isolated from Tk⁻143 cells infected with VVWT or VV mdm2 3.2.1.. 4 x 10^6 cells were infected over night with 5 pfu virus per cell or left uninfected. Total RNA was isolated, reverse transcribed and subjected to RT-PCR using mdm2 primers or murine HPRT primers as positive control.

Protein expression in cells infected with recombinant vaccinia virus mdm2 was examined by immunoprecipitation of cytosolic protein with a monoclonal antibody specific for human mdm2, Figure 3.1.4.

5 x 10^6 CHO cells were either left uninfected or infected for 2 hours with approximately 40 pfu per cell of recombinant vaccinia virus mdm2 3.2.1. or a control recombinant vaccinia virus p53 (see later). The cells were labelled with S^{35} methionine for 10 hours without removing the virus inoculum before being harvested and lysed at 12 hours post infection. Proteins from the pre-cleared cell lysate containing cytosolic proteins were incubated with a mouse monoclonal antibody 2A10 specific for amino acid 294-339 in the human mdm2 sequence. The 2A10 antibody also reacts with murine mdm2 (B. Elenbaas, Princeton University, personal communication). The corresponding murine sequence differs in only three amino acids in this sequence (corresponding to amino acid 292 to 337 in the murine sequence). The antibody protein conjugates were precipitated by reaction with Protein A Sepharose and separated by SDS-PAGE. In proteins precipitated from cells infected with recombinant vaccinia virus mdm2 a band of 90 kDa was more prominent than in the other samples. This could correspond to the 90 kDa mdm2 protein described in the literature (322). The level of mdm2 protein found in vaccinia mdm2 infected cells was not high compared to the background, but not detectable in uninfected cells. A 90 kDa band was also present but at lower levels in cells infected with control virus recombinant vaccinia virus p53. The presence of the band in vaccinia virus p53 infected cells is thought to be due to the fact that p53 positively controls the transcription of the mdm2 gene (242; 367; 368) and mdm2 and p53 can be co-precipitated with each other (322).

In conclusion, a vaccinia virus recombinant expressing mdm2 had been generated. Cells infected with the virus contained the full length mdm2 cDNA as shown by Southern blot analysis, the correct mRNA as determined by RT-PCR analysis and the protein as showed by immunoprecipitation.

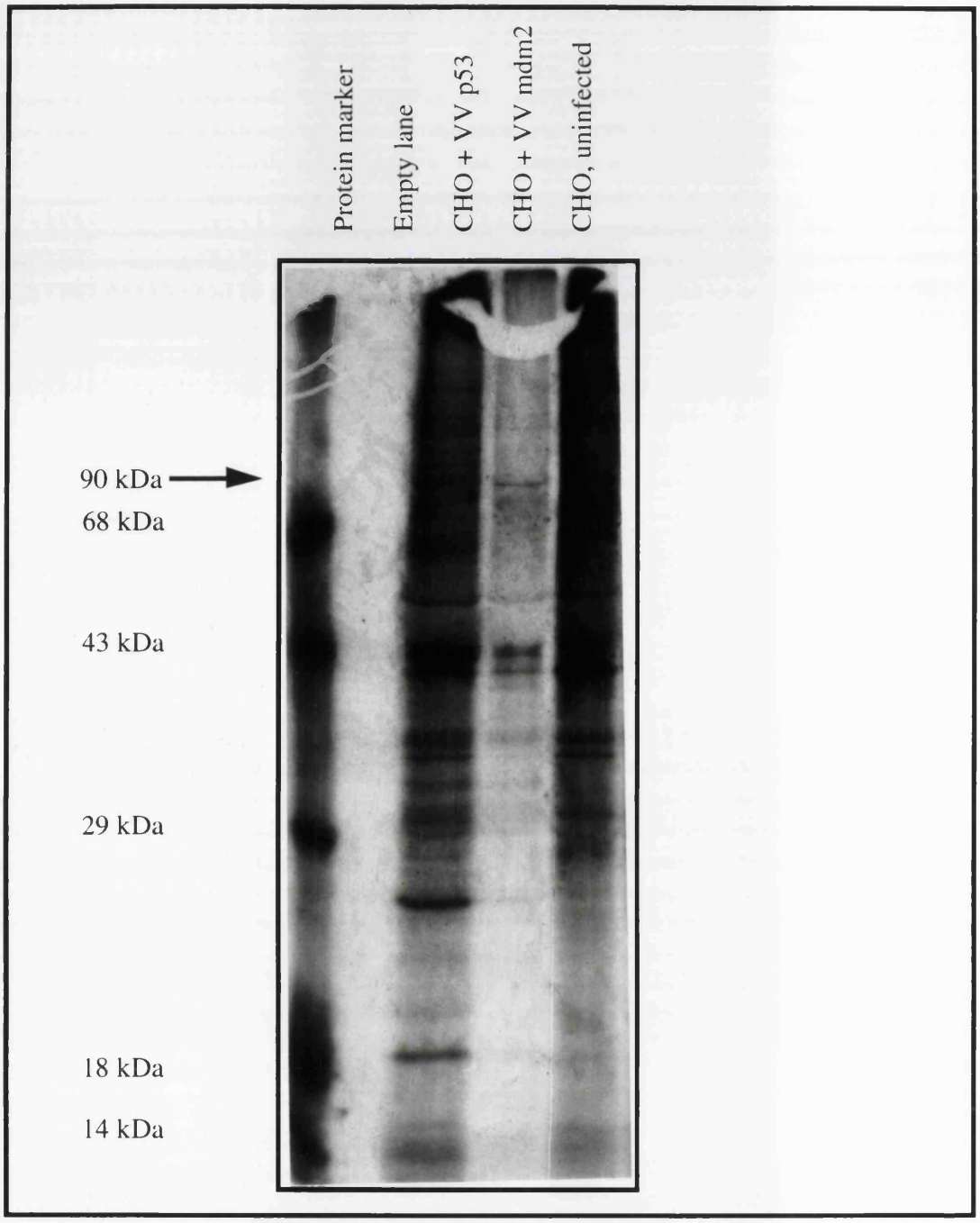


Figure 3.1.4.
Production of mdm2 protein in CHO cells infected with recombinant vaccinia virus mdm2, clone 3.2.1.

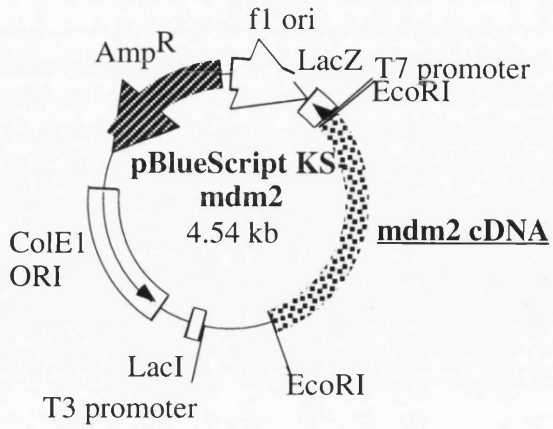
5 x10^6 CHO cells were infected for 2 hours with 40 pfu per cell of VV mdm2 3.2.1 or control recombinant vaccinia virus p53 (VV p53). Cells were labelled with S^{35} methionine for additional 10 hours. Cells were lysed, pre-cleared and proteins were immunoprecipitated with 100 µl 2A10 supernatant. The precipitates were collected, washed and separated by SDS-PAGE using a 12% (T) separating gel.
The gel was dried and exposed to autoradiography.

3.1.2. Generation of recombinant adenovirus expressing mdm2

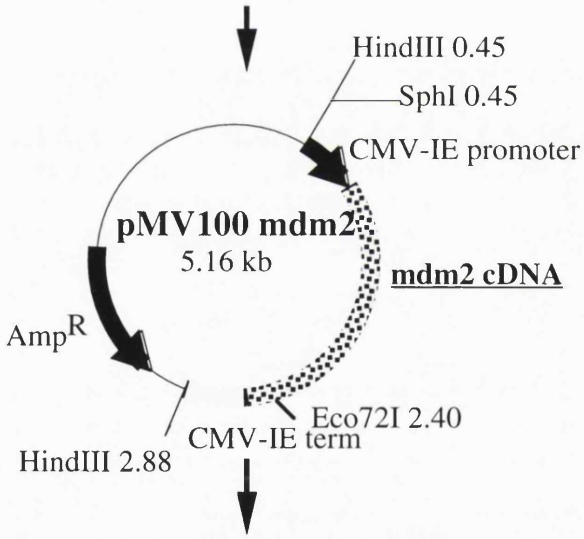
The procedure used to construct recombinant adenovirus expressing mdm2 is outlined in Figure 3.1.5.

The entire murine mdm2 cDNA was cloned as a 1.7 kb fragment into the XbaI site in the CMV-IE expression cassette in vector pMV100 by blunt end ligation. *E. coli* XL1 Blue cells were transformed with the ligation mixture and recombinants were identified by isolation of plasmid DNA from transformed colonies and digestion of the DNA with HindIII. There are two HindIII sites flanking each side of the CMV-IE expression cassette, see Figure 3.1.5. To determine the correct orientation of the insert, recombinant DNA was subjected to restriction enzyme digestion with SphI and Eco72I. The mdm2 cDNA contains a unique Eco72I site at position 1584 in the cDNA and pMV100 contains a unique SphI site at the beginning of the CMV-IE expression cassette, approximately 370 bp upstream of the original Xba I site, see Figure 3.1.5. Clones which yielded a 2.0 kb fragment upon SphI and Eco72I digestion were considered to have the insert in the correct orientation. One such clone was used to subclone the 2.4 kb CMV-IE mdm2 cassette into the HindIII site of pMV60. *E. coli* XL1 Blue cells were transformed with the ligation mixture and recombinants were identified by digestion of plasmid DNA with HindIII. The orientation of the CMV-IE expression cassette within the pMV60 vector is irrelevant with respect to the amount of protein produced by recombinant virus (311).

Recombinant adenovirus was generated by homologous recombination of vector pJM17 and pMV60 mdm2 in 293 cells by using the $CaPO_4$ transfection method. The transfection was carried out in T75 flasks and the whole content of the flask was harvested at day 8 after transfection. A dilution series of recombinant virus was subsequently made in 6 well plates containing 293 cells. A dilution series was also made with a control adenovirus Rad35, which expresses β- galactosidase (311). The control virus provided an important help in the identification of recombinant adenovirus on basis of the morphology of the cells. The 293 cells looked rounded up and some clumped together and eventually the whole cell layer started detaching. Adenovirus was harvested and extracted from wells with cells with this characteristic morphology and which had been infected with the highest possible dilution of the virus. Stocks were prepared by using the extracted virus to infect 293 cells in T75 flasks. These stocks were used for analysis of the recombinant virus.



1) EcoRI digestion of pBlueScriptKS$^-$ mdm2.
2) Purification of 1.7 kb EcoRI fragment containing mdm2 cDNA
3) Fill in of 3'recessed ends on mdm2 fragment.
4) Digestion of pMV100 with XbaI.
5) Fill in of 3' recessed ends on pMV100.
6) Dephosphorylation of pMV100.
7) Ligation of 1.7 kb mdm2 fragment to pMV100.



1) Digestion of pMV100 mdm2 with HindIII.
2) Digestion of pMV60 with HindIII.
3) Dephosphorylation of pMV60
4) Subcloning of 2.4 kb HindIII mdm2 expression casette into pMV60
5) Generation of recombinant adenovirus by homologous recombination with pJM17in 293 cells.
6) Analysis of recombinant Ad mdm2.

Figure 3.1.5.
Outline of the procedure used to generate recombinant adenovirus expressing murine mdm2.

To determine whether the recombinant adenovirus contained full length mdm2 cDNA, cellular DNA from infected cells was analysed by Southern blot analysis, Figure 3.1.6.

Preliminary experiments had established that the chinese hamster ovary cell line CHO and the murine thymoma cell line EL4 could be productively infected with the control β-galactosidase expressing adenovirus Rad35 by assaying for β-galactosidase activity with the substrate X- Gal (not shown).

EL4 and CHO cells were infected overnight with the mdm2 recombinant adenovirus or control adenovirus Rad35. Cellular DNA was isolated, digested with HindIII, separated by agarose gel electrophoresis, transferred to a nylon membrane and probed with the entire murine mdm2 cDNA. As shown in Figure 3.1.6, DNA from cells infected with the recombinant adenovirus mdm2 contained a 2.4 kb HindIII band detected with the mdm2 cDNA probe corresponding to the mdm2 expression cassette. The 2.4 kb band was not seen in uninfected EL4 cells or uninfected CHO cells or CHO cells infected with the control adenovirus.

To confirm that mdm2 mRNA was being produced, CHO cells were either left uninfected or infected for 14 hours with control adenovirus (Rad35) or Ad mdm2. Total RNA was isolated and analysed by RT-PCR using specific mdm2 primers spanning bp 704 to 1261 in the cDNA sequence. HPRT primers were used as positive control and were added together with the mdm2 primers. As shown in Figure 3.1.7, only cells which had been infected with Ad mdm2 contained the expected 558 bp band. The band was not seen in uninfected CHO cells or in CHO cells infected with control virus. Since EL4 cells express endogenous mdm2 cDNA they were not used for this experiment.

To confirm that full length mRNA was being produced in adenovirus mdm2 infected cells, CHO cells and EL4 cells were infected with Ad mdm2. Total RNA was isolated and subjected to Northern blot analysis, Figure 3.1.8. In all cells, the mdm2 probe cross-hybridised specifically to the endogenous 3.3 kb transcript (241). An approximately 2.1 kb transcript corresponding in size to the expected recombinant mdm2 transcript was seen only in CHO cells infected with Ad mdm2. Surprisingly, the transcript was not present in the Ad mdm2 infected EL4 cells although the cloned mdm2 DNA had been shown to be present in these cells, see Figure 3.1.6. It is possible that EL4 cells are not infected as efficiently as CHO cells are.

In conclusion, recombinant adenovirus mdm2 had been produced. The full length cDNA was produced in EL4 cells and CHO cells infected with the virus. The correct mRNA was being produced in CHO cells as determined by RT-PCR and Northern blot analysis.

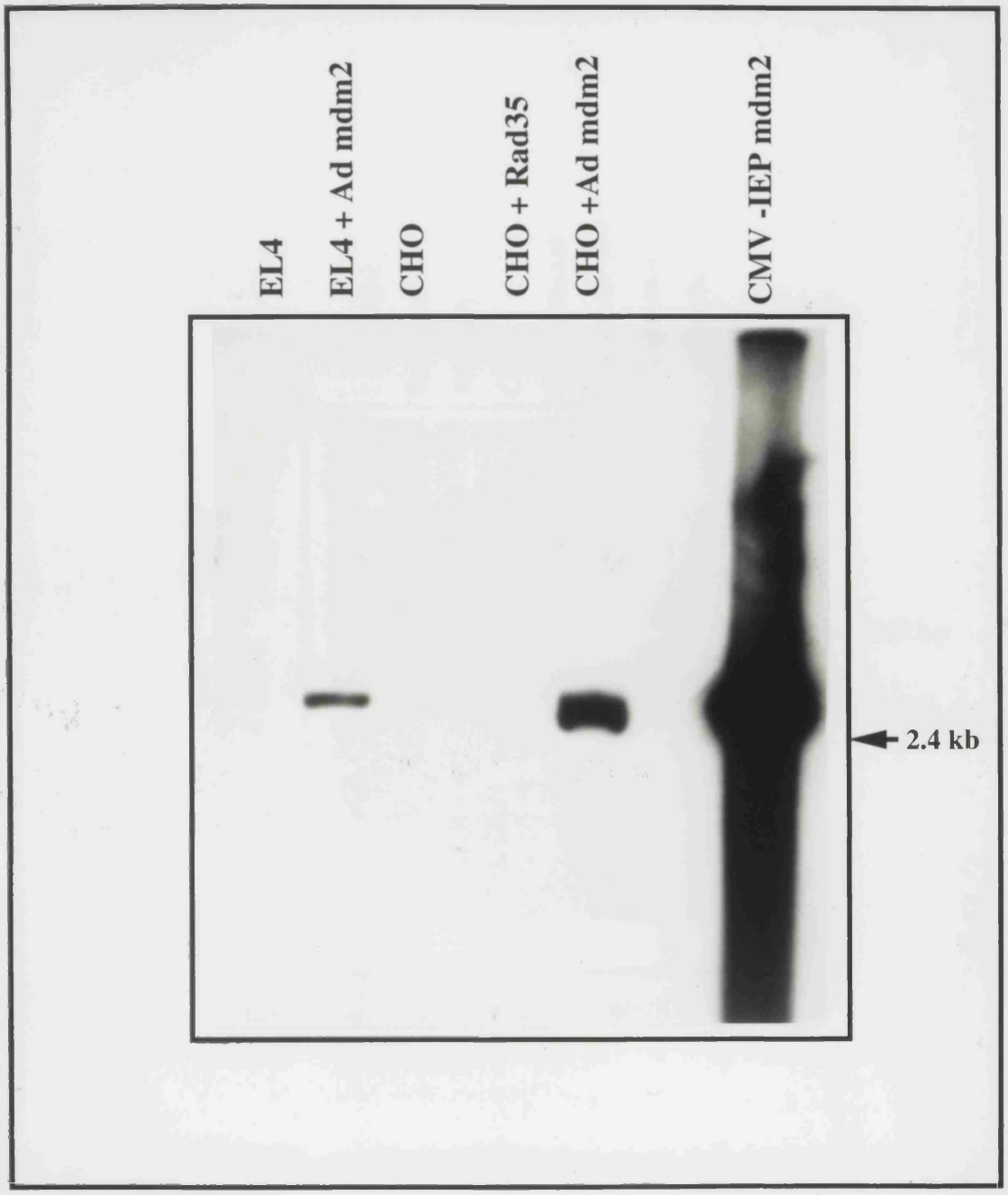


Figure 3.1.6.
Presence of mdm2 cDNA in CHO cells and EL4 cells infected with recombinant adenovirus mdm2 (Ad mdm2).
Southern blot of DNA from EL4 or CHO cells infected with Ad mdm2 or a control adenovirus (Rad 35). 4 x 10^6 cells were infected overnight with recombinant virus or left uninfected. Cellular DNA was isolated, digested with HindIII, separated in a 1 % agarose gel, transferred to a nylon membrane and probed with the entire murine mdm2 cDNA in a 1.7 kb EcoRI fragment. A lane containing the 2.4 kb CMV-IE mdm2 expression cassette was used as positive control.

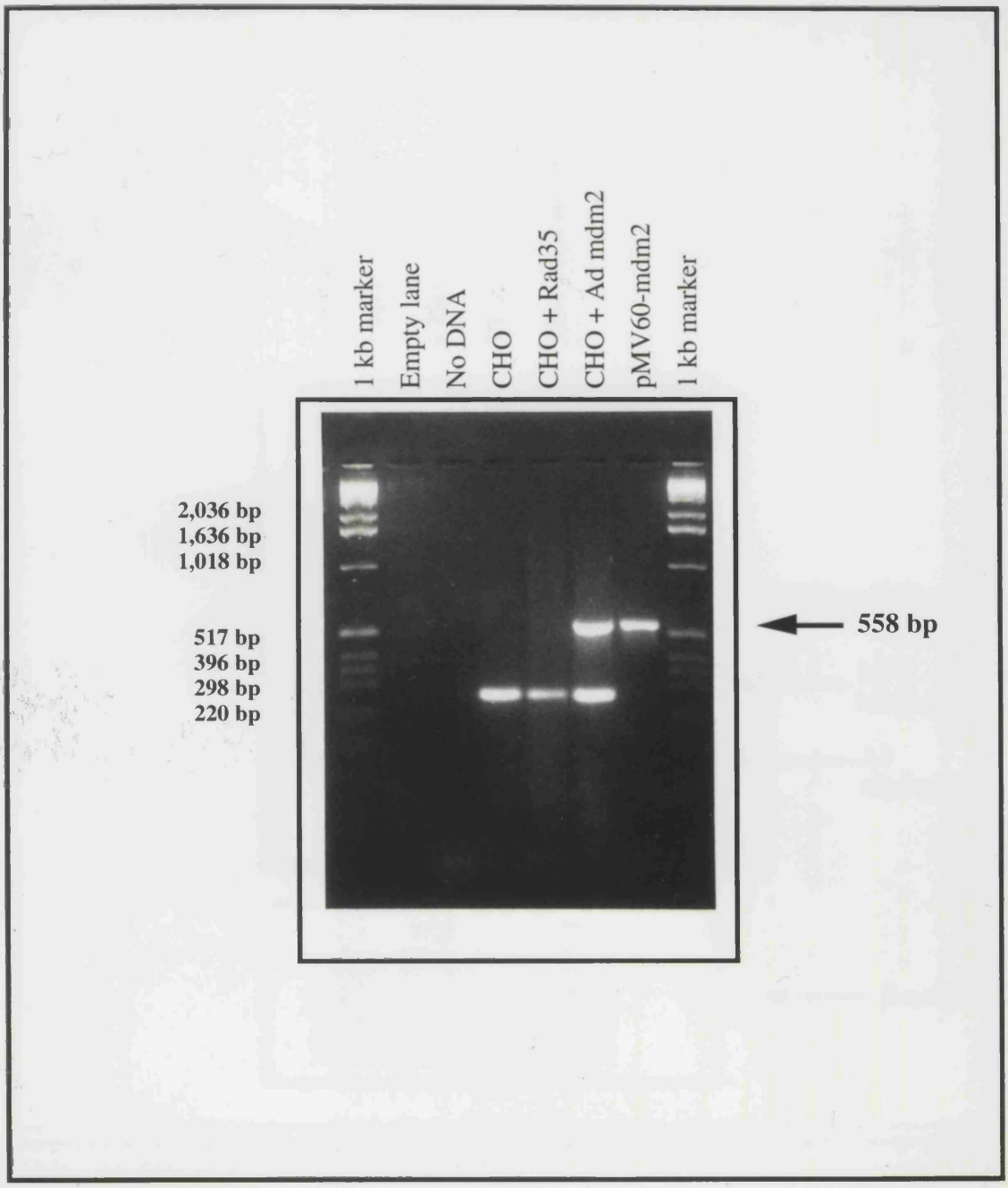


Figure 3.1.7.
Production of mdm2 mRNA in CHO cells infected with recombinant adenovirus mdm2.
RT-PCR of total RNA isolated from CHO cells infected with Ad mdm2 or control Rad35. 5 x 10^6 CHO cells were infected for 14 hours with Ad mdm2 or Rad35 or left uninfected. Total RNA was isolated, reverse transcribed and subjected to PCR using mdm2 specific primers and HPRT primers as positive control primers. pMV60 mdm2 plasmid DNA was used as positive control in the PCR reaction.

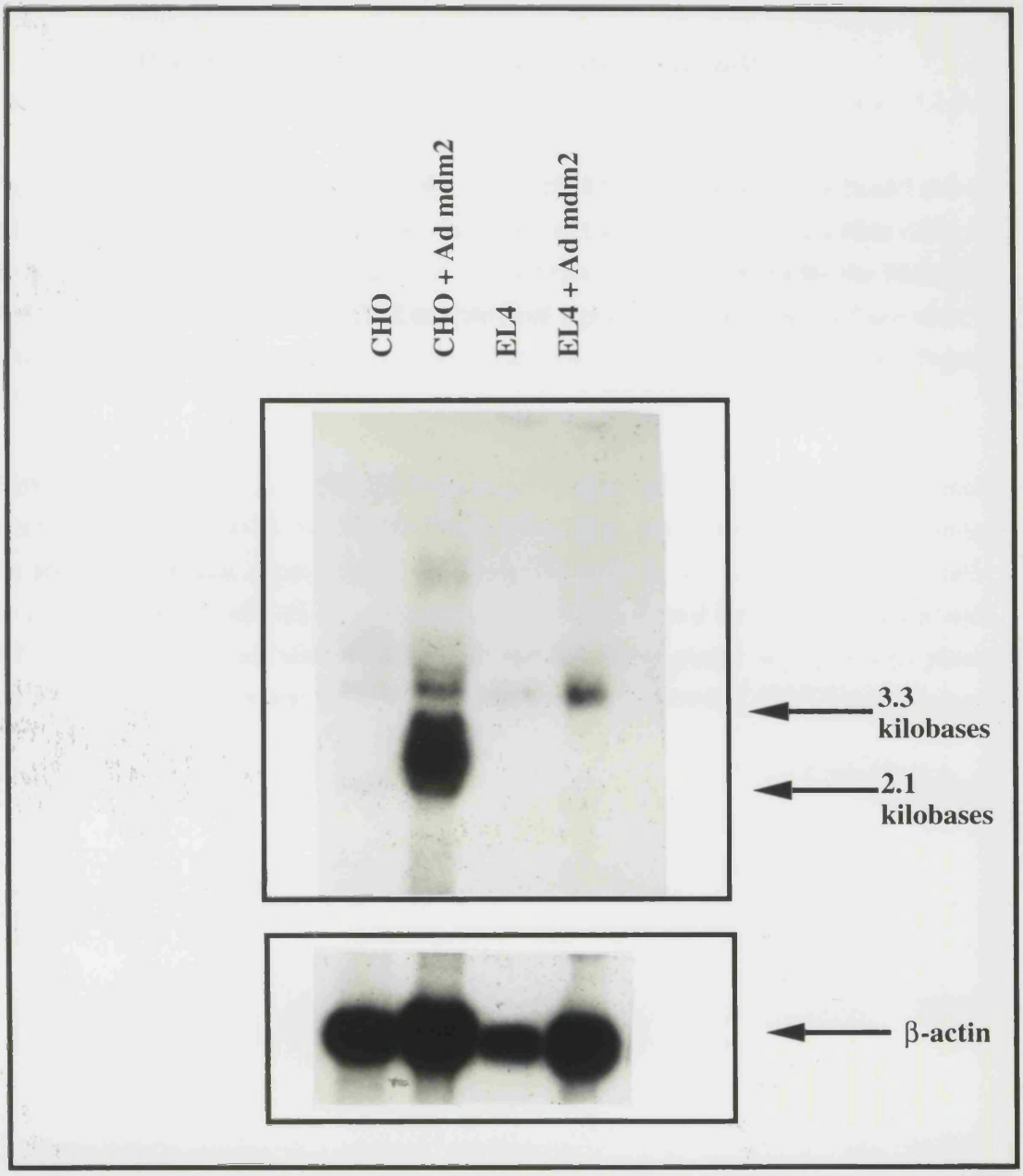


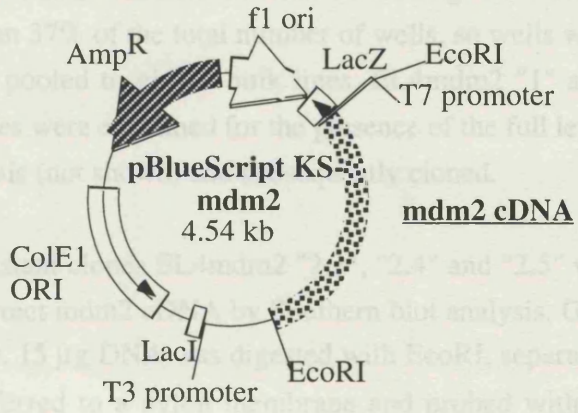
Figure 3.1.8.
Production of endogenous and exogenous mdm2 mRNA transcripts in adenovirus mdm2 infected CHO cells.
Northern blot of total RNA isolated from EL4 or CHO cells infected with Ad mdm2. 5 x 10^6 CHO cells or EL4 cells were infected for 8 hours with Ad mdm2 or left uninfected. The cells were harvested. Total RNA was isolated, separated in a 1% agarose gel with formaldehyde and transferred to a nylon membrane. The RNA on the blot was probed with the entire murine mdm2 cDNA and later reprobed with the murine β-actin cDNA. The endogenous mdm2 transcript is 3.3 kilobases and the exogenous mdm2 transcript is 2.1 kilobases.

3.1.3. Generation of cell lines with stable overexpression of mdm2

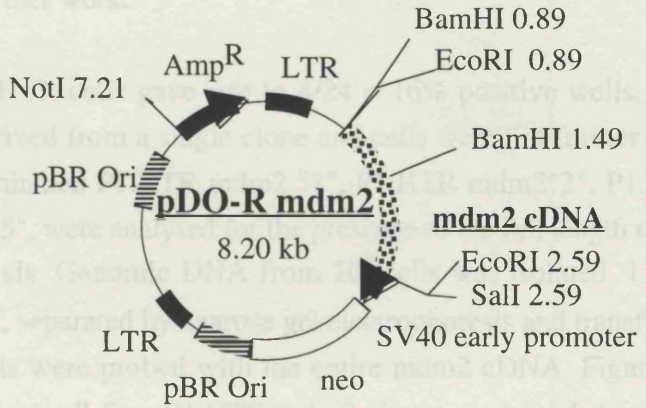
The procedure for generating transfectants expressing mdm2 is outlined in Figure 3.1.9.

The 1.7 kb EcoRI fragment containing the mdm2 cDNA was cloned into the EcoRI site of the pDO-R neo vector (330) which was then used to transform *E. coli* XL1 Blue cells. In the pDO-R neo vector, transcription of the foreign cDNA is driven by the Moloney murine Leukemia virus (Mo MuLV) Long terminal repeat (LTR) promoter and selection is achieved by the presence of the neo gene which confers resistance to G418, see Figure 3.1.9.

Plasmid DNA from transformed colonies was isolated and analysed by digestion with BamHI. There is a BamHI site in pDO-R neo immediately upstream of the EcoRI cloning site and a BamHI site at position 601 in the mdm2 cDNA, see Figure 3.1.8. EL4 cells (H-2^b) and P1HTR cells (H-2^d) respectively were transfected by electroporation with pDO-R mdm2 linearised with NotI. Transfected cells were plated out in 24 well plates and selection with G418 was initiated at 48 hours post transfection.



1) EcoRI digestion of pBlueScript KS$^-$ mdm2.
2) Purification of 1.7 kb EcoRI fragment containing mdm2 cDNA.
3) Digestion of pDO-R neo with EcoRI.
4) Dephosphorylation of pDO-R neo.
5) Ligation of 1.7 kb mdm2 fragment to pDO-R neo to generate pDO-R mdm2.



1) Linearisation of pDO-R mdm2 with NotI.
2) Transfection of P1.HTR cells and EL4 cells.
3) Cloning of transfectants.
4) Analysis of EL4 mdm2 (EM) clones 2.1.,2.2 and 2.4. and P1.HTR mdm2 (PM) clones PM 1, 2, 4 and 5.

Figure 3.1.9.
Outline of the procedure used to generate transfected cells expressing murine mdm2.

After 15 days on selection, the number of wells containing G418 sensitive cells (dead cells) only was less than 37% of the total number of wells, so wells with G418 resistant cells (live cells) were pooled to give 2 bulk lines, EL4mdm2 "1" and EL4mdm2"2". These two bulk cell lines were examined for the presence of the full length mdm2 cDNA by Southern blot analysis (not shown) and subsequently cloned.

Three stable G418 resistant clones EL4mdm2 "2.2", "2.4" and "2.5" were examined for the presence of the correct mdm2 cDNA by Southern blot analysis. Genomic DNA was isolated from 10^7 cells. 15 µg DNA was digested with EcoRI, separated by agarose gel electrophoresis, transferred to a nylon membrane and probed with the entire mdm2 cDNA. As shown in Figure 3.1.10., EcoRI digested DNA from one of the three clones, EL4 mdm2 "2.2" contained a 1.7 kb band hybridising to the mdm2 cDNA. The size of the band corresponded to the size of the intact mdm2 cDNA. The two other clones seemed to have lost the mdm2 cDNA despite the fact that they were G418 resistant. The 1.7 kb band was not present in the DNA derived from untransfected EL4 cells.
Both untransfected and transfected EL4 cells gave rise to some big DNA fragments which hybridised to the mdm2 cDNA. These fragments were presumably derived from the endogenous mdm2 gene or due to the presence of repeats in the probe.
In summary, as EL4 mdm2 "2.2" contained the correct mdm2 cDNA, it was decided to use this clone for further work.

Transfection of P1.HTR cells gave rise to 4/24 = 16% positive wells. Each well was considered to be derived from a single clone and cells were not further subcloned. The transfectants, denominated P1.HTR mdm2 "1", P1.HTR mdm2"2", P1.HTR mdm2"4" and P1.HTR mdm2"5", were analysed for the presence of the full length mdm2 cDNA by Southern blot analysis. Genomic DNA from 10^7 cells was isolated. 15 µg DNA was digested with EcoRI, separated by agarose gel electrophoresis and transferred to a nylon membrane. The blots were probed with the entire mdm2 cDNA. Figure 3.1.11 shows that DNA derived from all four P1.HTR mdm2 clones contained the expected 1.7 kb band hybridising to the mdm2 cDNA probe. This band was not seen in DNA derived from untransfected P1.HTR cells. The presence of other bands hybridising to the probe was again thought to be due to the presence of endogenous genomic mdm2 and repetitive sequences in the probe. It was decided to use clone P1.HTR mdm2 "1" for further analysis.

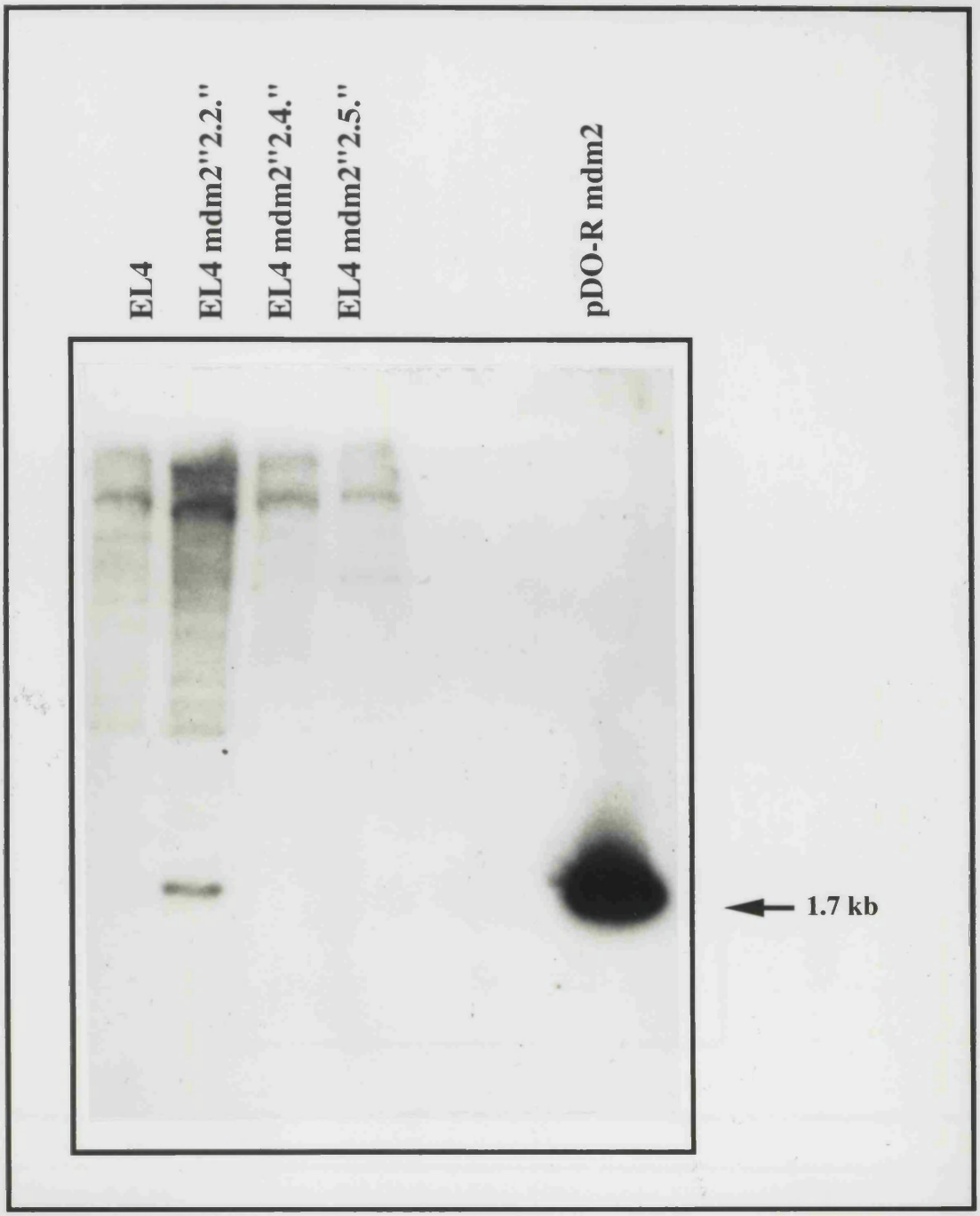


Figure 3.1.10.
Integration of the entire mdm2 cDNA in the EL4 mdm2 "2.2." clone.
Southern blot of genomic DNA isolated from untransfected EL4 cells or clones derived from EL4 cells transfected with plasmid pDO-R mdm2: EL4 mdm2 "2.2.", EL4 mdm2 "2.4." and EL4 mdm2 "2.5.".

Genomic DNA was isolated from 10^7 cells. 15 µg DNA was digested with EcoRI, separated in a 1% agarose gel and transferred to a nylon membrane.
The blot was probed with the 1.7 kb fragment containing the entire murine mdm2 cDNA. Positive control was pDO-R mdm2 DNA digested with EcoRI.

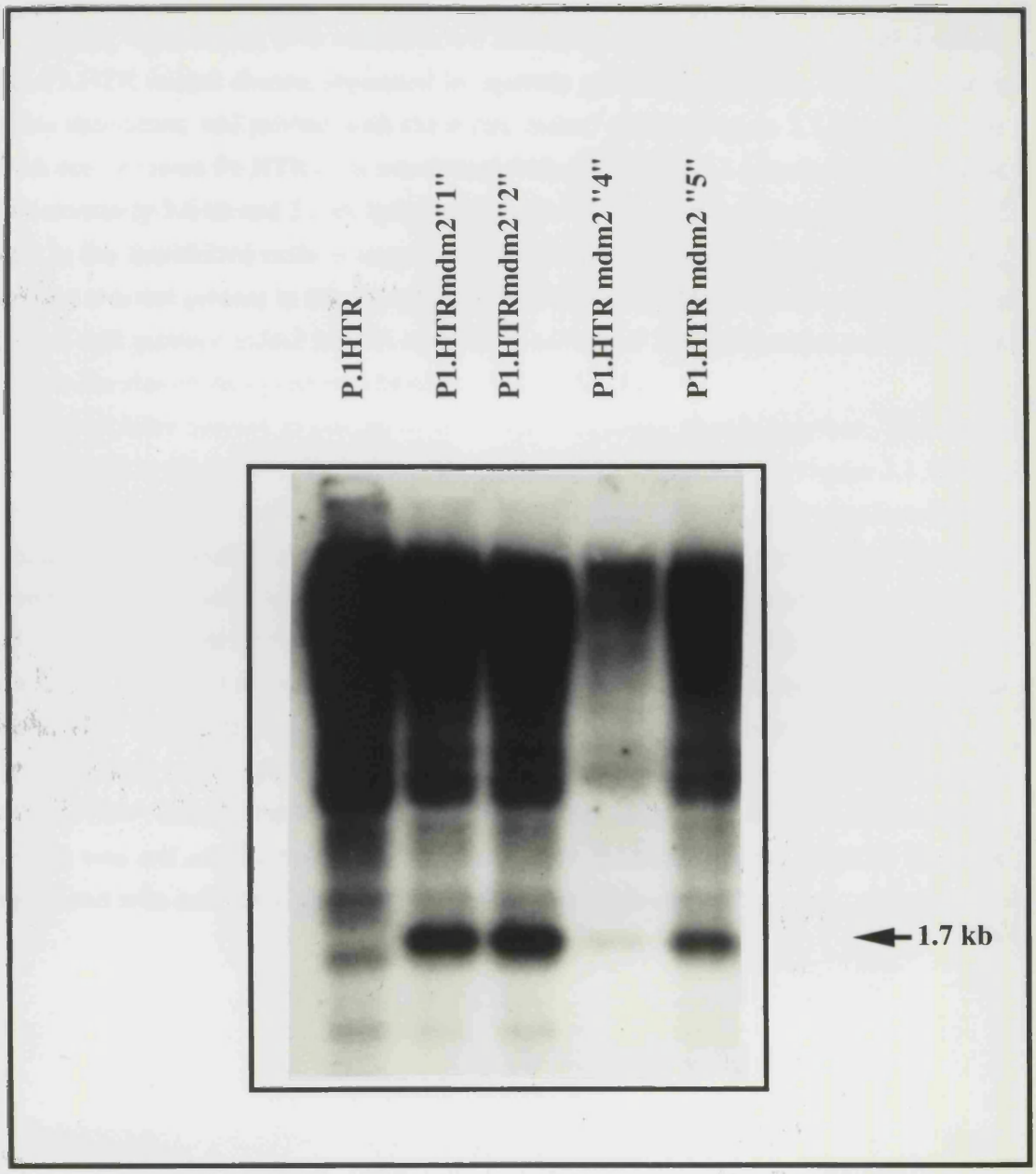


Figure 3.1.11.
Integration of the entire mdm2 cDNA in 4 different P1.HTR mdm2 clones. Southern blot of genomic DNA isolated from untransfected P1.HTR cells or clones derived from P1.HTR cells transfected with pDO-R mdm2: P1.HTR mdm2"1", P1.HTR mdm2 "2", P1.HTR mdm2 "4" and P1.HTR mdm2 "5". Genomic DNA was isolated from 10^7 cells. 15 µg DNA was digested with EcoRI, separated in a 1 % agarose gel and transferred to a nylon membrane. The blot was probed with the 1.7 kb EcoRI fragment containing the entire murine mdm2 cDNA. Positive control was Bluescript mdm2 digested with EcoRI (not shown in the figure).

Total RNA was isolated from untransfected EL4 or P1.HTR cells or from the EL4 mdm2 and P1.HTR mdm2 clones, separated by agarose gel electrophoresis, transferred to a nylon membrane and probed with the entire mdm2 cDNA. Figure 3.1.12. shows that RNA derived from P1.HTR cells transfected with pDO-R mdm2 contained two bands at approximately 3.4 kb and 2.2 kb hybridising to the mdm2 cDNA probe. The 2.2 kb band seen in the transfected cells is supposedly derived from the exogenous mdm2 cDNA, because it is not present in the untransfected cells. Recombinant mdm2 generated from pDO-R will produce mdm2 mRNA containing additional 570 nucleotides and this could explain the size of the exogenous band, see Figure 3.1.12.

The 3.4 kb band seemed to consist of two bands migrating closely together. This could be observed in all samples depending of exposure time of the blot. In Figure 3.1.12 it is most clearly visible in the sample representing RNA from EL4 cells transfected with mdm2. mdm2 is predominantly found as a 3.3. kb transcript in most murine cell lines and tissues (241) and see Figure 3.1.8. although a large number of alternative transcripts resulting in different size proteins have also been reported (241; 369). It is thought that the 3.4 kb transcript seen in all samples in Figure 3.1.12 is the same transcript as the endogenous transcript observed in the EL4 samples and CHO samples in Figure 3.1.8.

No exogenous mdm2 transcript could be observed in the EL4 cells, but as the overall level of RNA derived from EL4 as well as the EL4 mdm2 transfectant was low, that finding was not conclusive. In contrast, it was established that the clone of P1.HTR transfected with mdm2 did produce the correct length mdm2.

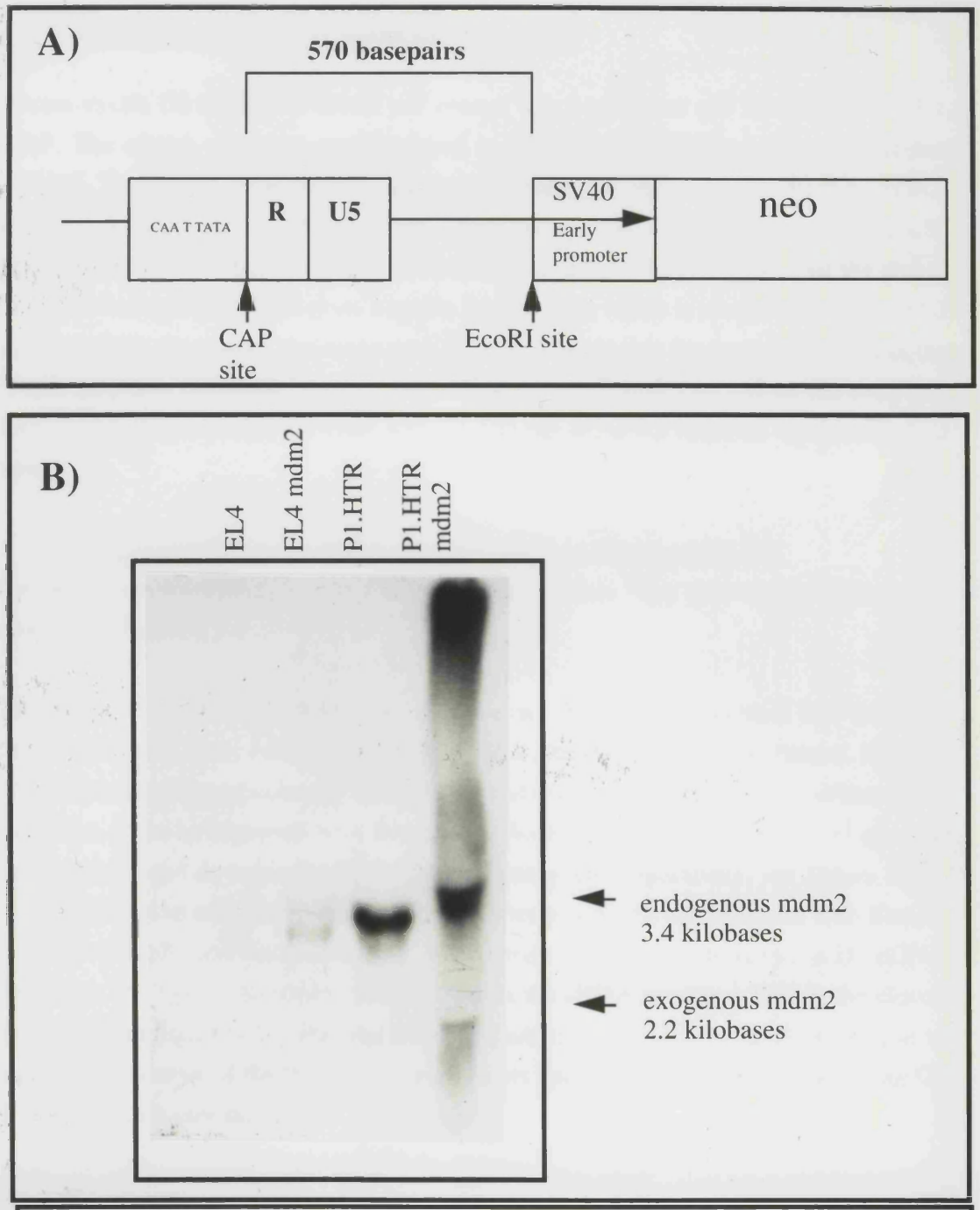


Figure 3.1.12.
Transcription of exogenous murine mdm cDNA cloned into the EcoRI site of pDO-R neo and transfected into EL4 cells or P1.HTR cells.
A) Schematic diagram of a fragment of the pDO-R neo vector containing the 5' LTR with the EcoRI cloning site (into which the murine mdm2 cDNA was cloned).
The distance from the Cap site to the EcoRI cloning site is approximately 570 basepairs.
B) Northern blot of total RNA isolated from untransfected EL4 cells, P1.HTR cells , or clones derived from EL4 cells or P1.HTR cells transfected with pDO-R mdm2, EL4 mdm2 (clone 2.2.) and P1.HTR mdm2 (clone 5) respectively.

3.2. Generation of cyclin D1 expression systems

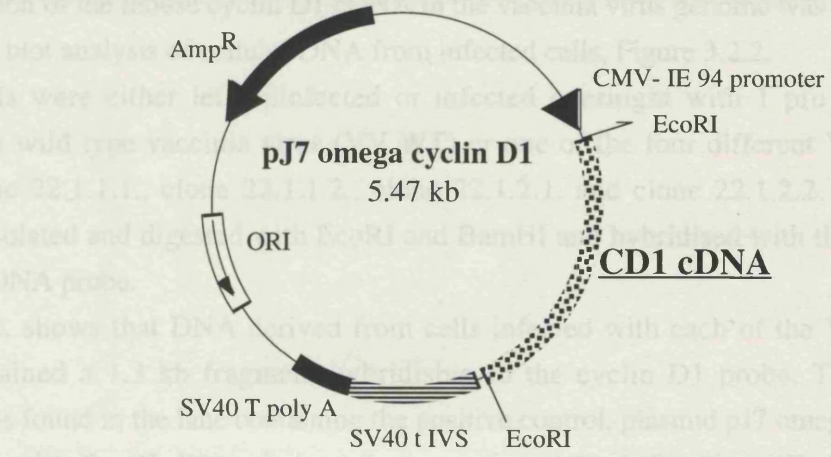
Murine cyclin D1 cDNA in vector pJ7 omega was a generous gift from Dr. V. Fantl, ICRF. The cDNA sequence was retrieved in the EMBL database, accession number M64403. The cDNA cloned in pJ7 omega starts with nucleotide 93 in the 1075 bp cDNA sequence found in the EMBL data base. There are additional 13 bp between the EcoRI cleavage site and the start of the cyclin D1 sequence and there are 282 bp after the end of the open reading frame. This gives a cyclin D1 fragment which is altogether 1278 bp (1.3 kb) long. This also means that restriction sites as numbered in the sequence found in the EMBL data base will differ by 79 bp from the sequence cloned here. Unless anything else is specified, the sequence numbers will refer to the sequence found in the EMBL data base.

3.2.1. Generation of recombinant vaccinia virus expressing cyclin D1.

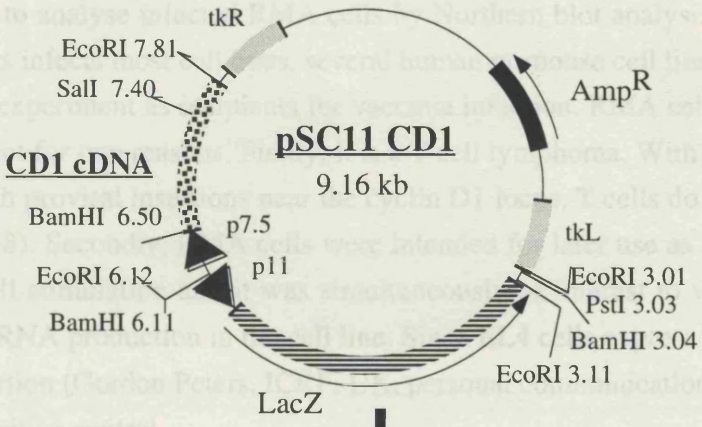
The procedure used for generating recombinant vaccinia virus expressing cyclin D1 is outlined in Figure 3.2.1.

The cDNA for murine cyclin D1 was cloned as a 1.3 kb EcoRI fragment into the SmaI site of pSC11 by blunt end ligation. *E. coli* XL1 Blue cells were transformed. Plasmid DNA from transformed colonies was isolated and analysed for the presence of the cyclin D1 cDNA insert by digestion with BamHI and EcoRI which will cut the pSC11 plasmid just upstream and downstream of the SmaI cloning site respectively, see Figure 3.2.1. Plasmids found to contain the cyclin D1 cDNA were subsequently digested with BamHI and SalI or EcoRI and SalI to identify the orientation of the inserts as cyclin D1 cDNA contains a SalI site at nucleotide position 983 in the cDNA sequence (904 in the cloned sequence), see Figure 3.2.1. Plasmid DNA was additionally subjected to PCR analysis to confirm the identity of the insert by using primers spanning bp 281-651 in the cyclin D1 cDNA sequence (not shown).

Recombinant vaccinia virus expressing cyclin D1 (VV CD1) was made by homologous recombination of pSC11 CD1 plasmid DNA with wild type vaccinia virus in Tk^-143 cells. The virus was subjected to 4 rounds of plaque purification. Four clones, VV CD1 22.1.1.1.,VV CD1 22.1.1.2.,VV CD1 22.1.2.1. and VV CD1 22.1.2.2., were selected for further analysis.



1) EcoRI digestion of pJ7 omega cyclin D1.
2) Purification of 1.3 kb EcoRI fragment containing cyclin D1 cDNA.
3) Fill in of 3' recessed ends with Klenow fragment of DNA polymerase I.
4) Digestion of pSC11 with SmaI.
5) Dephosphorylation of pSC11.
6) Ligation of 1.3 kb cyclin D1 fragment to pSC11.



1) Generation of recombinant virus by homologous recombination of pSC11-CD1 with wild type vaccinia virus (VV WT) in Tk$^-$143 cells.
2) Plaque purification.
3) Isolation and analysis of VV-CD1 clones: VV CD1 22.1.1.1, 22.1.1.2, 22.1.2.1 and 22.1.2.2.

Figure 3.2.1..
Out line of the procedure used to generate recombinant vaccinia virus expressing cyclin D1.

The integration of the mouse cyclin D1 cDNA in the vaccinia virus genome was analysed by Southern blot analysis of cellular DNA from infected cells, Figure 3.2.2.
Tk^-143 cells were either left uninfected or infected overnight with 1 pfu per cell recombinant wild type vaccinia virus (VV WT) or one of the four different VV CD1 clones, clone 22.1.1.1., clone 22.1.1.2., clone 22.1.2.1. and clone 22.1.2.2. Cellular DNA was isolated and digested with EcoRI and BamHI and hybridised with the 1.3 kb cyclin D1 cDNA probe.
Figure 3.2.2. shows that DNA derived from cells infected with each of the VV CD1 clones contained a 1.3 kb fragment hybridising to the cyclin D1 probe. The same fragment was found in the lane containing the positive control, plasmid pJ7 omega cyclin D1 digested with EcoRI. DNA derived from uninfected Tk^-143 cells or Tk^-143 cells infected with wild type vaccinia virus did not contain this band. It was therefore concluded that the entire cyclin D1 cDNA had been integrated in the genomes of the recombinant viruses. Clone VV CD1 22.1.1.1. was selected for further analysis.
The human cyclin D1 cDNA is 90% homologous to the murine cyclin D1 cDNA. The stringent washing conditions used here reduced the background derived from endogenous human cyclin D1 DNA in the Tk^-143 cells, so it was only visible on prolonged exposure of the blot.

To analyse the production of the cyclin D1 mRNA from the recombinant vaccinia virus, it was decided to analyse infected RMA cells by Northern blot analysis, Figure 3.2.3. As vaccinia virus infects most cell lines, several human or mouse cell lines could have been used for the experiment as recipients for vaccinia infection. RMA cells were chosen for the experiment for two reasons. Firstly, it is a T cell lymphoma. With the exception of T cell lines with proviral insertions near the cyclin D1 locus, T cells do not express cyclin D1 (226; 358). Secondly, RMA cells were intended for later use as antigen presenting cell for T cell stimulation and it was simultaneously of interest to verify the status of cyclin D1 mRNA production in the cell line. Since EL4 cells express cyclin D1 due to a proviral insertion (Gordon Peters, ICRF, UK, personal communication), these cells were chosen as positive control.

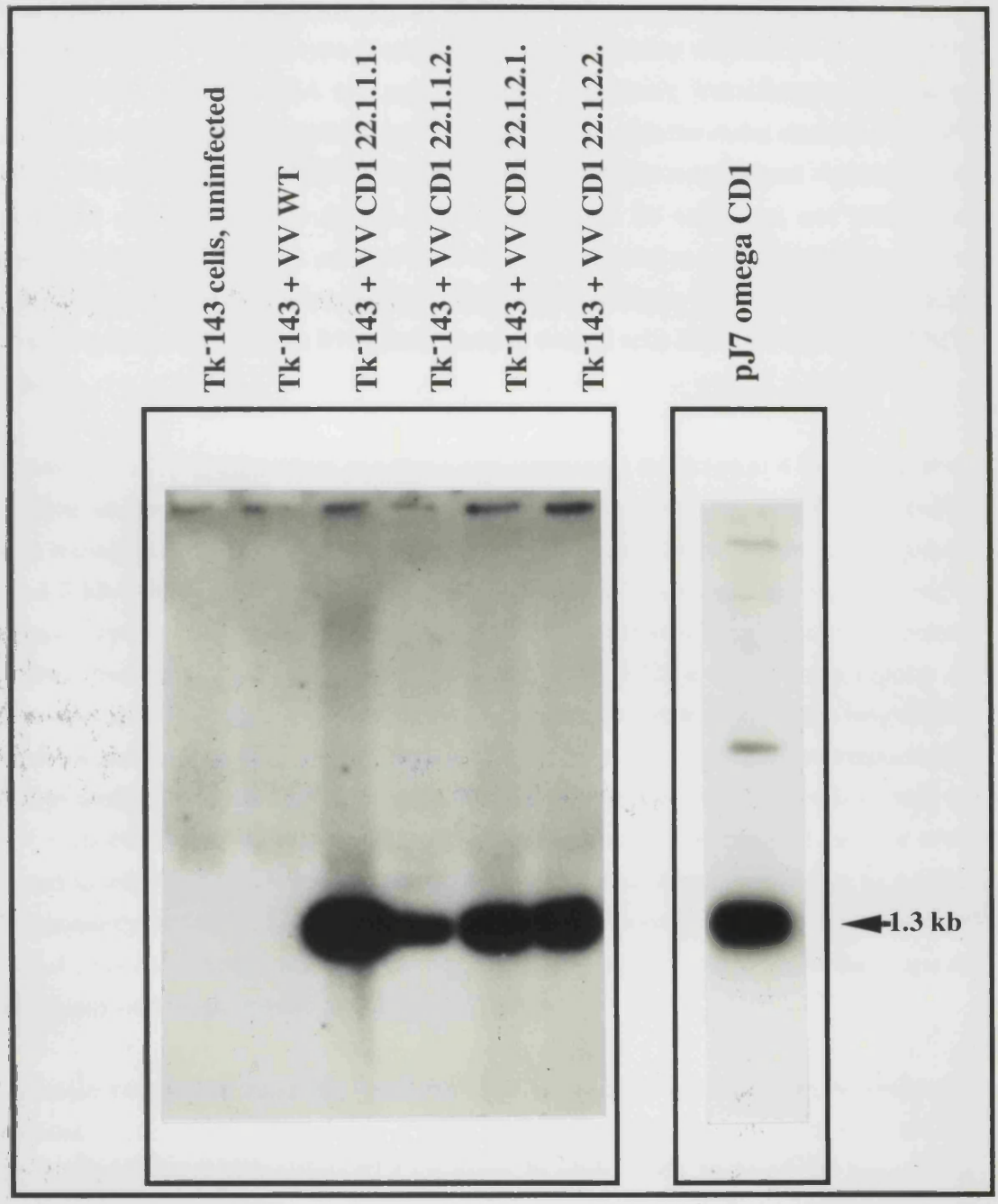


Figure 3.2.2.

Presence of cyclin D1 cDNA in Tk$^-$143 cells infected with recombinant vaccinia virus cyclin D1.

Southern blot of DNA from uninfected Tk$^-$143 cells, Tk$^-$143 cells infected with vaccinia virus wild type (VV WT) or one of four recombinant vaccinia virus cyclin D1 clones (VV CD1).

Tk$^-$ 143 cells were infected with 1 pfu virus per cell over night. Cellular DNA was isolated, digested with EcoRI and BamHI, separated in a 1 % agarose gel, transferred to a nylon membrane and probed with a fragment containing the entire murine cyclin D1 cDNA (1.3 kb). pJ7 omega CD1 plasmid DNA (see Figure 3.2.1.) was digested with EcoRI and used as positive control

Total RNA was isolated from uninfected EL4 cells and RMA cells and RMA cells which had been infected with wild type vaccinia virus or recombinant vaccinia virus cyclin D1, VV CD1 22.1.1.1. The RNA was separated in a denaturing fomaldehyde containing agarose gel, transferred to a nylon membrane and probed with the entire murine cyclin D1 cDNA. Figure 3.2.3 shows that RMA cells infected with the recombinant vaccinia virus cyclin D1 clone, VV CD1 22.1.1.1. produced a 1.6 kb transcript not present in uninfected RMA cells, RMA cells infected with control virus or in EL4 cells. The 1.6 kb presumably represented the exogenous cyclin D1. In addition to the 1.6 kb band, faint 2.0 kb bands could be seen in RNA from positive control cells EL4 as well as from RMA cells.

Murine cyclin D1 is transcribed as a major polyadenylated transcript of 4.0 kb along with variable amounts of a minor 3.5 kb in most murine tissues (370). Not unexpectedly, these transcript were not found in the T cell lines examined above. Shorter transcripts of 1.3.-1.7 kb with a longer half life than normal cyclin D1 transcripts and representing a truncated cyclin D1 transcript terminating shortly after the stop codon have been reported in some human tumour cell lines and tissues (371; 372) and there are reports of alternative spliced cyclin D1 transcripts leading to truncated proteins in human lung cancer cell lines and tissues (371). So far, similar shorter transcripts have not been reported in murine tissues. It seems likely that the 2.0 kb transcripts seen in RMA cells as well as EL4 cells could be a shorter transcript. However, it should be pointed out that with respect to mRNA production there seem to be no difference between RMA and EL4 cells. Consequently, it can not be excluded that RMA cells harbour the same proviral insertion as EL4 cells do, although the issue has not been investigated. It was beyond the scope of the present work to determine the identity of the bands.

The main conclusion from the Northern blot is that cells infected with recombinant vaccinia virus cyclin D1 produced a mRNA of the length expected from a cyclin D1 cDNA cloned under the vaccinia P7.5 promoter. In addition, the analysed cell lines which are all T cell lines seemed to produce an endogenous not characterised transcript which hybridised to a murine cyclin D1 probe. The transcript seemed to be produced to similar extents in RMA cells and EL4 cells.

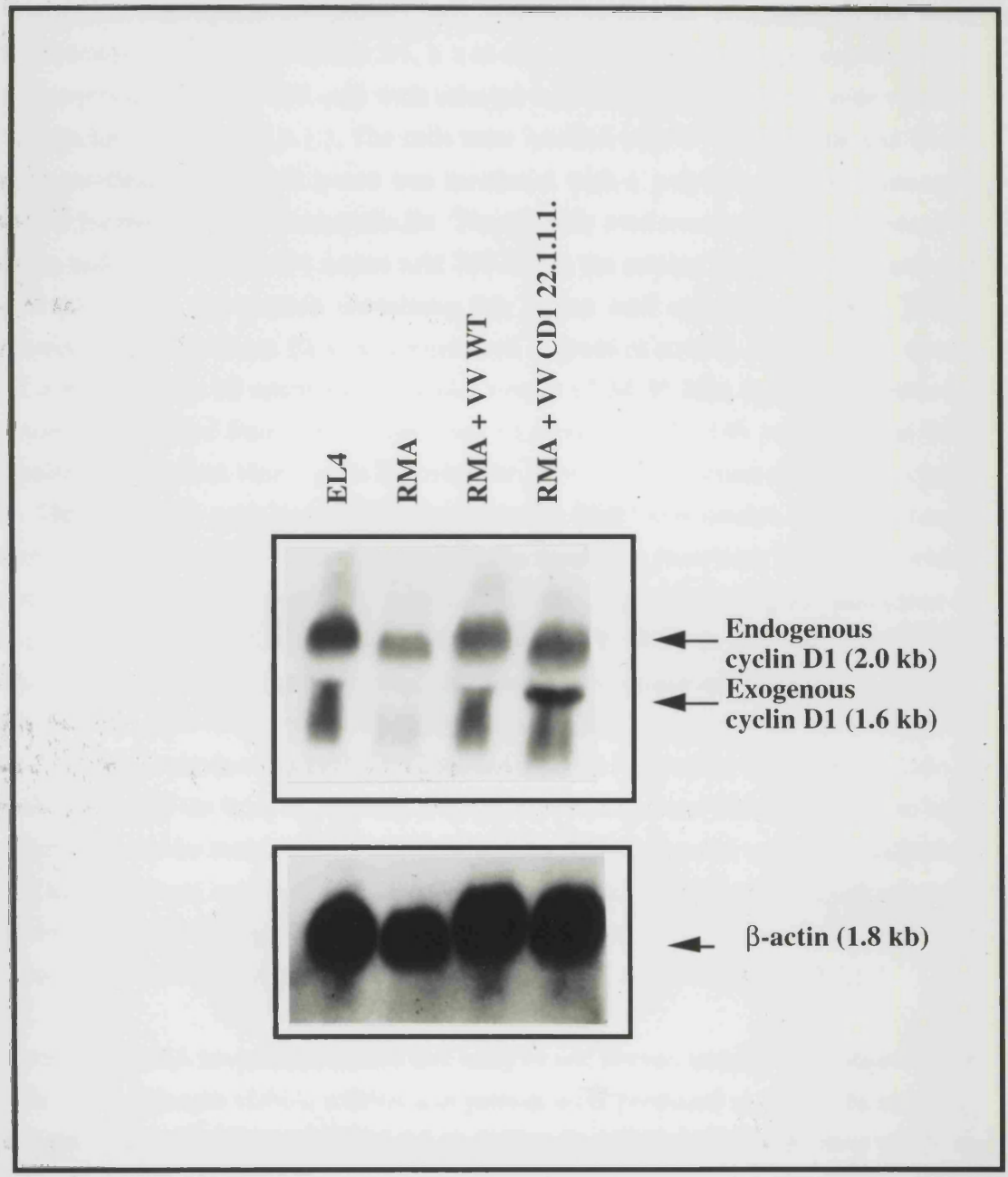


Figure 3.2.3.
Production of murine cyclin D1 messenger RNA in RMA cells infected with vaccinia virus cyclin D1 (VV CD1) clone 22.1.1.1.
5 x 10^6 RMA cells were infected over night with 10 pfu/cell VV WT or VV CD1, clone 22.1.1.1. Total RNA was extracted, separated in a 1% agarose gel containing formaldehyde and analysed by Northern blot analysis. A 1.3 kb EcoRI fragment containing the cyclin D1 cDNA was used as a probe. After exposure to autoradiography, the blot was stripped and reprobed with a 1.8 kb mouseβ-actin cDNA probe to compare the relative amounts of RNA loaded in each lane.

To confirm that cyclin D1 protein was actually produced in cells infected with recombinant vaccinia virus cyclin D1, it was decided to examine protein expression by immunoprecipitation. Tk$^-$143 cells were infected with wild type vaccinia virus or vaccinia virus cyclin D1, clone 22.1.1.1. The cells were labelled with S^{35} methionine and lysed. After pre-clearing, the cell lysate was incubated with a polyclonal rabbit antiserum specific for murine and human cyclin D1. The antibody used reacts with the C terminal of human and murine cyclin D1 amino acid 283-295 in the murine sequence (321) and can be blocked with the peptide containing this amino acid sequence (S.Bates, ICRF, personal communication). EL4 cells were used as positive control. Figure 3.2.4. shows that a protein with an apparent molecular weight of 34-35 kDa could be selectively immunoprecipitated from cell lysates from EL4 cells and Tk$^-$143 cells infected with recombinant vaccinia virus cyclin D1 using the polyclonal antiserum specific for cyclin D1. The size of the protein could correspond to the 36-37 kDa murine cyclin D1 bands reported in the literature (321). The protein was much less prominent in samples which have been pre-incubated with the C terminal cyclin D1 peptide blocking the interaction of the polyclonal rabbit antiserum with cyclin D1 and in Tk$^-$143 cells infected with vaccinia wild type. An additional band at around 30 kDa was also prominent in EL4 cells, Tk$^-$143 cells infected with wild type vaccinia virus or vaccinia virus cyclin D1 but not in cells infected with vaccinia virus cyclin D1 blocked with the C terminal cyclin D1 peptide. It seems likely that the band represented a cyclin dependent kinase which is known to bind to cyclin D1 and be immunoprecipiated with cyclin D1 with the anti cyclin D1 antibodies used in the present experiment (321) and S. Bates, ICRF, personal communication. In conclusion, the immunoprecipitation indicated that cyclin D1 protein was being produced at low levels in Tk$^-$143 cells infected with vaccinia virus cyclin D1 clone 22.1.1.1.

In summary, DNA analysis, Northern blot analysis and immunoprecipitation showed that cyclin D1 full length cDNA, mRNA and protein were produced in cells infected with vaccinia virus cyclin D1 clone 22.1.1.1 as well as in cells infected with other vaccinia cyclin D1 clones. On the basis of this data, it was decided to use clone VV CD1 22.1.1.1. for subsequent T cell work.

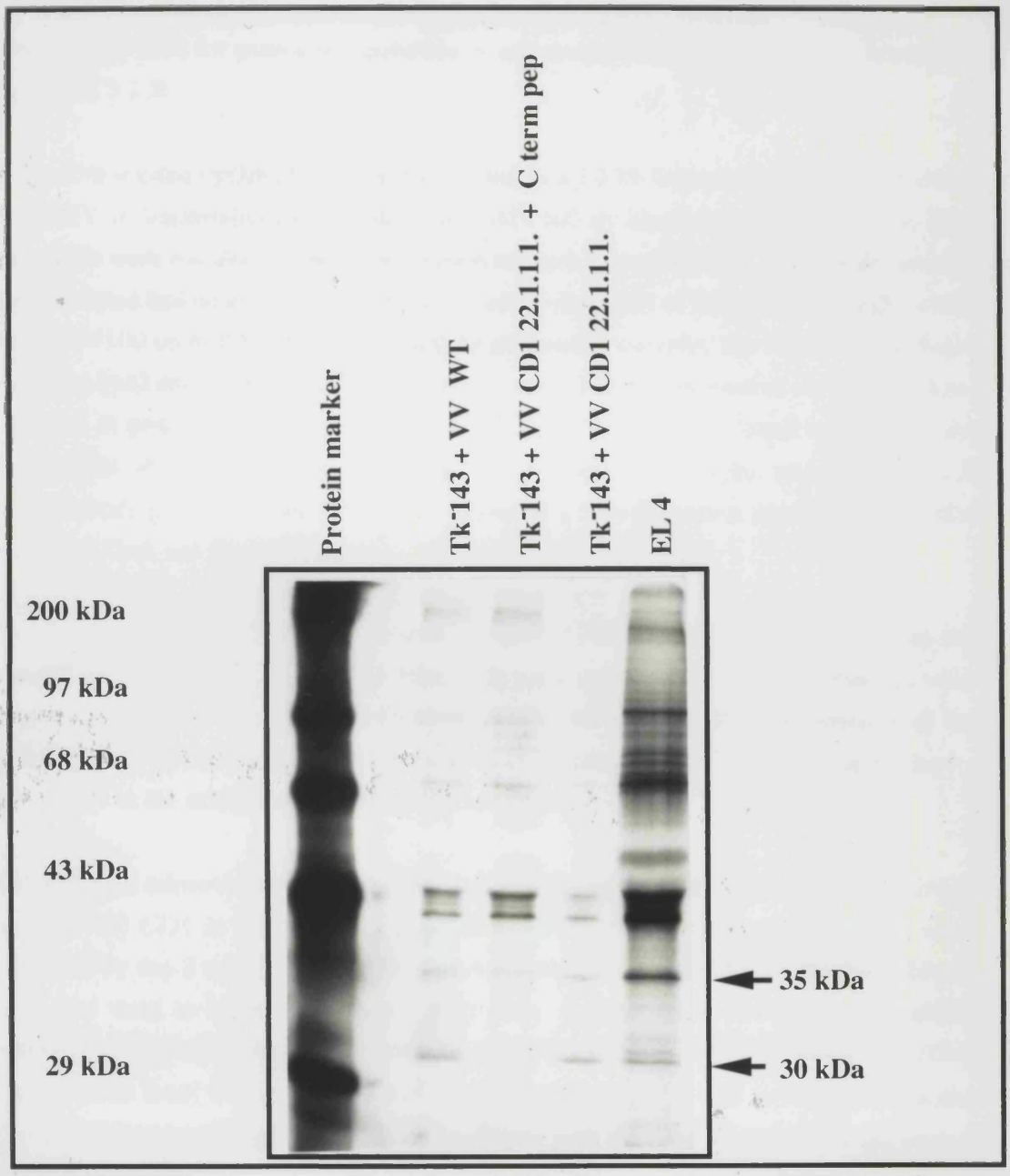


Figure 3.2.4.

Production of cyclin D1 protein in Tk$^-$ 143 Cells infected with recombinant vaccinia virus cyclin D1, clone 22.1.1.1. (VV CD1 22.1.1.1).

4 x 10^6 Tk$^-$143 cells were infected for 2 hours with 30 pfu/cell VV WT or VV CD1 22.1.1.1. Positive control cells were EL4 cells. Cells were labelled with S^{35} methionine for additional 10 hours. Cells were lysed, precleared and proteins were immuno precipitated with 12 µl of a polyclonal rabbit antiserum directed against the CD1 carboxy terminal. One sample, "+ C term pep " had blocking CD1 C-terminal peptide added together with the antiserum. The precipitates were collected, washed and separated by SDS-PAGE using a 12 % separating gel. The gels were dried and exposed to radiography.

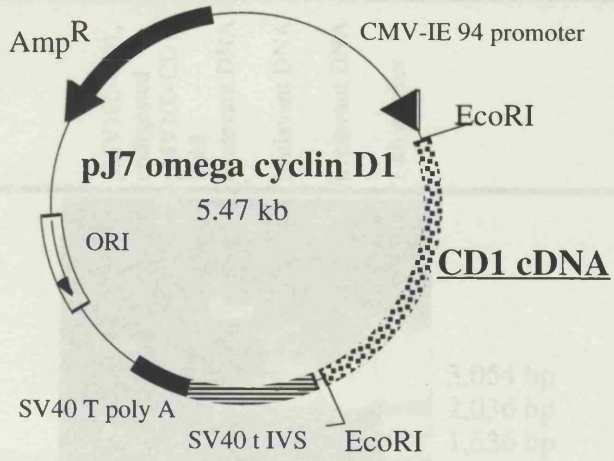
3.2.2. Generation of recombinant adenovirus expressing cyclin D1

The strategy used for generating recombinant adenovirus expressing cyclin D1 is outlined in Figure 3.2.5.

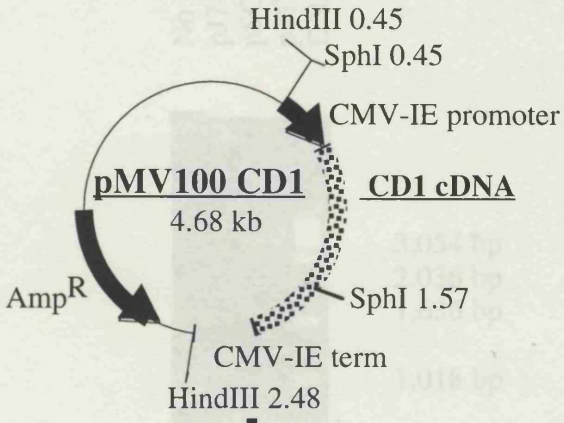
The entire murine cyclin D1 cDNA was cloned as a 1.3 kb fragment into the XbaI site in the CMV-IE expression cassette in vector pMV100 by blunt end ligation. *E. coli* XL1 Blue cells were transformed with the ligation mixture. Plasmid DNA from viable colonies were isolated and recombinants were identified by digestion of DNA with HindIII, which cuts pMV100 on both sides of the CMV-IE expression cassette, see Figure 3.2.5. Since there is a SphI site in vector pMV100 approximately 370 bp upstream of the original XbaI site and at position 826 in the cyclin D1 cDNA (747 in the cloned sequence), the orientation of the insert was determined by digestion with SphI, see Figure 3.2.5. Recombinant plasmid which yielded the expected 1.1 kb fragments upon SphI digestion was identified, see Figure 3.2.6.a.

The 2.0 kb CMV-IE CD1 cassette was cut out of pMV100 CD1 and cloned into the HindIII site of pMV60. *E. coli* XL1 Blue cells were transformed and recombinants were identified by isolation of plasmid DNA and digestion with HindIII. The identity of the insert in pMV60 was additionally checked by PCR using cyclin D1 primers spanning bp 281 to 651 in the murine cyclin D1 cDNA sequence, see Figure 3.2.6.b.

Recombinant adenovirus was generated by homologous recombination of vector pJM17 and pMV60 CD1 in 293 cells by using the $CaPO_4$ transfection method. Cells were harvested by day 8 and virus was released. A dilution series was made of the recombinant virus and used to infect cells in a 6 well plate. As discussed above for the mdm2-adenovirus, the β-galactosidase expressing adenovirus Rad35 was used as control. After 6 days, cells from wells infected with the recombinant virus and now possessing the characteristic morphology seen with cells infected with the control virus, were harvested. Virus was released from the cells. However, the recombinant virus failed to produce cyclin D1 mRNA in infected cells (data not shown) and the virus was not used. The frequency of the generation of wild type adenovirus is extremely low using the pJM17/pMV60 recombination system (316) and G. Wilkinson, Cardiff, personal communication. The most likely explanation for the observed phenomenon is that cells infected with recombinant virus producing cyclin D1 might not have survived due to the negative impact of the considerable amounts of cyclin D1 being produced. This might have favoured the outgrowth of virus without any insert, virus which most likely will have resulted from deletion of the pBRx insert in pJM17 (316).



1) EcoRI digestion of pJ7 omega cyclin D1.
2) Purification of 1.3 kb EcoRI fragment containing cyclin D1 cDNA.
3) Fill in of 3' recessed ends on cyclin D1 fragment with Klenow fragment of DNA polymerase I.
4) Digestion of pMV100 with XbaI.
5) Fill in of 3' recessed ends on pMV100.
6) Dephosphorylation of pMV100.
7) Ligation of 1.3 kb cyclin D1 fragment to pMV100.



1) Digestion of pMV100 CD1 with HindIII.
2) Isolation of 2.0 kb HindIII fragment containing cyclin D1 cDNA.
3) Digestion of pMV60 with HindIII.
4) Dephosphorylation of pMV60.
5) Ligation of 2.0 kb cyclin D1 fragment to pMV60.
6) Generation of recombinant adenovirus by homologous recombination with pJM17 in 293 cells.
7) Isolation and analysis of recombinant adenovirus cyclin D1 (Ad CD1).

Figure 3.2.5.
Out line of the procedure used to generate recombinant adenovirus expressing cyclin D1.

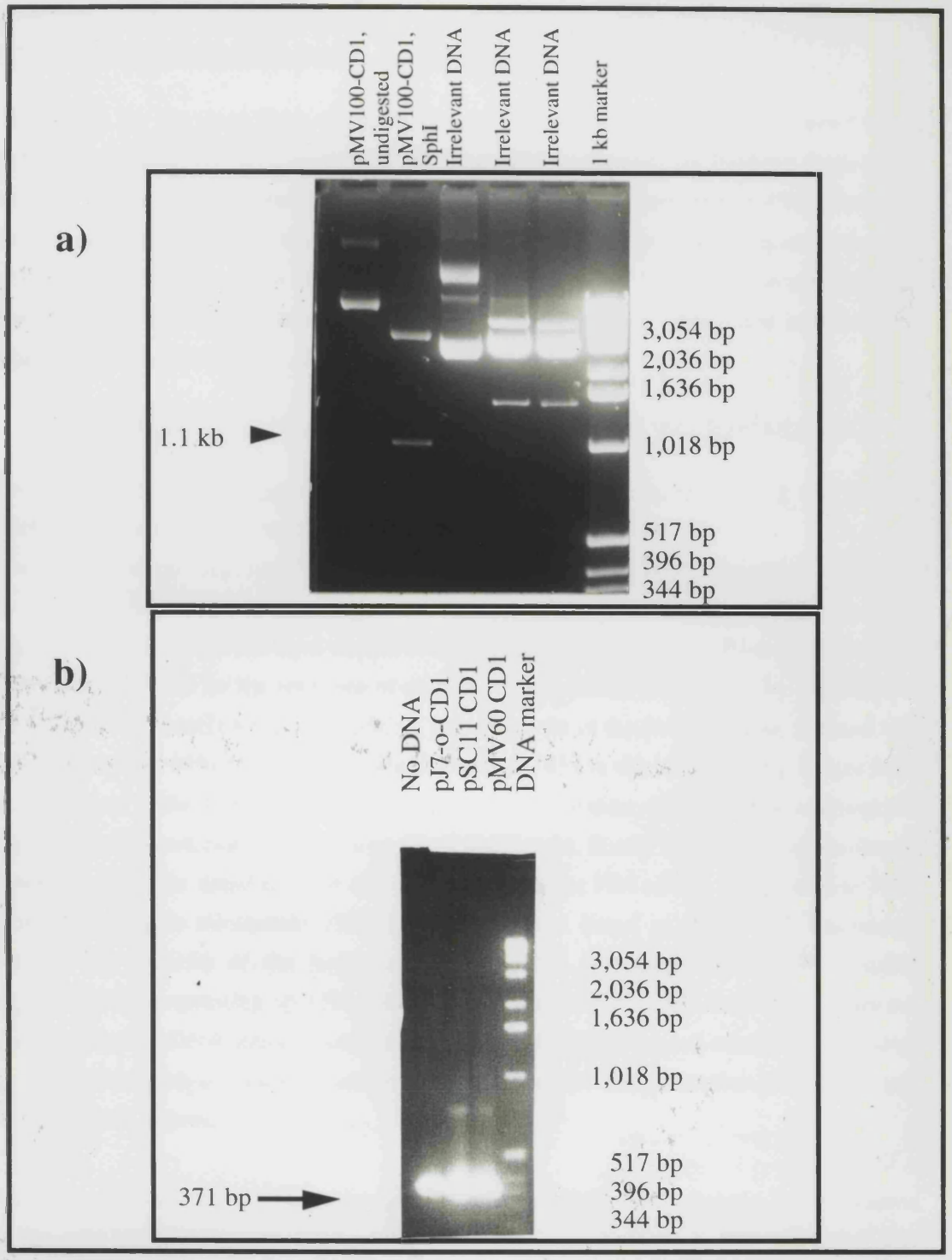


Figure 3.2.6.
Presence of correctly orientated cyclin D1 cDNA in the pMV60 plasmid.
a) Cyclin D1 cDNA is cloned in the correct orientation in the pMV100 plasmid. Plasmid DNA was digested with SphI and separated in a 1 % agarose gel.
b) Presence of cyclin D1 cDNA in the final pMV60 CD1 plasmid. Plasmid DNA was amplified using CD1 specific primers spanning bp 281 to 651 in the murine cyclin D1 cDNA sequence. pJ7 omega CD1 (pJ7-o-CD1) as well as pSC11 CD1 plasmid DNA served as positive controls in the PCR reaction.

3.3. Generation of fibroblast growth factor receptor I expression systems

The cDNA for the short form of murine basic fibroblast growth factor receptor I was a generous gift from Dr. N. Lemoine, ICRF. The cDNA sequence for the long form of the receptor was retrieved from the EMBL database, accession number M28998. The short form is not listed in the database but is identical to the long form but without nucleotide 148 to 414. This means that restriction sites (post nucleotide 148) found in the fragment cloned here, will differ with 267 basepairs from the restriction sites found in the cDNA sequence listed in the EMBL database.

3.3.1. Generation of recombinant vaccinia virus expressing the fibroblast growth factor receptor I (FRI)

The procedure used to generate recombinant vaccinia virus expressing the murine fibroblast growth factor receptor I is outlined in Figure 3.3.1.

The cDNA for the short form of the murine fibroblast growth factor receptor I (FRI) was cut out as an EcoRI/Sal I fragment from the vector pDO-R FRI and cloned into the SmaI site of pSC11 by blunt end ligation and used to transform *E. coli* XL1 Blue cells. Plasmid DNA was analysed for the presence of an insert in the correct orientation by digestion of the DNA with BamHI which cuts pSC11 just upstream of the SmaI cloning site and FRI DNA at position 1192 (this corresponds to position 1459 in the in the 267 bp longer form of FRI found in the EMBL database). The correct orientation of inserts was additionally confirmed by restriction mapping with XhoI and EcoRI. EcoRI cuts pSC11 immediately downstream of the SmaI cloning site and XhoI cuts the FRI cDNA at nucleotide 1725 (corresponding to nucleotide 1992 longer FRI form listed in the EMBL database). Finally, the identity of the insert in plasmid DNA was confirmed by PCR using oligonucleotides spanning bp 1291-1930 in the sequence of the FRI short form. (data not shown). Plasmid DNA found to contain the FRI cDNA in the correct orientation based on the restriction analysis and PCR analysis was selected for the generation of recombinant vaccinia virus expressing FRI.

Recombinant vaccinia virus expressing FRI was generated by homologous recombination of the pSC11 FRI plasmid DNA with wild type vaccinia virus in Tk^-143 cells. The recombinant virus was subjected to four rounds of plaque purification. 3 plaques were picked from the last round of purification and small stocks were prepared. The stocks were found to contain more than 95% recombinant virus as judged from the expression of β-galactosidase in infected cells. For this reason the stocks were considered to be clonal. The stocks will be referred to as VV FRI 1.1.1.1., VV FRI 1.1.1.2. and VV FRI 3.2.1.1.

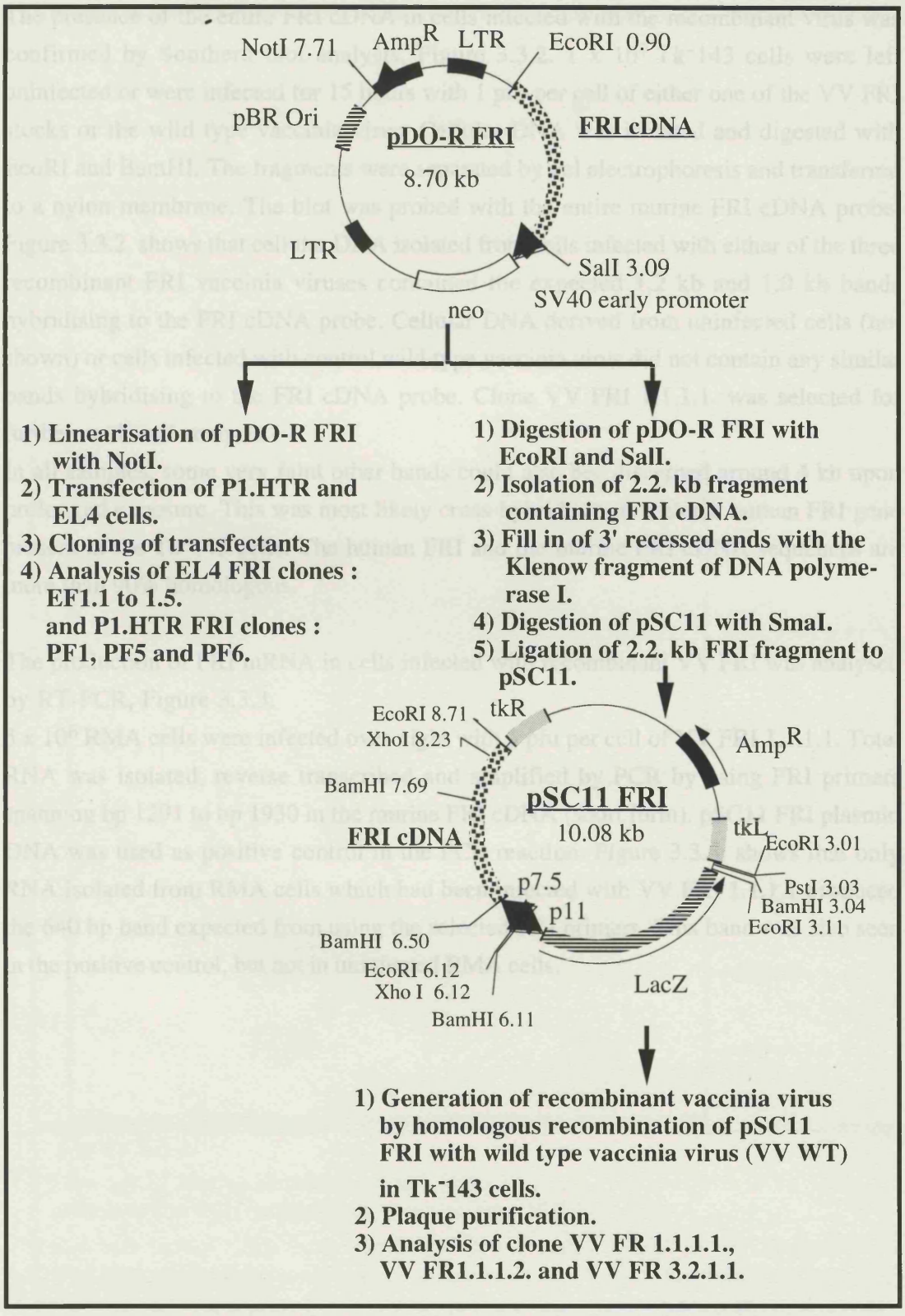


Figure 3.3.1.
Outline of the procedure used to generate recombinant vaccinia virus and transfectants expressing the murine fibroblast receptor I (FRI).

The presence of the entire FRI cDNA in cells infected with the recombinant virus was confirmed by Southern blot analysis, Figure 3.3.2. 1 x 10^7 Tk^-143 cells were left uninfected or were infected for 15 hours with 1 pfu per cell of either one of the VV FRI stocks or the wild type vaccinia virus. Cellular DNA was isolated and digested with EcoRI and BamHI. The fragments were separated by gel electrophoresis and transferred to a nylon membrane. The blot was probed with the entire murine FRI cDNA probe. Figure 3.3.2. shows that cellular DNA isolated from cells infected with either of the three recombinant FRI vaccinia viruses contained the expected 1.2 kb and 1.0 kb bands hybridising to the FRI cDNA probe. Cellular DNA derived from uninfected cells (not shown) or cells infected with control wild type vaccinia virus did not contain any similar bands hybridising to the FRI cDNA probe. Clone VV FRI 1.1.1.1. was selected for further work and analysis.
In all samples, some very faint other bands could also bee discerned around 4 kb upon prolonged exposure. This was most likely cross-hybridisation with the human FRI gene present in the Tk^-143 cells. The human FRI and the murine FRI cDNA sequences are more than 90% homologous.

The production of FRI mRNA in cells infected with recombinant VV FRI was analysed by RT-PCR, Figure 3.3.3.
5 x 10^6 RMA cells were infected over night with 5 pfu per cell of VV FRI 1.1.1.1. Total RNA was isolated, reverse transcribed and amplified by PCR by using FRI primers spanning bp 1291 to bp 1930 in the murine FRI cDNA (short form). pSC11 FRI plasmid DNA was used as positive control in the PCR reaction. Figure 3.3.3. shows that only RNA isolated from RMA cells which had been infected with VV FRI 1.1.1.1 produced the 640 bp band expected from using the selected FRI primers. This band was also seen in the positive control, but not in uninfected RMA cells.

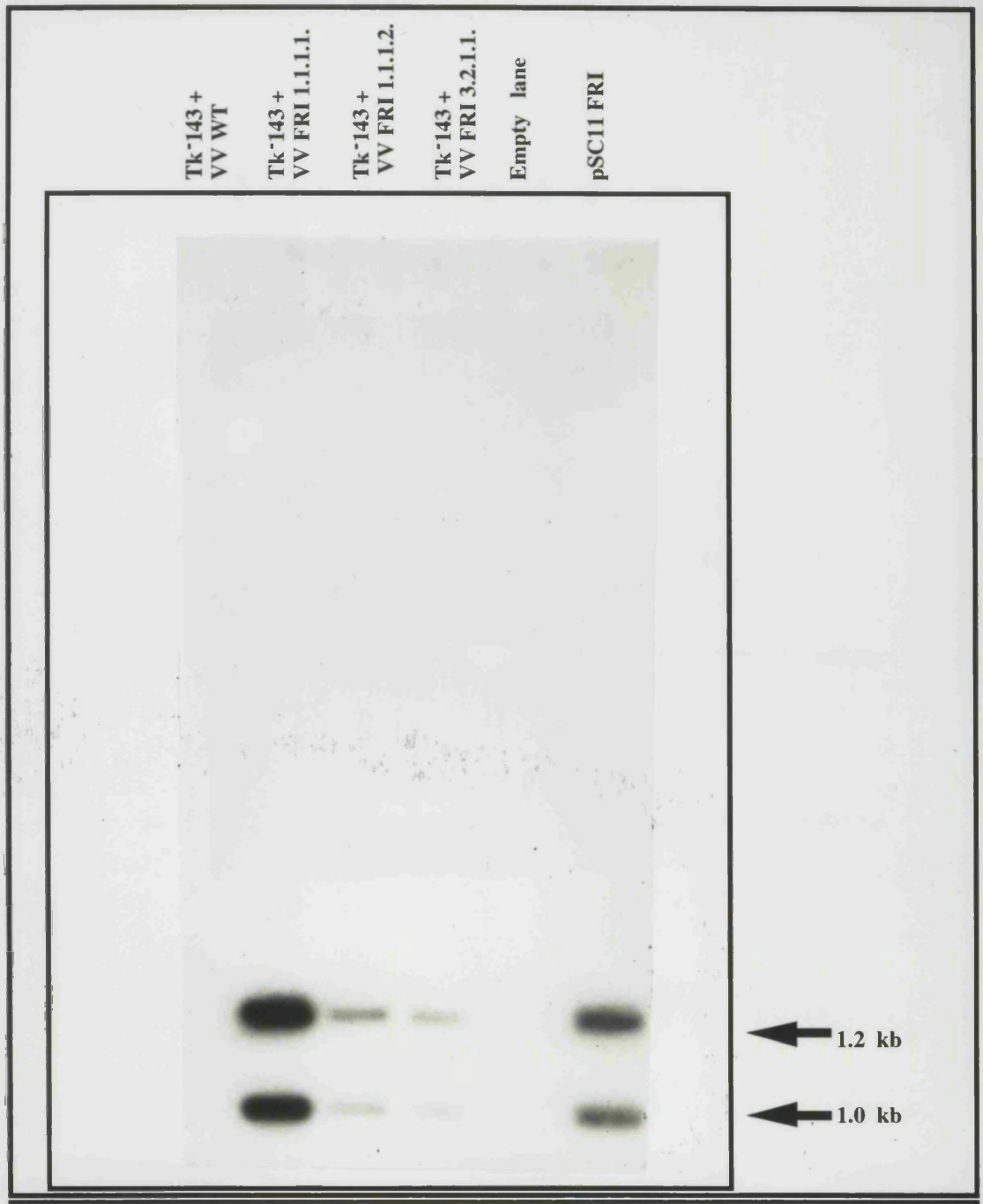


Figure 3.3.2.

Presence of murine fibroblast growth factor receptor I (FRI) cDNA in Tk$^-$143 cells infected with recombinant vaccinia virus FRI (VV FRI).

Southern blot of DNA from uninfected Tk$^-$143 cells or Tk$^-$143 cells infected with wild type vaccinia virus (VV WT) or one of three VV FRI recombinants.

TK$^-$143 cells were infected with 1 pfu per cell over night. Cellular DNA was isolated, digested with EcoRI and BamHI, separated in a 0.8 % agarose gel, transferred to a nylon membrane and probed with a fragment containing the complete murine FRI cDNA. Positive control was pSC11 FRI DNA digested with EcoRI and BamHI

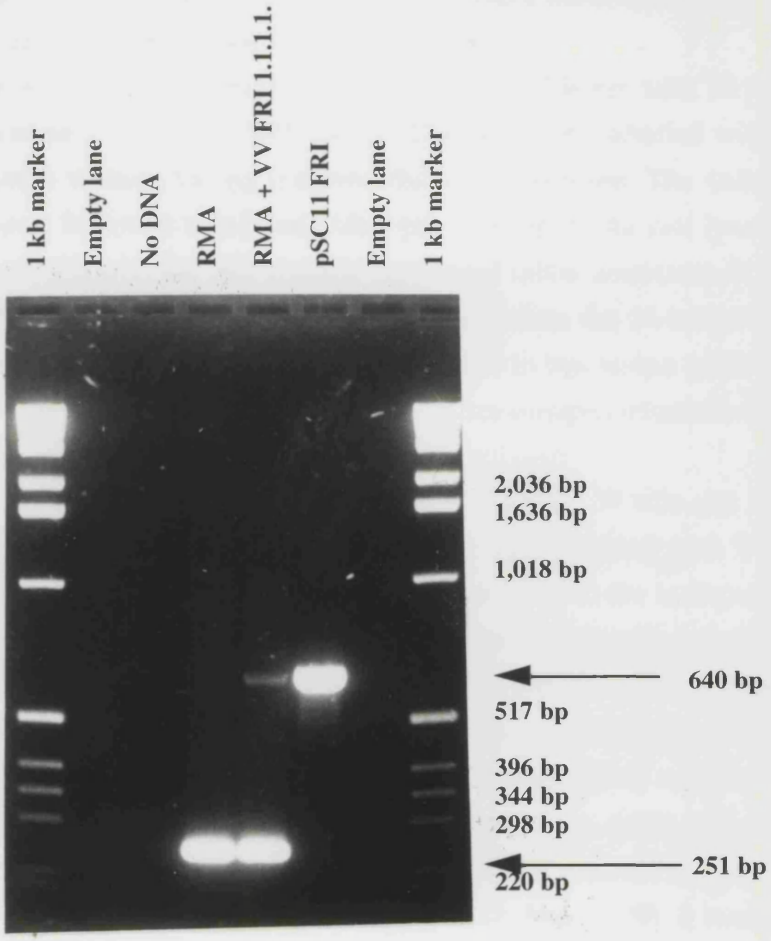


Figure 3.3.3.
Production of murine FRI mRNA in RMA cells infected with recombinant vaccinia virus FRI, clone VV FRI 1.1.1.1.
RT-PCR of total RNA from uninfected RMA cells or RMA cells infected with VV FRI 1.1.1.1 .5 x 10^6 RMA cells were infected with 10 pfu /per cell VV FRI for 12 hours or not infected as control. The RNA was reverse transcribed and subjected to PCR. The positive control in the PCR reaction for FRI cDNA was pSC11 FRI plasmid DNA. The amplified FRI fragment is 640 bp, the amplified HPRT fragment is 251 bp.

Expression of FRI protein in cells infected with recombinant VV FRI was analysed by immunoprecipitation of S^{35} methionine labelled proteins from a whole cell lysate with a polyclonal antibody raised against human FRI, Figure 3.3.4.

5 x 10^6 Tk^-143 cells were either left uninfected or infected for 2 hours with 30 pfu per cell of wild type vaccinia virus or VV FRI 1.1.1.1. The cells were labelled with S^{35}-methionine for 10 hours without having removed the virus inoculum. The cells were harvested 12 hours post infection and lysed. After pre-clearing of the cell lysate, the lysate was either incubated with the FRI specific polyclonal rabbit antiserum BG90 or with a pre-immune rabbit serum. The BG90 antiserum recognises the 14 amino acid C terminal of the protein. Although the murine sequence differs in two amino acids within this sequence, the serum cross reacts with murine FRI in immunoprecipitations (Dr. H. Leung, ICRF, personal communication).

Figure 3.3.4. shows the presence of three distinct bands at 175 kDa , 74 kDa and 37 kDa among the proteins derived from Tk^-143 cells which had been infected with VV FRI 1.1.1.1. The intensity of these bands were not very high compared to the background of other bands, but higher than amongst the proteins derived from Tk^-143 cells infected with VV WT and immunoprecipitated with BG90 or amongst the proteins derived from Tk^-143 cells infected with VV FRI 1.1.1.1. and immunoprecipitated with the rabbit pre-immune serum. The short form of the murine fibroblast growth factor receptor I has previously been precipitated as an approximately 85 kDa band from rabbit reticulocyte lysates (270) or as a 130 kDa band from transfected hamster CHO cells (271). The native protein is reported to precipitate as an approximately 150 kDa band (270). It seems that different bands can be expected depending on the glycosylation and thus the cell line used for transfection. It is possible that the bands seen in Figure 3.3.4. represent different glycosylated forms. The 37 kDa band might represent an incomplete translation product.

In summary, a recombinant vaccinia virus stock, VV FRI 1.1.1.1. was generated which produced FRI cDNA, FRI mRNA and FRI protein in infected cells as demonstrated by Southern blot of cellular DNA, RT-PCR of total RNA and immunoprecipitation of total cellular protein respectively. On basis of these results, it was decided to use this virus stock for priming, restimulation and analysis of CTL specific for the murine fibroblast growth factor receptor I.

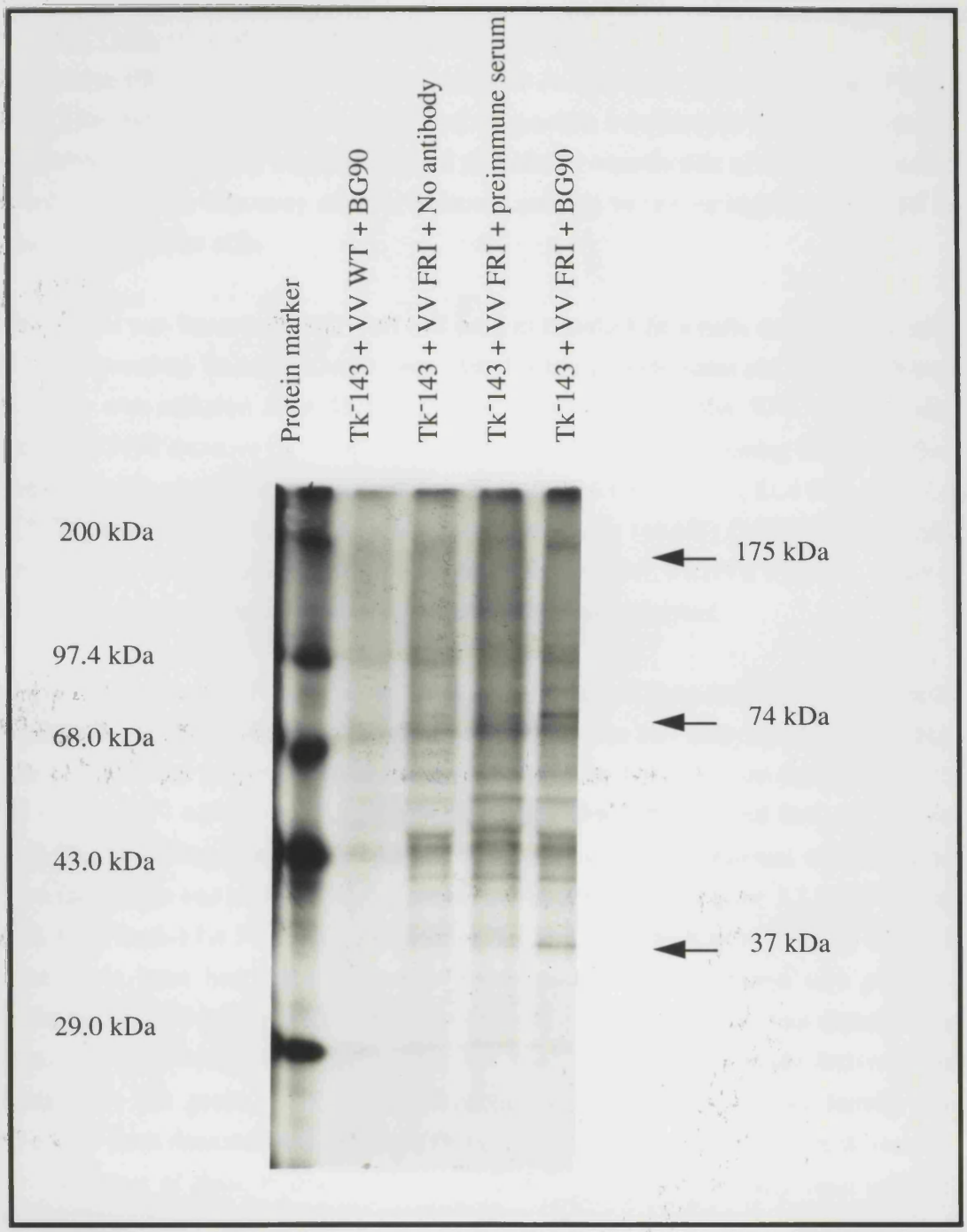


Figure 3.3.4.

Production of FRI protein in Tk⁻143 cells infected with recombinant vaccinia virus FRI, clone 1.1.1.1. (VV FRI 1.1.1.1.).

2 x 10^6 Tk⁻143 cells were infected for 2 hours with 30 pfu/cell VV WT or VV FRI 1.1.1.1. Cells were labelled with S^{35} methionine for additional 10 hours. Cells were lysed, precleared and proteins were immunoprecipitated with 12 µl polyclonal rabbit anti mouse FRI serum or 12µl preimmune serum. The precipitates were collected, washed and separated by SDS-PAGE using a 12% separating gel. The gels were dried and exposed to radiography.

3.3.2. Generation of transfectants expressing FRI

The murine FRI cDNA was provided in the pDO-R expression vector (330), see Figure 3.3.1. This expression vector was also used to generate transfectants expressing mdm2, see section 3.1.9. As this vector is in itself suitable for transfection of mammalian cells, no subcloning was necessary in order to have a suitable vector for expression of FRI in selected mammalian cells.

pDO-R FRI was linearised with NotI and used to transfect EL4 cells and P1.HTR cells by electroporation. Transfected cells were plated out in 24 well plates and selection based on G418 was initiated after 48 hours. After 2 weeks, less than 37% of the wells contained G418 sensitive EL4 cells (dead cells) only. The wells containing G418 resistant (live) EL4 cells were pooled to give three bulk lines EL4 FRI 1 (EF1), EL4 FR4 and EL4 FR7. Transfection of P1.HTR cells resulted in only four (16.6%) G418 positive wells after 2 weeks on selection. The cells from the positive wells, P1.HTR FRI 1, 4, 5 and 6 (PF 1, 4, 5 and 6) respectively were considered clonal and analysed.

Integration of the entire FRI cDNA in the genome of the cell lines derived from EL4 cells transfected with FRI cDNA was demonstrated by Southern blot analysis. Genomic DNA from 10^7 cells was isolated from EL4 cells and two of the bulk EL4 line transfected with FRI cDNA (EF1 and EF4). 15 µg DNA was digested with EcoRI and SalI, see Figure 3.3.1. The DNA fragments were separated by agarose gel electrophoresis, transferred to nylon membrane and probed with the entire murine FRI cDNA. Figure 3.3.5 shows that DNA from both EL4 FRI lines contained a 2.2 kb band which hybridised to the FRI probe. This band had the expected size for the FRI cDNA cloned into pDO-R. Untransfected EL4 cells did not contain this band. In all samples at least four distinct high molecular weight bands could be distinguished. These bands were probably derived from endogenous FR genes. The murine fibroblast growth factor receptor family has previously been demonstrated to belong to a multigene family by Southern blot analysis (270) and two of these FGFRI (FRI) and FGFRII (FRII) have already been isolated (269).

The EL4 FRI 1 cell line was cloned and gave rise to five clones EL4 FRI 1.1., 1.2., 1.3. and 1.4. and 1.5. The presence of the FRI cDNA was analysed and confirmed in clone EL4 FRI 1.3. by Southern blot analysis (not shown) and this clone was used for later CTL stimulation. One of the cell lines derived from transfecting P1.HTR cells with pDO-R FRI was equally analysed and found to contain the FRI cDNA (not shown). This cell line, PF5, was used for later CTL stimulation.

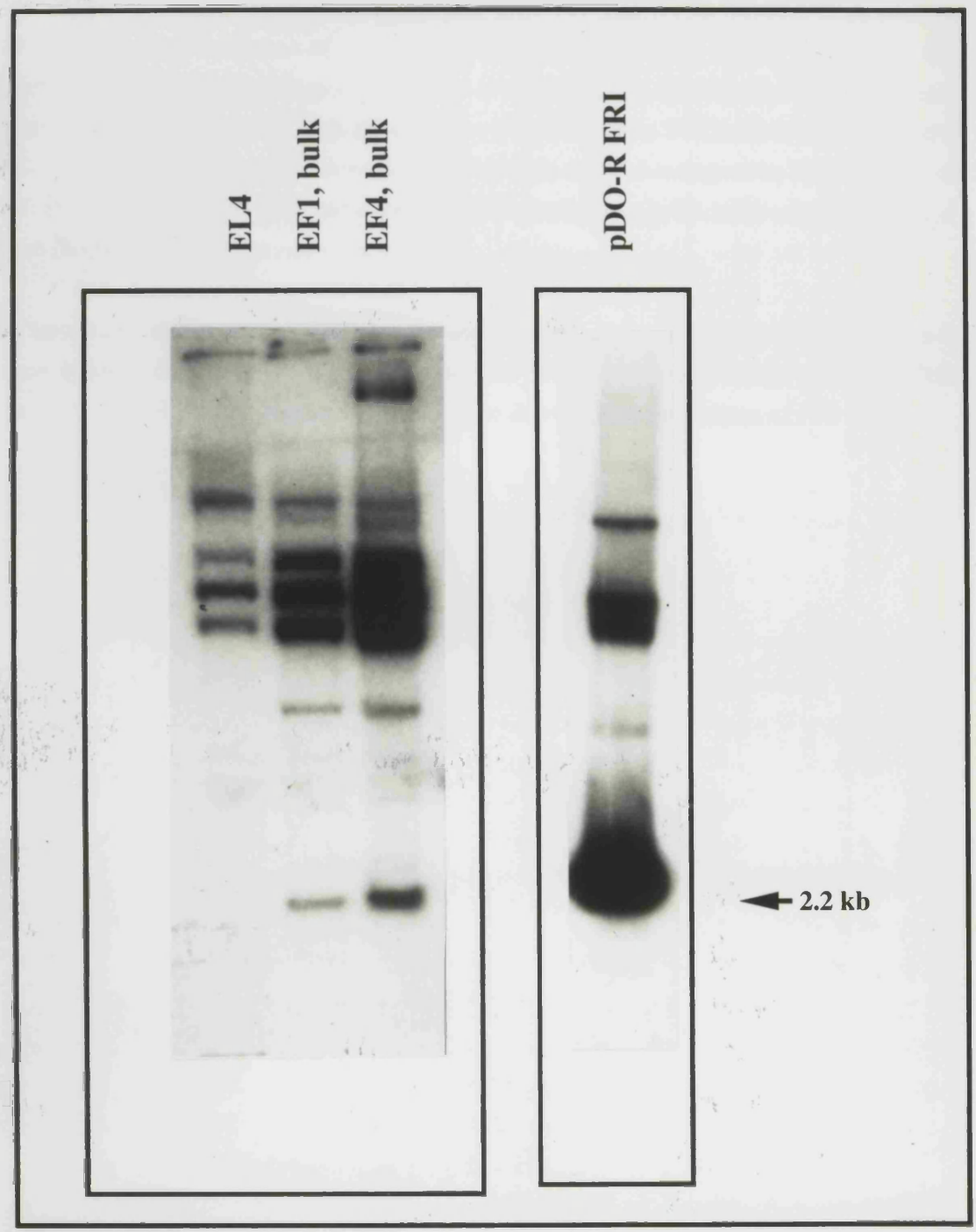


Figure 3.3.5.
Presence of FRI cDNA in EL4 cells transfected with pDO-R FRI.
Southern blot of total cellular DNA isolated from untransfected EL4 cells and from cell lines derived from EL4 transfected with PDO-R FRI. 15 µg cellular DNA was digested with EcoRI and SalI, separated in a 0.7% agarose gel, transferred to a nylon membrane. and probed wit the complete murine FRI cDNA. Positive control was pDO-R FRI plasmid DNA digested with EcoRI and SalI .After hybridisation, the blots were exposed to autoradiography. Due to excess loading of positive control DNA, the lane containing this sample had to be exposed separately.

The EL4 thymoma does not express endogenous FRI mRNA so RT PCR was used to demonstrate the production of exogenous FRI mRNA in the EF clones. mRNA for the fibroblast growth factor receptor I is present at only very low levels in the murine thymus and this could explain the lack of detection of endogenous FRI mRNA the EL4 cells (270). P1.HTR cells is a mastocytoma line found to express endogenous FRI mRNA, so RT-PCR was not used to demonstrate mRNA production in P1.HTR cells transfected with the FRI cDNA (data not shown).

In summary, stable clones of EL4 cells and P1.HTR cell transfected with FRI cDNA were generated. The clones had the entire FRI cDNA integrated in the genome. With respect to the EL4 FRI clone, it was possible to demonstrate production of FRI mRNA.

3.4. Generation of p53 expression systems

The murine wild type p53 cDNA in vector pT7-7 was a generous gift from Dr. L. Crawford, ICRF. The cDNA sequence was retrieved in the EMBL database, accession number X01237. The p53 cDNA cloned in the pT7-7 vector contains an exogenous ribosomal binding site at the 5' end. This means that the p53 ATG initiation codon is preceded by 41 basepairs of exogenous sequence and not the 157 bp derived from the p53 gene which are listed in the cDNA sequence in the EMBL data base. The cDNA listed in the EMBL data base contains 1773 nucleotides. The cloned cDNA sequence contain approximately 300 bp of p53 genomic sequence after the end of the open reading frame. This means that the cloned fragment is approximately 2.0 kb long.

3.4.1. Generation of recombinant vaccinia virus expressing p53.

The procedure followed for generating recombinant vaccinia virus expressing murine wild type p53 is outlined in Figure 3.4.1.

Murine WT p53 cDNA was isolated as a 2.0 kb XbaI/Hind III fragment from vector pT7-7 p53 and cloned into the SmaI site of pSC11 by blunt end ligation. Following transformation into *E. coli* XL1 Blue cells, plasmid DNA was isolated. The presence of the correct insert was examined by digestion of the plasmid DNA with EcoRI and BamHI, Figure 3.4.1. The orientation of the insert was determined by restriction mapping with BamHI and PstI or EcoRI and PstI, because there are two PstI sites in the cloned p53 sequence at nucleotide 246 and 377 (362 and 493 in the EMBL database cDNA sequence). There is one PstI site in pSC11 but it is only few basepairs from the EcoRI and BamHI sites around nucleotide 3010 in the pSC11 vector map, see Figure 3.4.1. The identity of inserts was additionally confirmed by PCR of the DNA using p53 primers spanning bp 546-1197 of the p53 cDNA sequence (not shown). Plasmid DNA found containing the p53 cDNA in the correct orientation, pSC11 p53, was amplified and used to generate recombinant vaccinia virus expressing p53.

Recombinant vaccinia virus expressing WT p53 was made by homologous recombination between pSC11 p53 and wild type vaccinia virus in Tk^-143 cells. The recombinant virus was subjected to 4 rounds of plaque purification. Virus from 3 plaques, VV p53 1.1.1.1., VV p53 1.2.1.1. and VV p53 1.2.1.2. were selected for analysis. The virus from each plaque was judged to contain more than 95% recombinant virus as estimated from the production of β-galactosidase by infected cells. The virus stocks derived from each of the three plaques will be referred to as clonal.

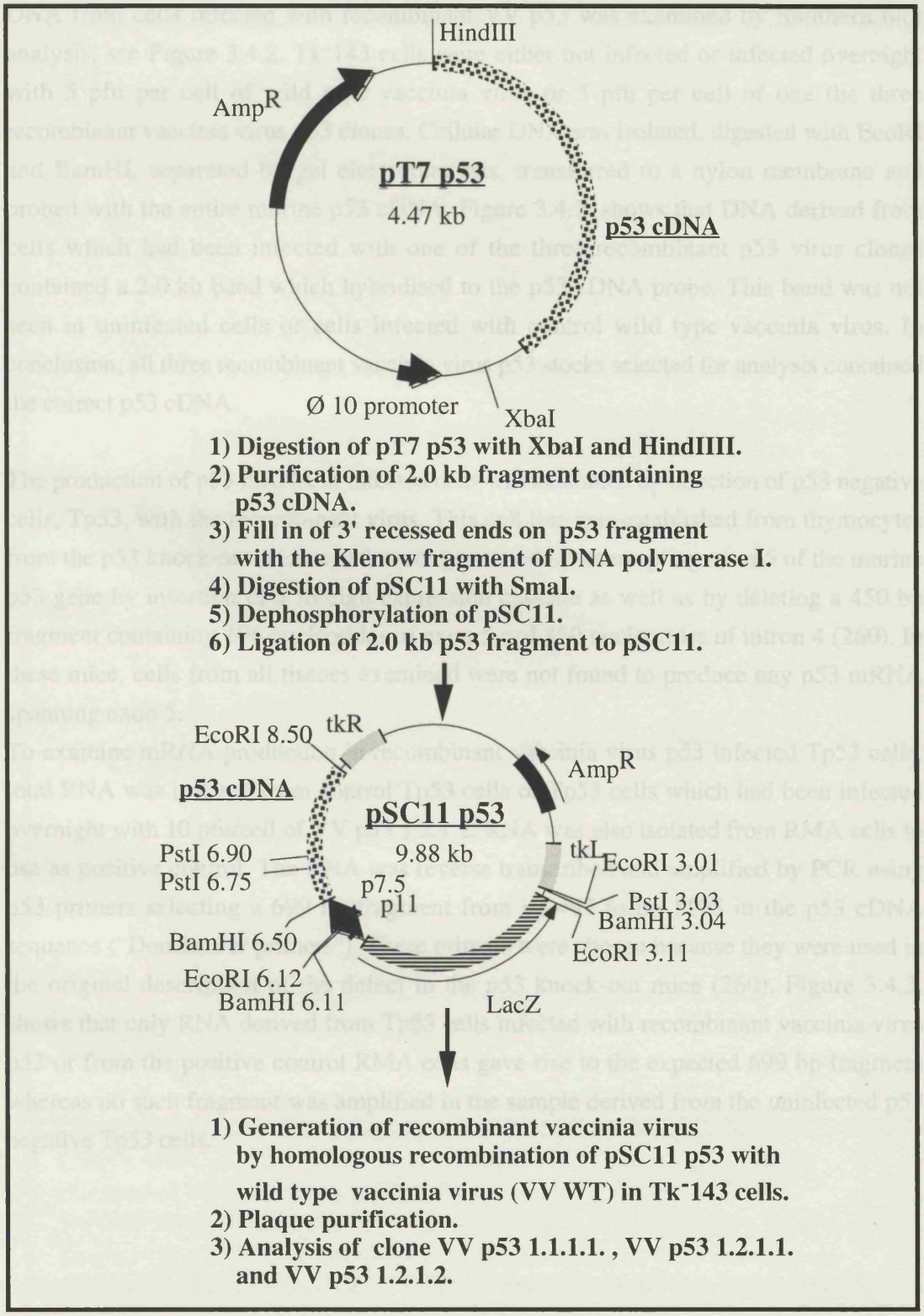


Figure 3.4.1.
Outline of the procedure used to generate recombinant vaccinia virus expressing murine wild type p53.

DNA from cells infected with recombinant VV p53 was examined by Southern blot analysis, see Figure 3.4.2. Tk$^-$143 cells were either not infected or infected overnight with 5 pfu per cell of wild type vaccinia virus or 5 pfu per cell of one the three recombinant vaccinia virus p53 clones. Cellular DNA was isolated, digested with EcoRI and BamHI, separated by gel electrophoresis, transferred to a nylon membrane and probed with the entire murine p53 cDNA. Figure 3.4.2. shows that DNA derived from cells which had been infected with one of the three recombinant p53 virus clones contained a 2.0 kb band which hybridised to the p53 cDNA probe. This band was not seen in uninfected cells or cells infected with control wild type vaccinia virus. In conclusion, all three recombinant vaccinia virus p53 stocks selected for analysis contained the correct p53 cDNA.

The production of p53 mRNA in infected cells was examined by infection of p53 negative cells, Tp53, with the recombinant virus. This cell line was established from thymocytes from the p53 knock-out mice which were generated by interrupting exon 5 of the murine p53 gene by insertion of a foreign expression cassette as well as by deleting a 450 bp fragment containing 106 nucleotides of exon 5 and 350 nucleotides of intron 4 (260). In these mice, cells from all tissues examined were not found to produce any p53 mRNA spanning exon 5.

To examine mRNA production in recombinant vaccinia virus p53 infected Tp53 cells, total RNA was isolated from control Tp53 cells or Tp53 cells which had been infected overnight with 10 pfu/cell of VV p53 1.2.1.2. RNA was also isolated from RMA cells to use as positive control. The RNA was reverse transcribed and amplified by PCR using p53 primers selecting a 699 bp fragment from bp 497 to bp 1195 in the p53 cDNA sequence ("Donehower primers"). These primers were chosen because they were used in the original description of the defect in the p53 knock-out mice (260). Figure 3.4.3. shows that only RNA derived from Tp53 cells infected with recombinant vaccinia virus p53 or from the positive control RMA cells gave rise to the expected 699 bp fragment whereas no such fragment was amplified in the sample derived from the uninfected p53 negative Tp53 cells.

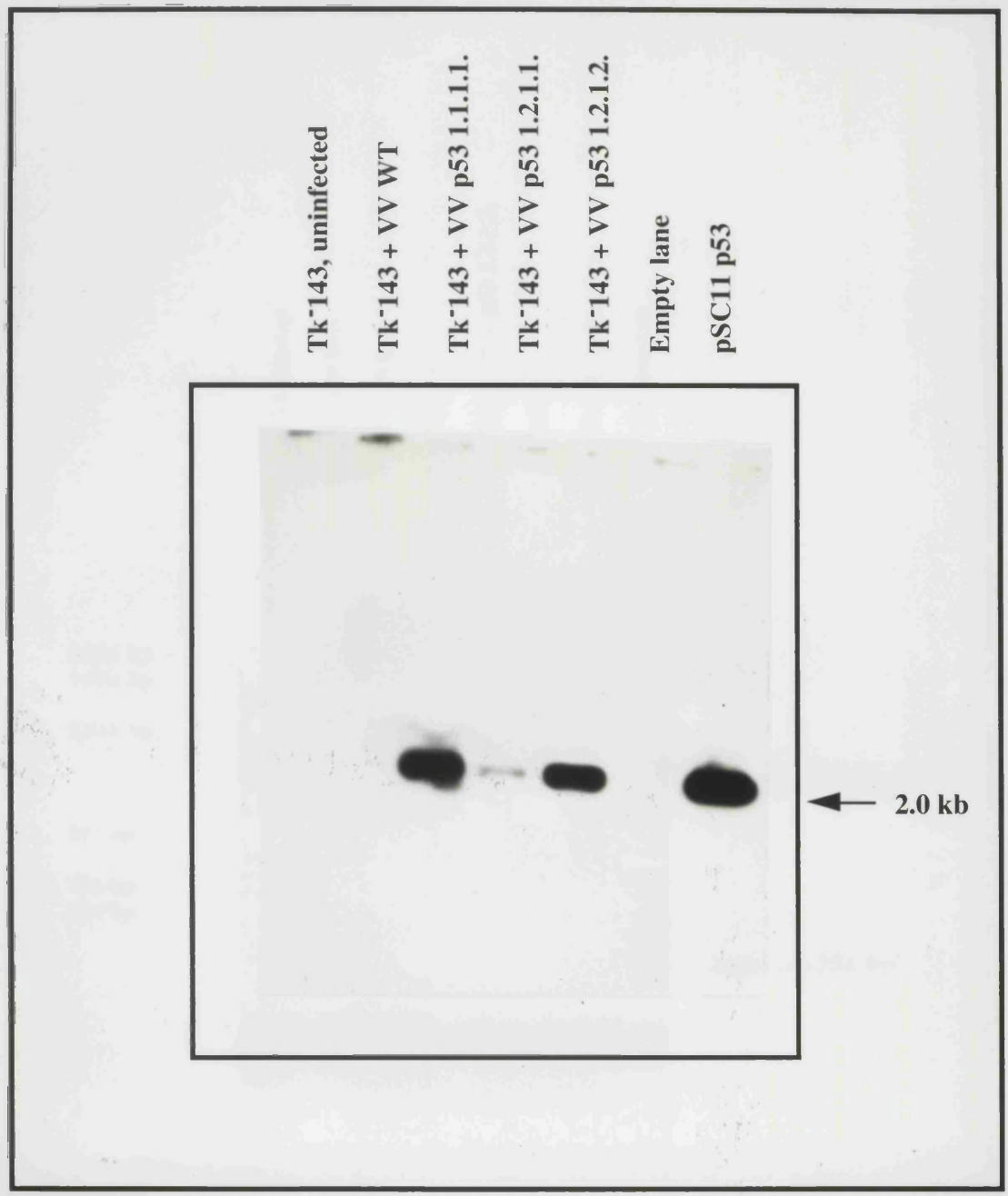


Figure 3.4.2.

Presence of WT p53 cDNA in Tk-143 cells infected with recombinant vaccinia virus p53.

Southern blot of DNA from uninfected Tk-143 cells, Tk-143 cells infected with vaccinia virus wild type (VV WT) or one of three recombinant vaccinia virus p53 stocks, VV p53 1.1.1.1. , VV p53 1.2.1.1. or VV p53 1.2.1.2

Tk-143 cells were infected with 5 pfu/cell virus overnight. Cellular DNA was isolated. 15 µ g DNA was digested with EcoRI and BamHI, separated in a 1 % agarose gel, transferred to nylon membrane and probed with the entire murine p53 cDNA in a 2.0 kb XbaI/Hind III fragment. A lane containing pSC11 p53 plasmid DNA digested with EcoRI and BamHI was used as positive control.

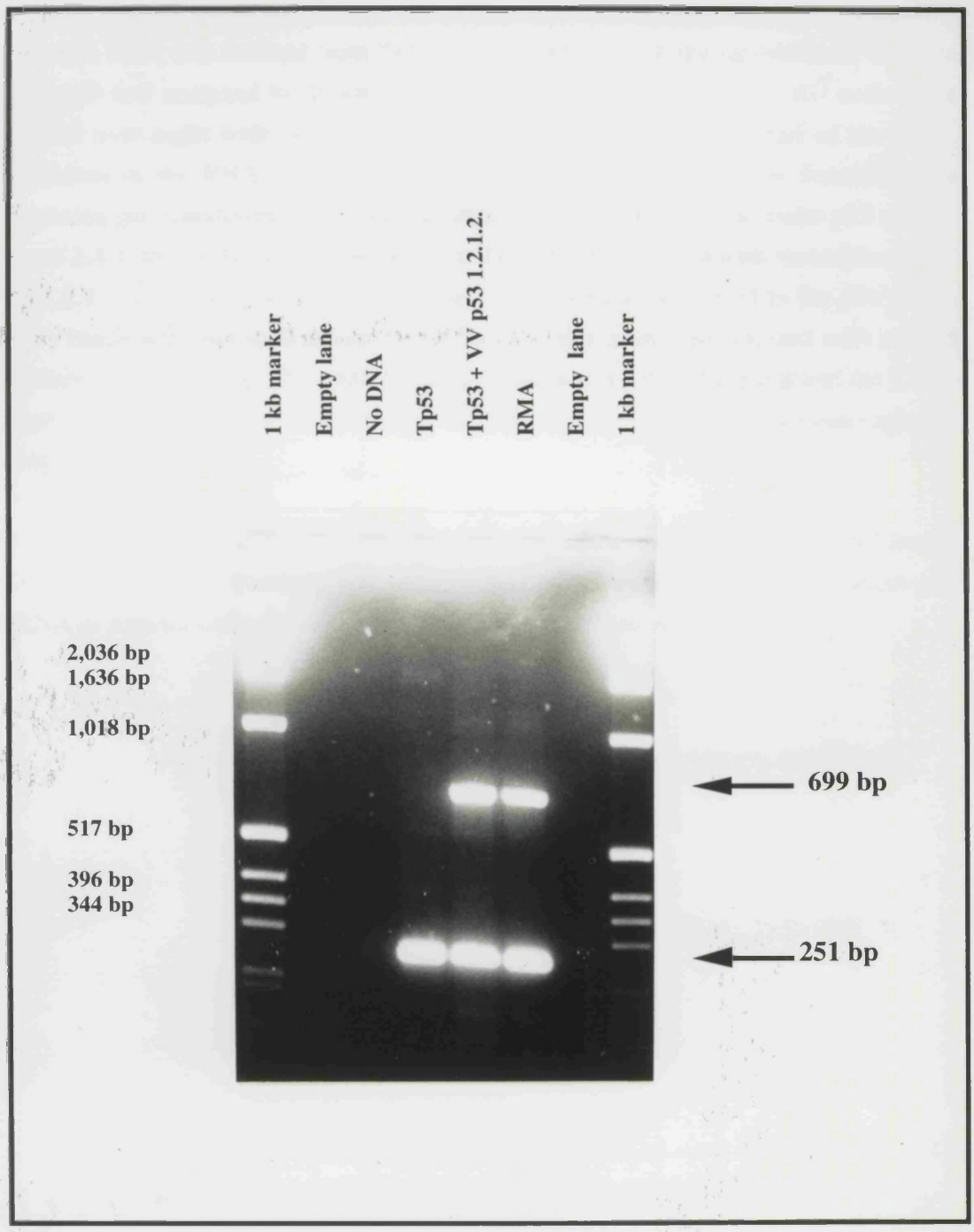


Figure 3.4.3.
Production of p53 mRNA in p53 negative cells infected with recombinant vaccinia virus p53 .
RT-PCR of total RNA from p53 negative Tp53 cells or Tp53 cells infected with VV p53 1.2.1.2. or , as positive control, RMA cells.
Tp53 were either uninfected or infected overnight with 10 pfu per cell VV p53 1.2.1.2. Total RNA was isolated, reversed transcribed and amplified by PCR by using p53 primers spanning a 699 bp fragment in the murine p53 sequence and HPRT primers spanning a 251 bp fragment in the murine HPRT sequence.

To verify that full length p53 mRNA was produced in cells infected with recombinant VV p53, total RNA was isolated from Tk$^-$143 cells infected with the recombinant vaccinia virus p53 and analysed by Northern blot analysis, Figure 3.4.4. 1 x 10^7 cells were infected over night with 10 pfu/per cell. Total RNA was isolated. Half of the RNA, equivalent to the RNA from 5 x 10^6 cells, was separated in a 1% formaldehyde containing gel, transferred to a nylon membrane and hybridised to the entire p53 cDNA. Figure 3.4.4. shows that RNA derived from Tk$^-$143 cells infected with recombinant VV p53 1.2.1.2. contained two bands of 2.0 and 2.4 kb which hybridised to the p53 probe. These bands were not seen in uninfected Tk$^-$143 cells or in cells infected with control vaccinia virus wild type. The p53 cDNA was cloned as a 2.0 kb fragment and the 2.4 kb fragment most likely represented the p53 mRNA with some additional vaccinia sequence at the 3' end.

In conclusion recombinant vaccinia virus p53 was made which contained a full length cDNA as shown by Southern blot analysis and which could produce the correct p53 mRNA in infected cells as shown by RT-PCR and Northern blot analysis.

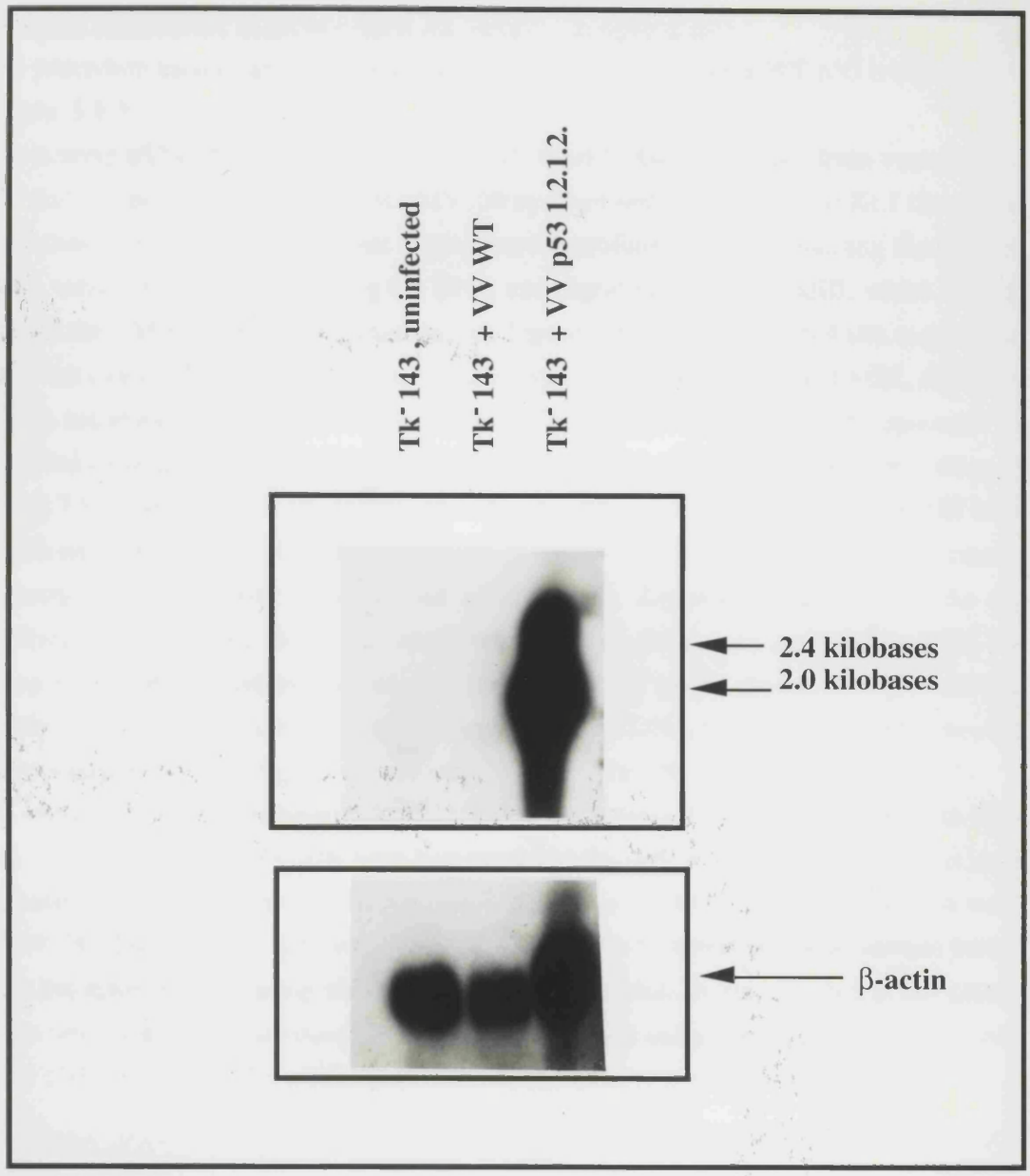


Figure 3.4.4.

Production of murine WT p53 mRNA in human Tk⁻143 cells infected with recombinant vaccinia virus p53.

Northern blot of total RNA isolated from Tk⁻143 cells infected with either VV p53 1.2.1.2 or control VV WT.

Tk⁻143 cells were left uninfected or infected with 10 pfu/cell VV WT or VV p53 1.2.1.2 over night. The cells were harvested and total RNA was isolated. The RNA was separated in a 1% agarose gel containing formaldehyde and transferred to a nylon membrane. The membrane was probed with the entire murine p53 cDNA in a 2.0 kb XbaI/HindIII fragment and subsequently with the entire murine β- actin cDNA.

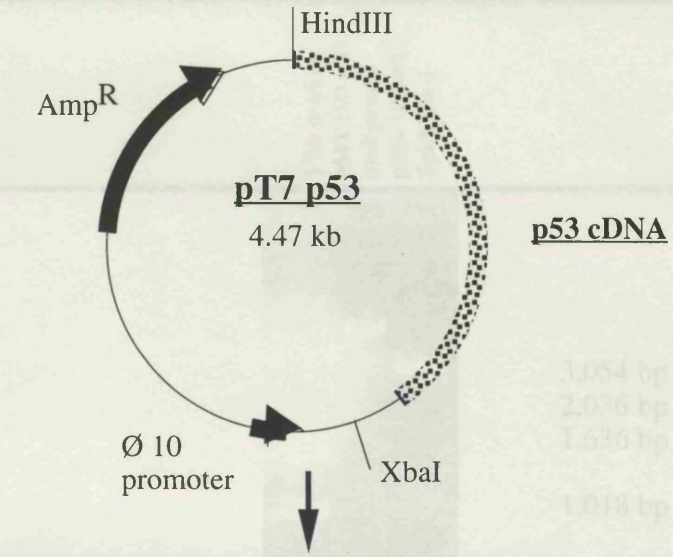
3.4.2. Production of recombinant adenovirus expressing p53.

The procedure used to generate recombinant adenovirus expressing WT p53 is outlined in Figure 3.4.5.

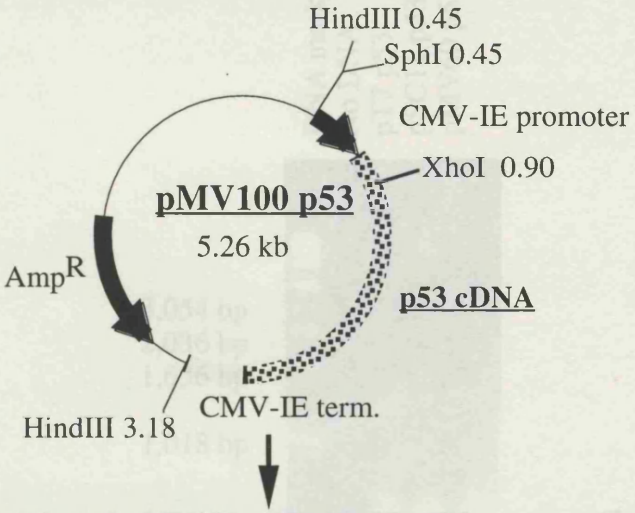
The murine p53 cDNA was cut out as a 2.0 kb HindIII/Xba I fragment from vector pT7-p53 and cloned into the XbaI site of pMV100 by blunt end ligation. *E. coli* XL1 blue cells were transformed and recombinant clones were identified. Clones containing the correct DNA were identified by isolating the DNA and digesting it with HindIII, which would cut out the CMV-IE expression cassette, see Figure 3.4.1. A unique XhoI site at position 78 in the cloned WT p53 DNA sequence (corresponding to bp 195 in the EMBL database cDNA sequence) and the unique SphI site in pMV100, approximately 370 bp upstream of the XbaI cloning site was used to determine the orientation of the insert, Figure 3.4.6.a.

The 2.7 kb CMV-IE p53 expression cassette was cut out of pMV100 with HindIII and subcloned into the HindIII site of pMV60. *E. coli* XL1 Blue cells were transformed. Recombinant DNA was isolated and identified by digestion with HindIII. As an additional proof of the identity of the inserts, plasmid DNA was analysed by PCR by using p53 specific primers spanning bp 546 to bp 1197 of the murine WT p53 cDNA sequence. As it can be seen in Figure 3.4.6.b., plasmid DNA from pMV60 p53 give rise to the expected 652 bp fragment when amplified with the p53 specific primers.

To make recombinant adenovirus p53, pMV60 was cotransfected with pJM17 into 293 cells. After 8 days the 293 cells were harvested and the cells were extracted with Arklone to isolate recombinant virus. The Arklone extract was used to infect 293 cells in 6 well plates. No plaques ever appeared. The $CaPO_4$ transfection was repeated several times with the same disappointing result. Due to time constraints, it was decided to not spend more time making adenovirus p53 and instead focus on using the recombinant vaccinia virus p53 as well as p53 peptides for CTL induction and restimulation.



1) Digestion of pT7 53 with XbaI and HindIII.
2) Purification of 2.0 kb fragment containing p53 cDNA.
3) Fill in of 3' recessed ends on p53 fragment with the Klenow fragment of DNA polymerase I.
4) Digestion of pMV100 with XbaI.
5) Fill in of 3' recessed ends on pMV100 with the Klenow fragment of DNA polymerase I.
6) Ligation of 2.0 kb p53 fragment to pMV100



1) Digestion of pMV100 p53 with HindIII.
2) Purification of 2.73 kb CMV- IE expression casette containing the p53 cDNA.
3) Digestion of pMV60 with HindIII.
4) Dephosphorylation of pMV60.
5) Ligation of 2.73 kb CMV-IE p53 cDNA fragment to pMV60.
6) Generation of recombinant adenovirus by homologous recombination of pMV60 p53 with pJM17 in 293 cells.

Figure 3.4.5.
Outline of the procedure used to generate recombinant adenovirus expressing murine wild type p53.

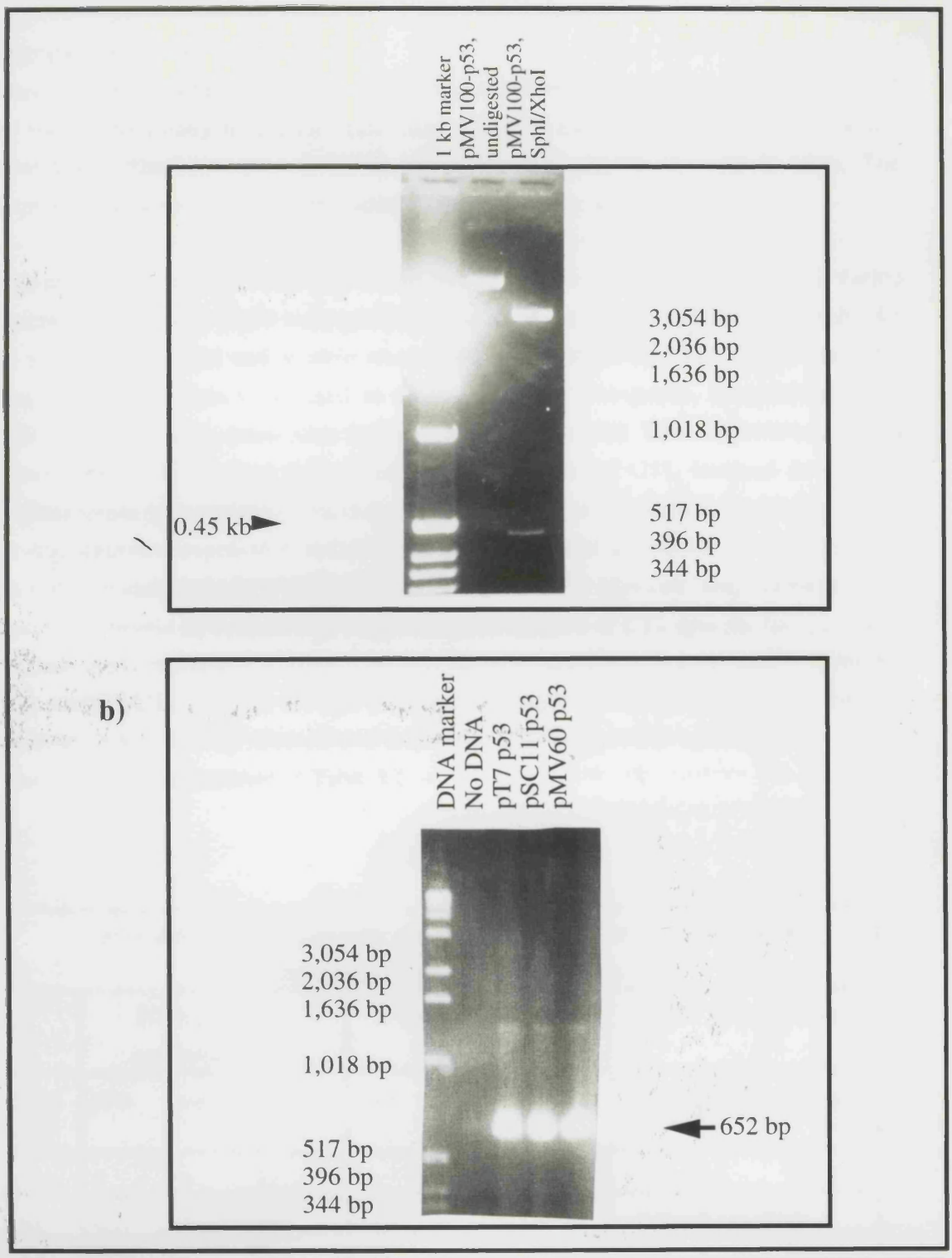


Figure 3.4.6.
Presence of correctly orientated p53 cDNA in pMV60.
a) p53 cDNA is cloned in the correct orientation in the CMV-IE expression cassette in pMV100. 1% agarose gel with pMV100 p53 DNA digested with XhoI and SphI.
b) Presence of p53 cDNA in the final pMV60-p53 plasmid. Approximately 100 pg of plasmid DNA was amplified using p53 specific primers spanning bp 546 to 1197 of the murine WT p53 cDNA sequence. pT7 p53 as well as pSC11 p53 plasmid DNA served as positive controls in the PCR reaction.

Discussion

This chapter describes the construction of three different expression systems which can be used individually to induce expression of one of the four murine proteins mdm2, cyclin D1, fibroblast growth factor receptor I and p53 *in vivo* and *in vitro*. The expression systems are useful for stimulation and analysis of CTL.

Recombinant vaccinia virus expressing mdm2, cyclin D1, fibroblast growth factor receptor I (FRI) or WT p53 were generated and analysed. These constructs are useful for *in vivo* immunisation and *in vitro* stimulation and analysis of CTL. To prevent CTL responses to vaccinia virus itself to dominate the CTL response, alternatives to the vaccinia expression systems were also searched and generated. These expression systems would primarily be used for stimulation or analysis of CTL induced *in vivo* by immunisation by recombinant vaccinia virus. Recombinant adenovirus expressing mdm2 or transfectants expressing mdm2 were generated and aimed for use for *in vitro* stimulation and analysis of CTL specific for mdm2. The EL4 cell line, overexpressing cyclin D1, would be available for stimulation and analysis of CTL specific for cyclin D1. Transfectants expressing fibroblast growth factor receptor I were generated to stimulate and analyse CTL specific for this protein. With respect to wild type p53, it was not possible to generate any alternative to the vaccinia virus expression system.
The results are summarised in Table 3.2.

Table 3.2.

DNA, mRNA and protein production obtained with different mammalian expression systems									
	Recombinant vaccinia virus			**Recombinant adenovirus**			**Stable transfectants**		
Cloned cDNA	DNA	mRNA	Protein	DNA	mRNA	Protein	DNA	mRNA	Protein
mdm2	+	+	+	+	+	N.D.	+	+	N.D.
CD1	+	+	+	-	-	N.D.	construct not made		
FRI	+	+	+	construct not made			+	+	N.D.
p53	+	+	N.D.	N.D.	N.D.	N.D.	construct not made		

Analysis and characterisation of the system had to circumvent the problems of the presence of endogenous proteins in the analysed cells. This was particularly the case for cyclin D1 and mdm2. cDNA analysis was uncomplicated in this respect, whereas mRNA analysis usually had to proceed via Northern blot analysis to be able to distinguish

between endogenous and exogenous transcripts. Analysis of constructs expressing p53 was aided by the availability of a p53 negative cell lines derived from p53 knock-out mice. Constructs expressing the fibroblast growth factor receptor could mostly be analysed in cell lines not expressing the growth factor receptor due to more limited tissue distribution of this protein or in human cell line.

Firstly, recombinant vaccinia virus expressing each of the proteins were produced and characterised. All recombinant vaccinia virus constructs were analysed and found to produce the relevant cDNA and mRNA in infected cells. Protein expression by the recombinant vaccinia virus was also demonstrated for vaccinia virus cyclin D1, vaccinia virus fibroblast growth factor receptor I and vaccinia virus mdm2. Protein production was not determined for recombinant vaccinia virus p53. Cells infected with this recombinant virus was found to produce abundant amounts of p53 mRNA as established by RT-PCR and Northern blot analysis, but it can not automatically be assumed that p53 protein was produced as a result of infection with the recombinant vaccinia virus. There is no strict correlation of the presence of the correct mRNA and protein production. The presence of the mRNA in itself is an indicator of the corresponding production of protein as translation elongation is a relatively accurate process with a missense rate of 5×10^{-4} per codon (373), but variation in RNA transport from the nucleus to the cytosol (374; 375), translation rate and stability of the mRNAs (376) can introduce variation in the rate and magnitude of protein synthesis. Variation in RNA transport from the nucleus to the cytosol should not be important as the production of vaccinia virus encoded mRNAs takes place in the cytosol. In addition, synthesis of virally produced proteins is usually favoured and known to completely supplant the synthesis of host protein in cells infected with viruses such as vaccinia virus or adenovirus. This is either because the viral mRNAs are more abundant or better designed for initiating translation and there is a simultaneous reduction in the overall translational capacity of the cell (376).

Protein analysis of cells infected with recombinant vaccinia virus generally revealed that protein was produced in cells infected by recombinant vaccinia virus, but that the level of protein production was low. It is thought that the apparent low levels of protein seen in vaccinia virus infected cells was either due to technical details surrounding the immunoprecipitations such as the use of antibodies which were all raised against the human and not the murine sequences or due to the proteins being rapidly degraded.

The level of intact protein does not tell anything in itself of the level of protein being produced and processed. The latter is a more relevant parameter with respect to CTL induction. In this respect, unstability and increased degradation of a protein has been found to increase antigen presentation to CTL (377). The proteins examined with the exception of murine fibroblast growth factor receptor I all have high turnover rates *in vivo*

due to their involvement in the cell cycle. In addition, there are examples where cDNAs have been cloned out of frame and still been used to stimulate CTL when introduced into antigen presenting cells without any detectable protein or peptides being produced in the cells (378; 379). This phenomenon is presumably due to the use of alternative initiation codons (380).

Altogether, there was suitable proof that recombinant vaccinia virus expressing mdm2, cyclin D1, fibroblast growth factor receptor or p53 had been produced and could be used for induction of CTL *in vivo* and *in vitro*. The availability of the vaccinia virus expression system would allow transient production of protein in antigen presenting cells of different haplotypes. These properties would be useful for induction and stimulation of CTL *in vivo* and *in vitro*.

Recombinant adenovirus expressing mdm2 was also produced and characterised. Cells infected with the recombinant virus were found to contain the full length mdm2 cDNA and produce the correct mdm2 mRNA. Attempts to generate adenovirus cyclin D1 and adenovirus p53 failed. With respect to adenovirus cyclin D1, virus was generated but the virus did not contain the cyclin D1 cDNA. The difficulty in generating adenovirus expressing cyclin D1 or p53 may be due to the effect of high levels of cyclin D1 and p53 on cell viability. There are reports in the literature about recombinant adenovirus expressing p53, but they are under the control of other promoters. The CMV-IE promoter is a very strong promoter (317). Since high levels of cyclin D1 or p53 are known to have a detrimental effect on cell growth (231; 232; 233; 317; 360; 361; 362; 381), it is possible that cells producing recombinant adenovirus expressing either of these two proteins were not able to survive. Analysis of the levels of protein expressed in cells infected with recombinant vaccinia virus cyclin D1, revealed that the levels of protein produced by this virus were not high (see above). This could explain why it was possible to generate recombinant vaccinia virus expressing this protein.

Transfectants expressing mdm2 or fibroblast growth factor receptor I were also generated and analysed. The transfectants were found to have the respective cDNAs stably integrated in their genomes at low copy numbers. The low copy number would probably mimic the situation *in vivo* in many tumours which overexpress the proteins although at low levels. In addition to using the transfectants for *in vitro* stimulation of CTL induced by *in vivo* immunisation with recombinant vaccinia virus, the transfectants could be used for tumour challenge experiments *in vivo*.

To sum up, the aim of generating expression systems for *in vivo* and *in vitro* stimulation of CTL had been fulfilled. All four proteins under investigation could be expressed by recombinant vaccinia virus. In addition, three of the proteins, cyclin D1, mdm2 and

fibroblast growth factor receptor could also be expressed using alternative expression systems including recombinant adenovirus and/or tumour cell lines overexpressing the protein.

4. Stimulation of cytotoxic T lymphocytes specific for self proteins by *in vivo* immunisation with recombinant vaccinia virus expressing self proteins.

Introduction

The use of selected normal self proteins as targets for tumour specific CTL requires an initial investigation of whether such self protein specific CTL exist and can be activated. This central issue can be addressed in two ways. The first is to attempt to stimulate potential autoreactive CTL *in vivo* by immunisation with the whole self protein expressed in such a way that it gains access to the class I antigen presentation pathway. The second is immunisation *in vivo* or *in vitro* with selected peptides derived from the protein under investigation.

The two approaches were explored simultaneously so that results obtained using one could be used to complement results obtained using the other. The present chapter deals with the first method involving *in vivo* immunisation of mice with whole self protein expressed in a recombinant viral expression system. Chapter 5 deals with peptide immunisation.

The objective of *in vivo* immunisation with whole self proteins, expressed in a way favouring presentation to the class I pathway, was to investigate whether CTL specific for the proteins existed, could be activated and stimulated to lyse target cells expressing high levels of the self protein.

The rationale for *in vivo* immunisation with whole self proteins delivered to the class I pathway was based on the following experimental evidence suggesting the effectiveness of *in vivo* stimulation of precursor CTL specific for self or non-self antigen to detect specific CTL responses:

1) *In vivo* immunisation can stimulate naive CTL to develop into memory CTL which exist at a higher frequency and are more responsive to antigen (93).

2) Tolerance to self proteins can be broken by *in vivo* immunisation with constructs expressing the self protein (160; 382).

3) Immunisation with whole protein *in vivo* will result in whole protein being processed within antigen presenting cells in a manner resembling the processing of endogenous self protein *in vivo*, assuming that the activities of the proteolytic machinery in the cell are identical for identical proteins, independent of the source of the protein.

The strategy used to activate CTL specific for four selected murine self proteins, cyclin D1, mdm2, fibroblast growth factor receptor I and WT p53 was based on immunisation with recombinant vaccinia virus expressing the self proteins and to the widest extent possible restimulation of responder cells *in vitro* with either a non-vaccinia based system or with a vaccinia based system used in combination with a non-vaccinia based system.

The experimental approach was initially based on methods for *in vivo* immunisation established in the lab for activation of CTL to foreign antigens such as human papilloma viruses (286). In addition, variations in the immunisation schedules as well as the procedures used for *in vitro* restimulation would be tried.

Specificity of CTL is defined differently by different labs. In the present work, specificity was based on the following two criteria :

1) Lysis of target cells infected with recombinant vaccinia virus expressing the immunising protein but not of target cells infected with recombinant vaccinia virus expressing a control protein or uninfected target cells.

2) Minimum 8-10% higher lysis of cells infected with the relevant vaccinia virus for at least two different E/T ratios.

The reason for this definition was that occasionally CTL derived from mice immunised with a particular recombinant virus would kill a cell infected with irrelevant virus better than with relevant virus, but this lysis was never more than 5-8% and only observed for the highest E/T values. Whether this was due to manipulations performed during the CTL assay or that the proteins under investigation, and used as controls for each other, had an effect on each others expression was not investigated.

CTL assays were carried out in duplicate unless CTL were scarce. CTL lines derived from mice immunised with a different vaccinia construct were always tested in the same assays to control for biased lytic activity due to the *in vivo* immunisation procedure or the *in vitro* infection of target cells for the CTL assay.

4.1. Induction of CTL specific for cyclin D1

Cyclin D1 is ubiquitously expressed in murine cells except in T cells; with the exception of some T cell tumour cell lines with integrated retroviral genomic components able to influence cyclin D1 expression. In order to investigate whether it would be possible to activate CTL precursors to this cell cycle protein, C57BL/6 mice of the H-2^b haplotype were immunised with vaccinia virus cyclin D1 *in vivo*. EL4 cells overexpressing the cyclin D1 protein or dendritic cells infected with vaccinia virus cyclin D1 were used for *in vitro* stimulation of spleens cells from immunised mice.

Altogether 22 different CTL lines derived from 19 different groups of mice immunised with vaccinia cyclin D1 (VV CD1) were generated and analysed. Different conditions were used for the *in vivo* immunisation as well as for the *in vitro* restimulation. The number of immunisations was varied from one to up to four immunisations with the recombinant vaccinia virus. Three different protocols were used for activation and stimulation of cyclin D1 specific CTL:

1) C57BL/6 mice were immunised *in vivo* with VV CD1. Spleen cells from immunised mice were restimulated weekly with mitomycin C treated EL4 cells.

2) C57BL/6 mice were immunised *in vivo* with VV CD1.
Spleen cells from immunised mice were
a) stimulated with mitomycin C treated EL4 cells during the first 6 days in culture and subsequently with purified dendritic cells infected with VV CD1 or
b) stimulated with EL4 cells twice, at day 0 and day 6 or 7 and then with dendritic cells infected with VV CD1 from day 12/13.

3) C57BL/6 mice were immunised *in vivo* with VV CD1.
Spleen cells from immunised mice were restimulated *in vitro* with VV CD1 infected dendritic cells only.

The protocols used and their relative efficiency are listed in Table 4.1.1.

Table 4.1.1.

Conditions used for *in vivo* and *in vitro* stimulation of cyclin D1 (CD1) specific CTL lines				
Antigen presenting cells used for *in vitro* stimulation of CTL	**Number of *in vivo* immuni-sations with vaccinia virus cyclin D1 (VV CD1)**	**Days between last immuni-sation and removal of spleen**	**Total number of CTL lines set up and analysed**	**Number of CTL lines recognising specifically VV CD1 infected target cells.**
From day 0 and onwards: Mit. C treated EL4 cells	2	9	3	**0**
Day 0-day 6: Mit. C treated EL 4 cells. From day 6: DCs + VV CD1	2-4	7-14	10	**7**
Day0-day 12/13: Mit.C treated EL4 cells From day 12/13: DCs +VV CD1	3	10-13	4	**0**
From day 0 and onwards: DCs + VV CD1	2 (4 lines) or 5 (1 line)	7-10	5	**0**

A cell population enriched for dendritic cells and referred to as DCs was widely used as antigen presenting cells and is described in more detail in Chapter 2 and 5. Briefly, the cell population was purified from the non-adherent fraction of naive spleens by centrifugation on a layer of metrizamide. The main contaminant was found to be B cells, whereas macrophages were largely removed, see chapter 5. DCs infected with recombinant vaccinia virus have successfully been used to generate CTL to human papilloma virus type 11 E7 protein (383).

A procedure involving initial exposure of responder cells to mitomycin C treated EL4 cells followed by restimulation by day 6 with VV CD1 infected dendritic cells ("Protocol 2 a") was found to be efficient for generating cyclin D1 specific CTL. Out of 22 CTL lines established, 10 lines were generated using these conditions, see Table 4.1.1. Of these 10 lines, 7 lines recognised specifically cyclin D1 based on the finding that RMA target cells infected overnight with vaccinia cyclin D1 were lysed more efficiently than RMA target cells infected overnight with control vaccinia virus mdm2, see Figure 4.1.1. for an example.

Insufficient mitomycin C treatment of the EL4 cells used as APCs from day 0 to day 6 was thought to be responsible for the lack of specificity observed with the three remaining lines. These three cultures were dominated by day 13 by a high concentration of cells having a morphology closely resembling the morphology of EL4 cells.

It was found essential to start restimulating the CTL lines with DCs infected with VV CD1 by day 6 and not later. In addition to the 10 lines generated by initial stimulation with EL4 followed by stimulation with VV CD1 infected DCs by day 6 or 7 ("Protocol 2a"), 4 lines were generated which were stimulated with mitomycin C treated EL4 cells until day 12/13 and then with DCs infected with VV CD1 ("Protocol 2b"). These lines never developed any cyclin D1 specificity.

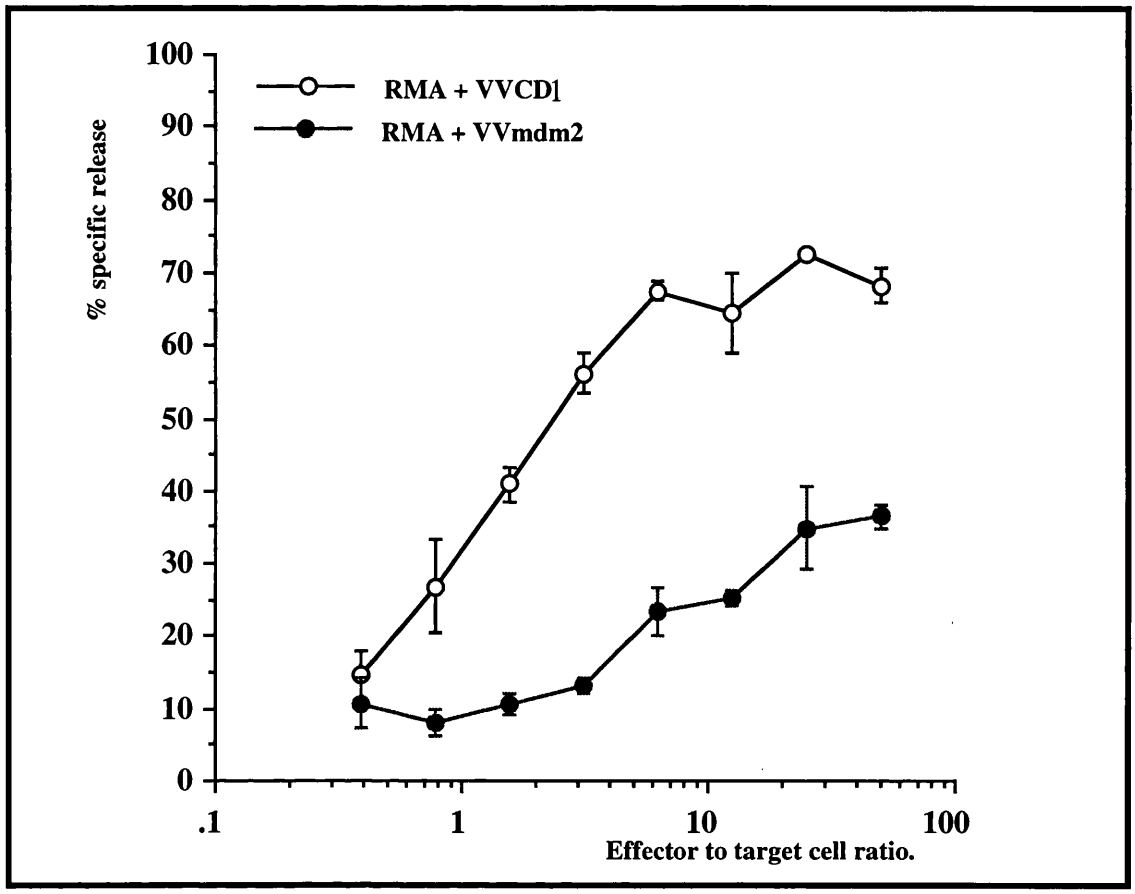


Figure 4.1.1.
Induction of cyclin D1 specific CTL by in vivo immunisation with recombinant vaccinia virus cyclin D1 (VV CD1).
^{51}Cr release assay using RMA cells infected over night with VV CD1 or control VV mdm2 as target cells. Responder cells were derived from C57BL/6 mice which had been immunised three times with VV CD1. The cells were restimulated in vitro with mitomycin C treated EL4 cells from day 0 to day 6 and with a cell population enriched for dendritic cells and infected with VV CD1 from day 6 to day 13. The assay shows the lytic activity of the responder cells at day 13 in culture. Each point represents the mean of two wells.

Continuous restimulation with mitomycin C treated EL4 cells only ("Protocol 1") or dendritic cells infected with VV CD1 only ("Protocol 3") was inefficient in stimulating cyclin D1 specific CTL lines, see Table 4.1.1.

Lines which were restimulated with EL4 cells only past day 6, developed non-specific activity apparently mostly directed against EL4 cells as EL4 cells were lysed better than RMA cells and RMA cells infected with different recombinant vaccinia viruses. This effect was significant independent of the source of IL-2 added to the culture, but more pronounced with purified recombinant IL-2 than with concanavalin A supernatant (not shown).

Restimulation with antigen presenting cells infected with vaccinia cyclin D1 from the very beginning of the culture resulted in lines which indiscriminately lysed RMA cells infected with vaccinia cyclin D1 and RMA cells infected with control vaccinia virus mdm2 (not shown).

In summary, the lines which were indeed cyclin D1 specific were all generated by restimulating lines with first EL4 cells then with vaccinia cyclin D1 infected DCs. It was thought that the epitopes recognised by the cyclin D1 immunised mice could be identified by testing cyclin D1 specific CTL lines against peptide fractions eluted from RMA cells infected with vaccinia cyclin D1 as well as control virus infected RMA cells. In order to do this experiment a considerable number of CTL were required. However, it was not possible to establish long term cyclin D1 specific CTL lines using dendritic cells infected with vaccinia virus as stimulators. The lines were highly specific 6-7 days after initial restimulation with VV CD1 infected DCs (at 12 to 13 days in culture). However, if the cells were restimulated with VV CD1 infected DCs again and tested 6 days later, they were mostly dead and the remaining cells had no lytic activity, Figure 4.1.2.

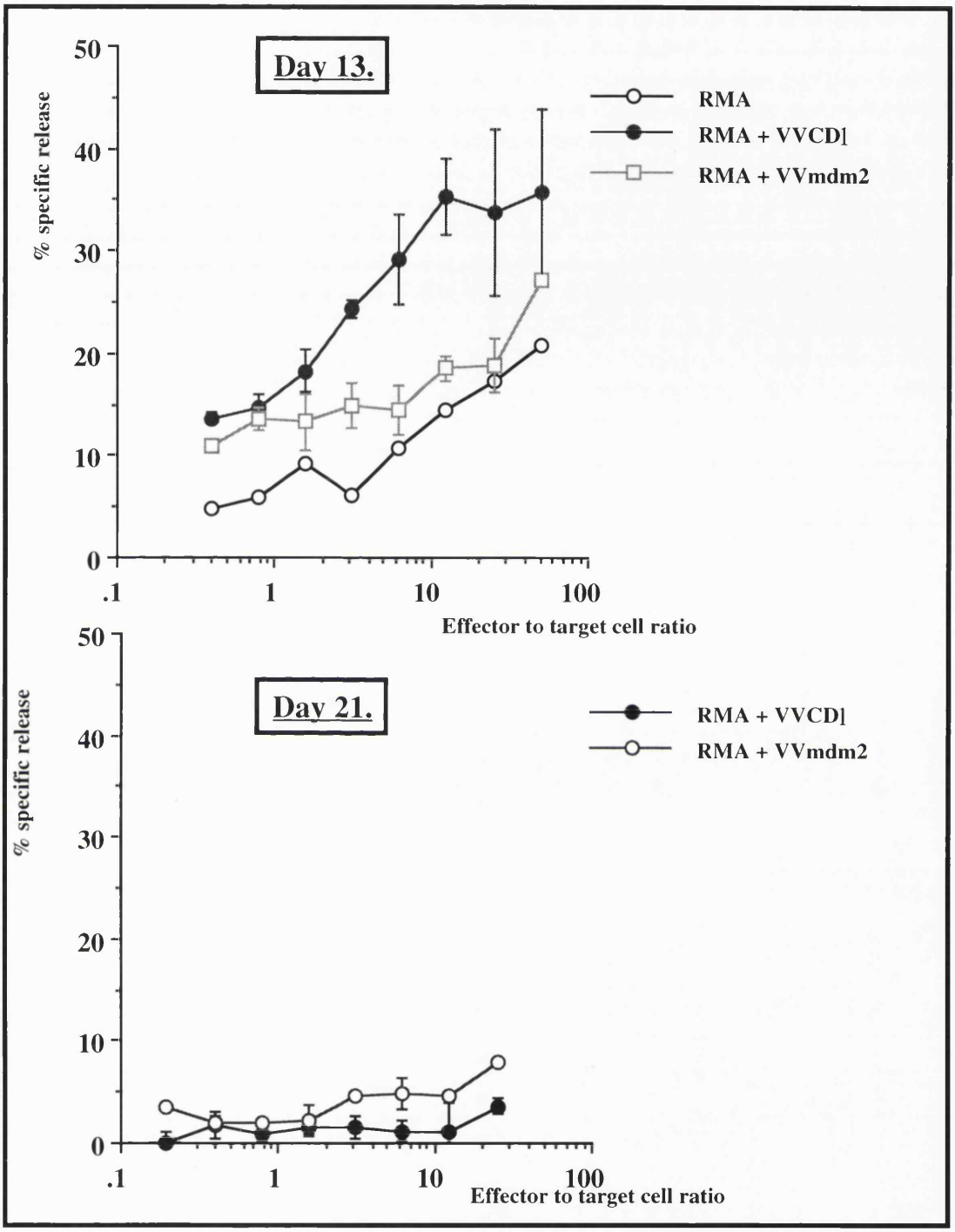


Figure 4.1.2.
Dendritic cells infected with VV CD1 can be used to restimulate a potent cylin D1 specific CTL response from mice immunised with VV CD1, but can not be used to generate a long term stable cell line.
^{51}Cr release assay using RMA cells infected with VV CD1 or VV mdm2 or uninfected RMA cells (day 13 only) as target cells. The responder cells line were derived from spleens from C57BL/6 mice which had been immunised four times with VV CD1. The responder cells were stimulated with mitomycin C treated EL4 cells on day 0 and with DCs infected with VV CD1 on day 6 and day 13.

The difficulty in establishing stable cyclin D1 specific CTL lines is most likely due to the effect of the vaccinia virus on cell viability. The infected DCs used in this work were neither irradiated (impossible for safety reasons) or mitomycin C treated so the virus would presumably be alive and spread unless kept under control by the CTL in the culture. The use of untreated vaccinia virus infected DCs for successful CTL induction was reported by (383) but this group did not report the maintenance of CTL lines beyond day 6 in culture.

Cyclin D1 specific CTL lines generated by using identical conditions for *in vitro* stimulation could differ with respect to the treatment of the mouse from which the lines were derived. This included the number of immunisations, the timing of immunisations as well as the time passed between the last immunisation and the *in vitro* boost. These factors did indeed seem to influence the magnitude of the response, although variation in the response of individual mice cannot be excluded. Between 2-4 immunisations could be used to stimulate cyclin D1 specific CTL. In one batch of mice, some of the mice had their spleens removed after 3 immunisations (Figure 4.1.1.), others had their spleens removed after 4 immunisations (Figure 4.1.2). Spleen cells derived from either group of mice developed into cyclin D1 specific effector cells.

Usually, specificity of cyclin D1 specific CTL lines was observed by day 12/13 in culture. By day 6 the CTL were only weakly specific for cyclin D1, in the sense that lysis of cyclin D1 infected targets was more than 10% higher than of the control infected target but only at the highest E/T ratio, usually 25 or 50. The only exception was a line which was derived from mice which had been immunised twice with VV CD1 with a months interval. The spleens were removed 14 days after the last immunisation, restimulated with EL4 cells and tested for lytic activity at day 6. This line showed specific cyclin D1 recognition at a very low E/T ratio. 28% lysis of RMA cells infected with VV CD1 compared to 4.3% lysis of RMA cells infected with VV mdm2 at an E/T ratio of 0.2 (not shown)

4.2. Induction of CTL specific for mdm2

Mdm2 is ubiquitiously expressed in the mouse and involved in the regulation of the cell cycle. To investigate the question whether mdm2 specific CTL exist and can be activated, C57BL/6 mice of the H-2^b haplotype were immunised with vaccinia virus mdm2 *in vivo*. A wide range of reagents described in detail in chapter 3 were available for the activation and stimulation of potential mdm2 specific CTL in mice *in vitro*. These reagents included recombinant vaccinia virus, recombinant adenovirus and transfectants expressing mdm2.

Altogether 23 different CTL lines from 14 different groups of mice were generated and analysed. The availability of three different reagents allowed for testing of different combinations of APCs for *in vitro* stimulation of CTL from immunised mice. In particular, it was necessary to test a variety of different cell types for *in vitro* restimulation with recombinant adenovirus expressing mdm2.

It was found that mdm2 specific CTL could be generated. 11 different protocols for *in vitro* stimulation of spleen cells derived from vaccinia virus mdm2 (VV mdm2) immunised mice were used. Of the different protocols investigated, three involving immunisation with VV mdm2 *in vivo* and stimulation of responder T cells with different combinations of antigen presenting cells *in vitro*, were found to stimulate mdm2 specific CTL. These three protocols are described in more detail below. All protocols are listed in Table 4.2.1.

Table 4.2.1.

Conditions used for *in vivo* and *in vitro* stimulation of mdm2 specific CTL lines				
Antigen presenting cells used for *in vitro* stimulation of CTL.	**Number of *in vivo* immunisations with vaccinia virus mdm2 (VVmdm2)**	**Days between last immunisation and spleen removal**	**Total number of CTL lines set up and analysed**	**Number of CTL lines recognising specifically VV mdm2 infected target cells**
From day 0 (and onwards) : Mit. C treated EL4 mdm2 cells	2	7	1	**1**
Day 0-6: Mit. C treated EL4 mdm2 cells From day 6: DCs+VV mdm2	3	14	2	**2**
Day 0-6: Mit. C treated EL4 mdm2 cells From day 12/13: DCs+VVmdm2	2-3	8-12 days	2	**0**
From day 0: DCs + VV mdm2	2-3	7-12	4	**0**
Day 0- 6 : Mit. C treated EL4 mdm2 cells From day 6 : EL4 +VV mdm2, Mit.C treated.	3	8	1	**0**
From day 0 : DCs+Ad mdm2	2-3	7	2	**0**
From day 0 : Whole spleen + Ad mdm2	2	10	4	**0**
From day 0 : C57+Ad mdm2	2	10	1	**0**
Day 0-6: Adherent spleen cells + Admdm2 From day 6: DCs + VV mdm2	1-2	14	2	**1**
From day 0: DCs + Admdm2	2-3	7-10	2	**0**
Day 0-6: HelaK^b +Ad mdm2 From day 6 : DCs + VV mdm2	2	14	2	**0**

The protocols which resulted in the generation of mdm2 specific CTL were the following:

1) C57BL/6 mice were immunised once with VV mdm2 *in vivo* and spleens were removed two weeks after the immunisation. Responder cells were restimulated in *vitro* with mouse splenic adherent cells as APCs. The adherent cells were infected with adenovirus mdm2 for 8 hours and used as APCs from day 0 to day 6. At day 6 responder cells were restimulated with dendritic cells infected with VV mdm2. Specificity was observed already by day 6, but was greatly improved by day 13 in culture, see Figure 4.2.1.

The T cells were tested at day 13 for recognition of two mdm2 peptides containing class I binding motifs and known to be either intermediate or strong class I binders, Figure 4.2.1. (for details of the peptides see chapter 5). RMA-S cells pulsed with these peptides were lysed inefficiently compared to RMA cells infected with VV mdm2. RMA cells infected with the control cyclin D1 virus were also lysed inefficiently compared to cells infected with VV mdm2. This indicates that the CTL generated are able to distinguish between the endogenous lower levels of mdm2 present in the lymphoma cell line RMA and the higher levels of mdm2 present in RMA cells infected with recombinant VV mdm2.

Immunisation of C57BL/6 mice twice instead of once and followed by culture of the spleen cells using the *in vitro* conditions described above did not give rise to any specific CTL by day 6, and the line was not available for testing by day 13.

2) C57BL/6 mice were immunised three times with vaccinia mdm2 and spleens were removed two weeks after the last immunisation. Responder cells were restimulated *in vitro* with mitomycin C treated EL4 cells transfected with mdm2 from day 0 to day 6 and subsequently with dendritic cells infected with VV mdm2.

Mdm2 specificity was observed at day 13 in culture. Two such lines were generated, one of which is shown in Figure 4.2.2. Lines from mice immunised with VV CD1 were tested in the same experiment and found to have cyclin D1 specificity (not shown). It is clear, that there is specific lysis of mdm2 infected target cells, but that there is also considerable lysis of cells infected with control virus, see Figure 4.2.2.

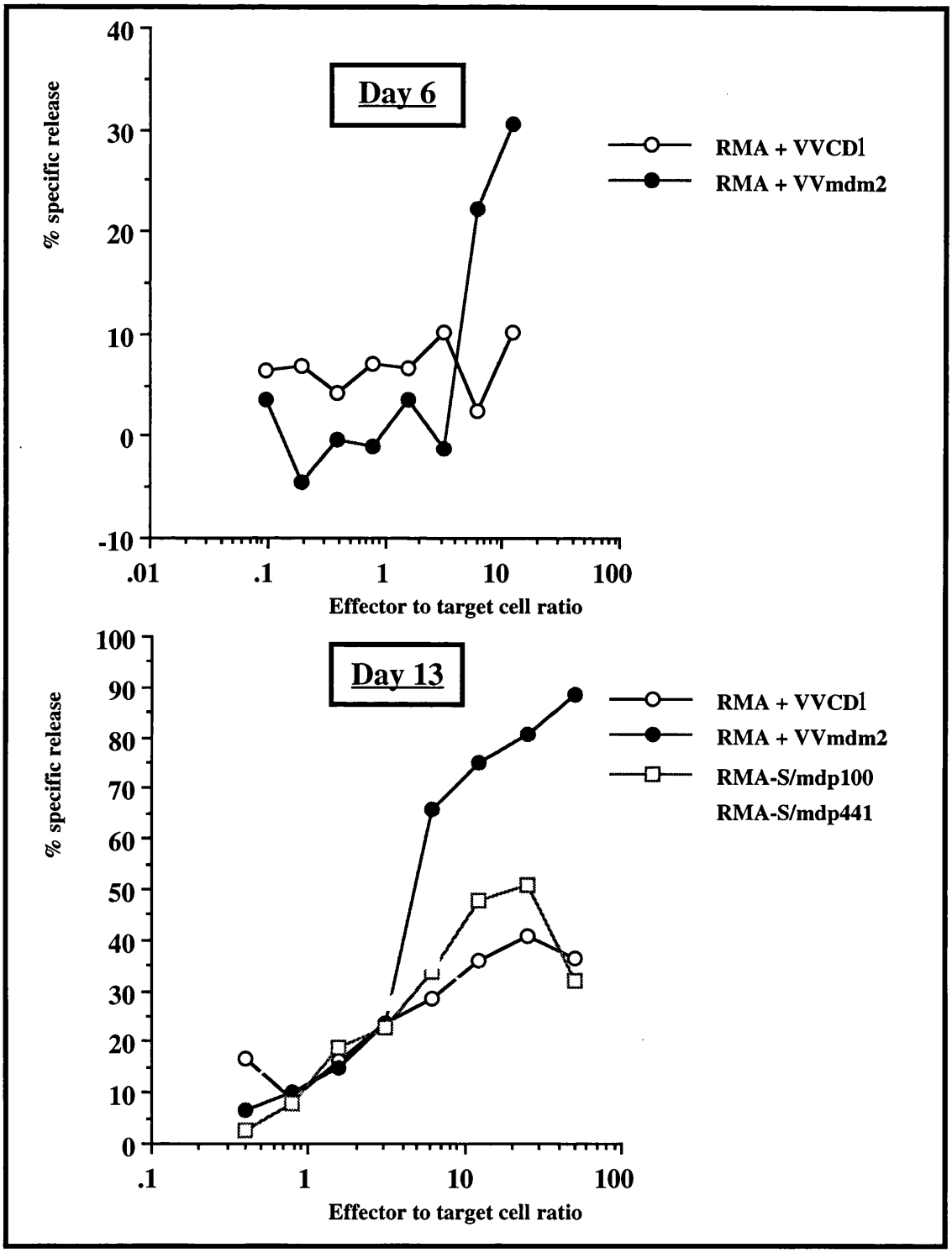


Figure 4.2.1.
Immunisation of C57BL/6 mice with vaccinia virus mdm2 (VV mdm2) stimulate mdm2 specific CTL.
10 weeks old C57BL/6 mice were immunised once with $2x10^7$ pfu VV mdm2. Spleens were removed two weeks later and restimulated with splenic adherent cells which had been infected for 8 hours with Ad mdm2. The responder cells were tested for lytic activity at day 6 and restimulated with DCs infected with VV mdm2. The responder cells were again tested for lytic activity on day 13.

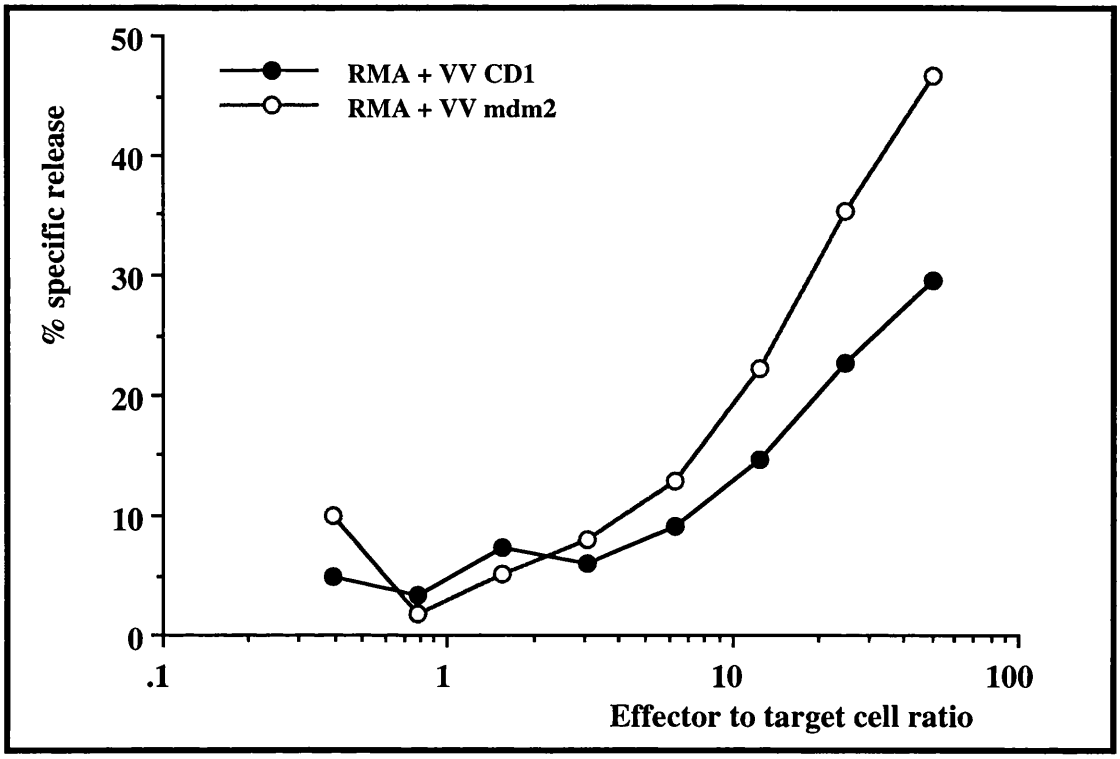


Figure 4.2.2.
In vivo primed mdm2 specific CTL can be restimulated in vitro with transfectants expressing mdm2 and dendritic cells infected with VV mdm2.
C57BL/6 mice were immunised with VV mdm2 in vivo and restimulated in vitro with EL4 cells transfected with mdm2 from day 0 to day 6 and with DCs infected with VV mdm2 from day 6 to day 13.
^{51}Cr release assay representing CTL activity of the T cell line at day 13 in culture against RMA cells infected overnight with VV mdm2 or control VV CD1.

3) C57BL/6 mice were immunised twice with VV mdm2 *in vivo* and spleens were removed one week after the last immunisation. Responder cells were restimulated *in vitro* with mitomycin C treated EL4 cells transfected with mdm2, see Figure 4.2.3. These lines had weak mdm2 specificity in early cultures, see Figure 4.2.3, top panel. There was considerable lysis of cells infected with control virus. On day 27, the CTL still lysed VV mdm2 infected target cells better than control vaccinia virus fibroblast growth factor receptor I (VV FRI) infected target cells, but there was still significant lysis of the control cells, Figure 4.2.3., bottom panel. CTL from mice immunised with VV FRI were tested against the same target cells in the same assay and found to have opposite specificity, see later (Figure 4.3.1.). Altogether, Figure 4.2.3. shows that the use of EL4 cells transfected with mdm2 to restimulate responder cells from VV mdm2 immunised mice did not generate as potent and specific CTL as T cells generated by *in vitro* restimulation with splenic adherent cells infected with adenovirus mdm2 followed by restimulation with DCs infected with VV mdm2 ("Protocol 1", Figure 4.2.1.). The advantage of using mitomycin C treated transfectants for restimulation of CTL would be the ability to create a stable T cell line.

In summary restimulation based on the initial use of adherent cells derived from a naive spleen and infected with adenovirus mdm2 *in vitro* as APCs followed by the use dendritic cells infected with VV mdm2 as APCs seemed to be the optimal way for restimulation of mdm2 specific CTL *in vitro*. However, this needs to be verified by the generation of more CTL lines to generate a statistically valid comparison.

Due to use of untreated DCs infected with VV mdm2 as stimulators *in vitro*, long term cell lines were not established. However, as the specific lysis of mdm2 was very high, future work to identify target epitopes could be based on day 13 T cells. This could also be done for the cyclin D1 specific CTL described in section 4.1.

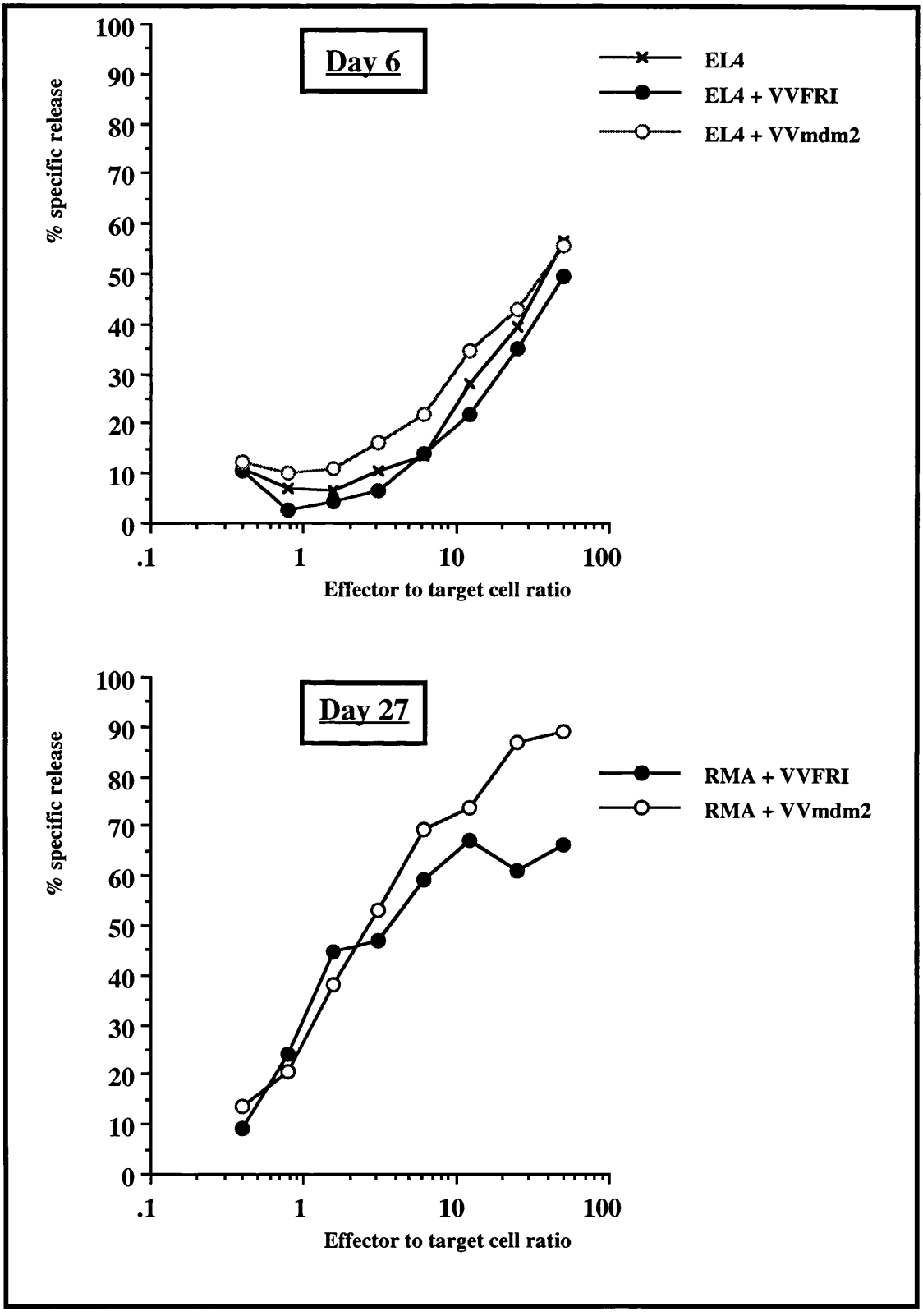


Figure 4.2.3.
In vivo primed mdm2 specific CTL can be restimulated in vitro exclusively with transfectants expressing mdm2.
C57BL/6 mice were immunised twice with VV mdm2 in vivo and restimulated with EL4 cells transfected with mdm2 in vitro. ^{51}Cr release assays showing lysis by the CTL line of RMA cells infected overnight with VV mdm2 or control virus VV FRI.

4.3. Induction of CTL specific for FRI

Fibroblast growth factor receptor I (FRI) represents a self protein which is not expressed in the thymus and spleen but abundantly in other tissues in the periphery including neuroepithelial cells, skeletal muscles and the heart.

C57BL/6 or BALB/c mice were immunised with recombinant vaccinia virus expressing fibroblast growth factor receptor I (VVFRI). Spleen cells from immunised mice were restimulated *in vitro* with transfectants or dendritic cells infected with VVFRI (DC + VV FRI). Altogether 3 lines were established from C57BL/6 mice and 2 lines were established from BALB/c mice. Only one line showed some specificity for FRI, Figure 4.3.1. This line was established from C57BL/6 mice immunised 3 times *in vivo* with recombinant virus and restimulated *in vitro* with EL4 cells transfected with FRI (EF). Specificity of the FRI stimulated cell lines was not apparent until after prolonged culture.

T cells derived from mice immunised with VV mdm2 and having been 27 days in culture were tested against the same target cells in the same assays. These CTL had oppositie specificity (see Figure 4.2.3). No lines of significant specificity were established from BALB/c mice immunised *in vivo* and restimulated *in vitro* with P1.HTR cells transfected with FRI.

Future experiments investigating the immunogenicity of FRI should involve the use of EL4 or P1.HTR cells transfected with FRI for the initial 6 days of culture followed by restimulation with dendritic cells infected with recombinant vaccinia virus expressing FRI. These conditions were found to stimulate CTL specific for two other self proteins, cyclin D1 and mdm2, see Section 4.1 and 4.2. Time constraints prevented investigation of this method for the FRI protein. The establishment of one line able to recognise cells expressing high levels of FRI is by no means a proof of the general feasibility of stimulating autoreactive CTL specific for FRI.

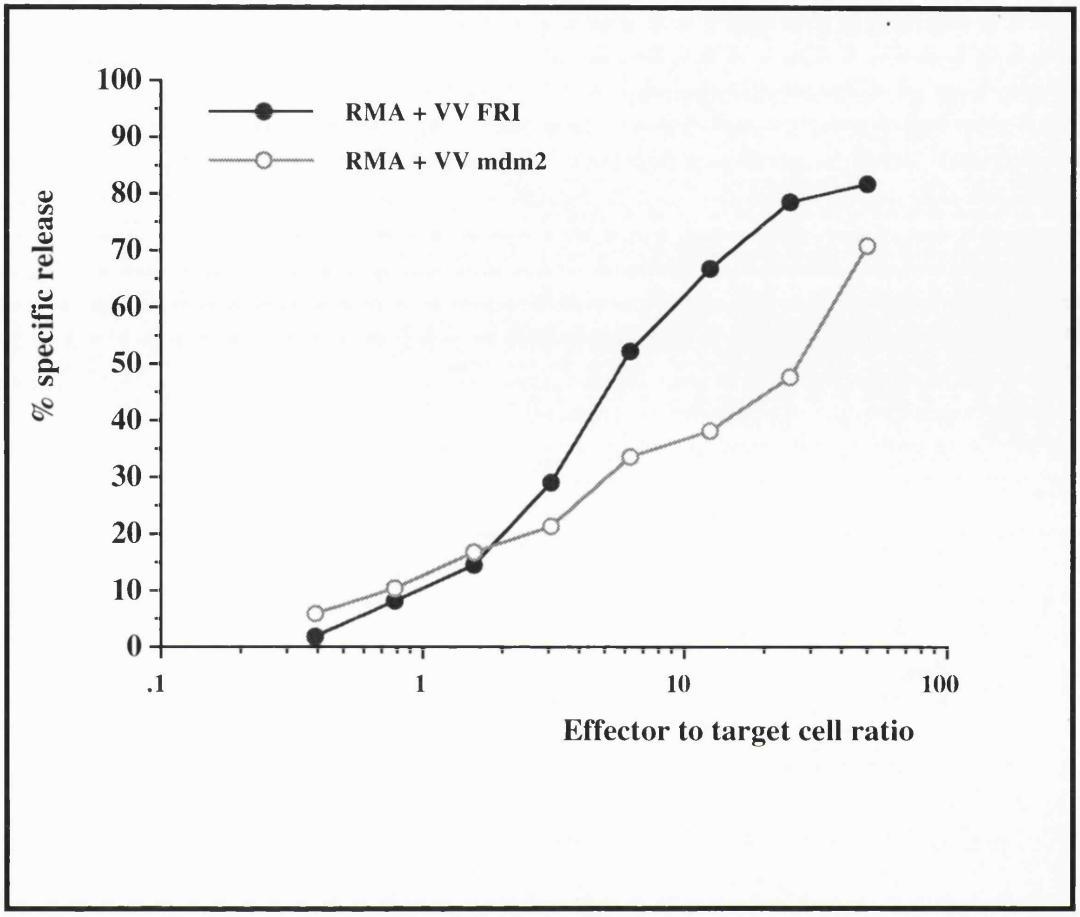


Figure 4.3.1.
FRI specific CTL can be generated by in vivo immunsation with vaccinia virus FRI (VV FRI) and in vitro restimulation with transfectants expressing FRI. C57BL/10 mice were immunised three times with VV FRI in vivo. Splenocytes from immunised mice were subsequently restimulated with EL4 cells transfected with FRI in vitro.
Chromium release assay showing lysis of RMA cells infected overnight with VV FRI or VV mdm2 by responder CTL at day 40 in culture.

4.4. Induction of CTL specific for p53

WT p53 is ubiquitiously expressed in the mouse. To investigate induction of CTL in the mouse, C57BL/6 mice were immunised with vaccinia virus p53. It had not been possible to generate transfectants expressing WT p53 or adenovirus expressing wild type p53, so spleen cells from immunised mice were stimulated *in vitro* with dendritic cells infected with vaccinia virus p53 or with untransfected EL4 cells, which express low levels of endogenous p53 (not shown). However, none of these methods of restimulation resulted in the generation of CTL lines able to recognise specifically WT p53. This lack of success was thought to be due to a lack of suitable reagents for stimulation of lines *in vitro* rather than a firm demonstration of the inability to break potential tolerance to this protein *in vivo*. The methods used, continuous restimulation with vaccinia infected cells or a tumour cell line, had not been optimal for the stimulation of CTL to two other self proteins, cyclin D1 and mdm2, whereas other methods of restimulation could be used to obtain CTL highly specific for either of these two proteins.

Discussion

Normal self proteins represent potential interesting tumour antigens for immunotherapy because several normal self proteins are found to be expressed at elevated levels in a wide variety of human cancers. The use of such normal self proteins to stimulate CTL responses directed towards tumours expressing high levels of the self protein in question requires an initial investigation of whether CTL directed against the self protein do exist and can be activated *in vivo*. The central issue is to what extent CTL tolerance to self proteins exist and in particular to what extent CTL can be activated to recognise and lyse target cells expressing elevated levels of self proteins specifically. This issue is closely linked to the issue of autoimmunity and whether it will be possible to generate CTL able to distinguish between normal tissues presenting low levels of individual self peptides on the cell surface and tumour cells presenting higher levels of the same peptide.

Two approaches can be used to investigate whether CTL specific for peptides derived from endogenously processed self proteins exist and can be stimulated to lyse target cells expressing these self proteins. One approach involves the delivery of self protein to the class I pathway *in vivo* by immunisation with an appropriate vehicle. The second approach involves the use of synthetic peptides derived from the self protein to stimulate CTL *in vivo* and *in vitro*. The present chapter describes results obtained using the first approach in a murine system.

Recombinant vaccinia virus expressing four selected murine self proteins, cyclin D1, mdm2, WT p53 and fibroblast growth factor receptor I were used to immunise mice, mostly of the H-2^b haplotype. Spleen cells from immunised mice were stimulated *in vitro* using a combination of different antigen presenting cells including transfectants expressing the protein under investigation as well as a cell population enriched for dendritic cells and infected with recombinant vaccinia virus or adenovirus.

The main findings were the following:

1) It is possible to induce CTL reproducibly to selected murine self proteins using *in vivo* immunisation with vaccinia virus and stimulation of CTL *in vitro* with APCs producing the self protein used for immunisation.

2) The method used for *in vitro* restimulation of CTL derived from mice immunised *in vivo* is crucial for the specificity and magnitude of the response directed towards the self protein as judged from lysis of cells producing elevated levels of self protein.

It is possible to induce CTL reproducibly to selected murine self proteins

The first major finding was that it was possible to stimulate CTL specific for selected self proteins by a combination of *in vivo* and *in vitro* stimulation of CTL:
Immunisation with recombinant vaccinia virus expressing cyclin D1 and mdm2 resulted in the generation of several CTL lines able to recognise cyclin D1 and mdm2 respectively. In one instance CTL lines specific for the murine fibroblast growth factor receptor I were also detectable. The findings indicate that tolerance to cyclin D1 and mdm2 (and possibly fibroblast growth factor receptor I) is not absolute. The data need to be complemented by additional data on the ability of activated CTL to recognise and eliminate tumours expressing high levels of the protein without concomitant destruction of healthy tissues expressing lower levels of the protein. However, the data suggest that self proteins may be used to activate CTL which might be exploited for immunotherapy.

The results need to be interpreted in the light of recent data on thymic selection and peripheral tolerance. Developing thymocytes are subjected to positive and negative selection in the thymus, for review see (120; 148). During the process of positive selection, T cell receptors on the developing $CD4^+CD8^+$ thymocyte interact with MHC molecules on antigen presenting cells. This delivers a survival signal to the T cell. It is known that peptides can contribute to positive selection, but not known whether peptides are important for the process *in vivo*. Peptides which are potent T cell agonist do not induce positive selection(154) indicating that if peptides are directly involved in the process of positive selection *in vivo*, the same peptides are unlikely to activate the mature T cell. It is therefore likely that T cells specific for epitopes derived from mdm2 or cyclin D1 have not been positively selected by these epitopes in the thymus. Current knowledge indicates that the T cells will have been selected either by an irrelevant peptide which can act as an antagonist for the T cell or no peptide at all.

Whereas the role of self peptides in positive selection *in vivo* is still equivocal, the role of self peptide in negative selection is clearer. T cells which bind with high avidity to MHC class I /self peptide complexes in the thymus will be deleted. The avidity of the interaction between thymocyte and antigen presenting cell is the result of the intrinsic affinity of the TCR for the peptide /MHC complex, the density of TCR on the T cell and the density of peptide/MHC complexes (384). If the combined effect of these factors is insufficient to cause deletion of the thymocyte, the self peptide specific thymocyte will potentially be able to leave the thymus and enter the circulation. Thus T cells specific for self proteins such as mdm2 and cyclin D1 could exist in the periphery, either because the epitopes recognised are not well presented *in vivo* or because the CTL are of low avidity due to low numbers of T cell receptors specific for the cyclin D1 or mdm2 derived epitopes or because the affinity of the T cell receptors specific for the generated self peptide/MHC complexes is low. This hypothesis is supported by data from several independent

transgenic models based on the use of T cell receptors, defined antigens or both as transgenes (158; 159; 160; 161; 163). These different models showed that high avidity T cells were deleted and that the remaining T cells were either of low avidity or specific for poorly presented, usually subdominant, epitopes.

The work described in the present chapter does not investigate the avidity of the T cells involved. This issue will be investigated in chapter 5 for self peptide specific CTL. The present work only establishes that CTL recognising the self proteins cyclin D1 and mdm2 are present and can be stimulated to recognise endogenously processed protein. At present work by others (100) and the work described in chapter 5 suggest that CTL able to recognise endogenously processed protein are of intermediate to high avidity. The most likely explanation for the presence of the autoreactive CTL detected by *in vivo* immunisation with the whole protein is that the epitopes recognised are presented at insufficient levels *in vivo* to induce negative selection of the T cells. This situation would be analogous to the situation observed in mice transgenic for hen egg lysozyme or LCMV nucleoprotein. In those experiments, high avidity T cells specific for immunodominant epitopes were deleted whereas T cells able to recognise endogenously processed proteins and specific for subdominant epitopes from lysozyme or other LCMV proteins respectively were detectable (158; 159; 161). In the hen egg white lysozyme transgenic model, even T cells specific for the subdominant epitopes were deleted when animals expressed very high levels of lysozyme, emphasising the effect of antigen dose on T cell selection (158).

In the work described in the present chapter, class I restricted CTL specific for the self proteins cyclin D1, mdm2 and on one occasion FRI were activated by *in vivo* immunisation with the self proteins. Activation of class I restricted CTL specific for a variety of self proteins has also been reported using other experimental approaches. In the mouse, self peptide specific CTL recognising endogenously processed TCR Vβ8.2. protein, were detected by *in vivo* immunisation of B6 mice with a Vβ8.2 derived peptide *in vivo* (385). Human CTL recognising endogenously processed self protein include HLA-A2.1 restricted CTL specific for glutamic acid decarboxylase and derived from PBMC of preclinical or recent onset insulin-dependent diabetes mellitus patients (386), HLA-A2 restricted CTL specific for myelin basic protein or myelin associated glycoprotein and derived from PBL of Multiple sclerosis patients as well as normal healthy volunteers (387) and finally CTL derived from melanoma patients and specific for either of a range of different melanoma antigens including tyrosinase, gp100, gp75, MART-1 and MAGE-1, for review see (388; 389). Overall, these results as well as the results presented in this chapter show that autoreactive CTL exist and can be activated under certain circumstances. However, without activation these CTL will not do any damage to normal tissue. This has also been confirmed in several transgenic models (160;

168; 382). In the present work, immunisation with recombinant vaccinia virus expressing cyclin D1, mdm2 and in one case fibroblast growth factor receptor I activated CTL specific for the immunising proteins to an extent that the CTL could kill target cells expressing the self protein in assays *in vitro*. The question arising is then whether these CTL shown to have lytic activity *in vitro* would do any damage *in vivo* towards the mice which had been immunised. In other words, would the CTL being able to lyse antigen presenting cells *in vitro* also do so *in vivo* and if the latter was the case would the CTL then discriminate between the low levels of antigen present in normal tissue and the higher levels supposedly present in cells infected with recombinant vaccinia virus expressing self protein?

All mice under investigation appeared healthy. It seems likely that either no or only minor autoimmune damage occurred, with the latter response not being able to be sustained due to low levels of expression of the relevant epitopes in normal tissue. Transient but minor autoimmunity has been reported by priming T cell receptor transgenic CBA mice containing high avidity T cells specific for K^b, with high avidity being defined in that case as high density of the transgenic T cell receptor (160). These high avidity T cells existed in the mouse without doing any harm to pancreatic β cells expressing a second transgene, K^b, the antigen recognised by the transgenic T cell receptor. However, when the mice were primed with spleen cells expressing K^b transient autoimmunity occurred, and it was suggested that the inability of the β cells to act as efficient APCs to sustain the immune response accounted for the transient nature of the response. Self proteins such as cyclin D1 and mdm2 are expressed in all tissues examined, except T cells in the case of cyclin D1. This means that even professional antigen presenting cells would be expected to present epitopes continuously from these self proteins. Lack of presentation of the proteins would consequently not be accounted for by the inability of normal tissue to act as APCs. Instead, it seems likely that the level of antigen presented is too low to sustain an immune response. The avidity of the interaction between activated T cells and antigen presenting cells might be high enough for T cell activation and lysis of the APC if high levels of peptide epitopes are produced from the infection of cells with a recombinant virus expressing the protein, but not if lower levels are produced such as is the case in normal tissue. Some of the mdm2 specific CTL were clearly shown to be able to distinguish between different levels of self protein, in as much as the CTL did not lyse RMA cells known to express endogenous mdm2 but did indeed lyse RMA cells infected with a recombinant vaccinia virus expressing mdm2.

The second explanation for lack of detectable activity of self reactive CTL *in vivo* post immunisation, could be that the T cells were anergised *in vivo* by the immunisation procedure used. Exposure of mature peripheral naive T cells to antigen by transfer of antigen specific CTL to recipient animals, expressing different levels of the target antigen,

has been found to result in either activation at low doses of antigen, exhaustion at intermediate doses of antigen and anergy with down regulation of CD8 co-receptors at high antigen concentration (175). This experimental evidence accounts not only for the fact that self protein specific CTL can exist *in vivo* despite having been initially activated but can also provide a second explanation for the difficulty in detecting CTL specific for self or non-self proteins after repeated immunisation. Rapid expansion followed by exhaustion of antigen reactive CTL has also been observed when mice were infected with lymphocytic choriomenigitis virus, LCMV (390).

In transgenic models, tolerance induction has been shown to be a multistep process with T cells already tolerant to certain stimuli being able to be rendered completely tolerant by exposure to high levels of antigen (166; 174; 391). TCR transgenic mice specific for K^b and crossed with mice expressing mutant K^b in several tissues including the thymus or normal K^b exclusively in the periphery, had T cells already expressing low levels of TCR specific for the antigen and were unable to reject skin grafts expressing the antigen *in vivo*. These T cells would further down-regulate the level of T cell receptor on encountering high levels of the antigen. In the present work, spleen cells from mice immunised *in vivo* with vaccinia virus mdm2 were restimulated *in vitro* from day 0 to day 6 with adherent cells infected with recombinant adenovirus expressing mdm2. Only spleen cells derived from mice which had received a single immunisation were specific for mdm2 by day 6 (see Figure 4.2.1). Mice which had been immunised twice did not give rise to any mdm2 specific CTL, showing that in this case increased exposure to antigen in vivo did not result in improved antigen specific CTL responses but the opposite. In contrast, activation of cyclin D1 specific CTL seemed to be optimal when several immunisations were involved. The main difference between the expression pattern of cyclin D1 and mdm2 is that cyclin D1 is not present whereas mdm2 is present at high levels in T cells. Whether murine T cells play a role as APCs in this context is unknown. Activated human T cells express MHC Class II molecules as well as various adhesion molecules important in interactions with T cells including ICAM-1 and B7-1 (392). Human T cells can act as antigen presenting cells able to activate human class II restricted resting T cells (392; 393), but human T cells as APCs have also been reported to be able to anergise T cells which were already activated (392).

With respect to the use of CTL specific for normal self proteins in tumour immunotherapy *in vivo*, it should be noted that the antigenic load of a tumour is expected to be very high. In addition to the lack of costimulatory molecules on some tumour cells, antigenic overload resulting in T cell exhaustion has been suggested to contribute to the inability of CTL to reject tumour cells (390).

The method used for *in vitro* restimulation of CTL derived from mice immunised *in vivo* is crucial for the specificity and magnitude of the response.

The second major result from the work described in this chapter was that detection of cyclin D1 and mdm2 specific CTL responses was highly dependent of the method of restimulation used *in vitro*.

The antigen presenting cells used included a cell population enriched for dendritic cells, EL4 cells (either transfected or untransfected), whole spleens (infected with recombinant adenovirus or vaccinia virus), the adherent fraction from spleens as well as different epithelial cell lines including Hela and C57 epithelial cell lines (all infected with recombinant adenovirus). Three different protocols for *in vitro* restimulation utilising a single type of APC or a combination of different APCs were clearly able to stimulate a CTL response specific for the self proteins used to immunise the mice. These were
1) the combination of EL4 cells for the initial stimulation of T cells (untransfected or transfected) followed by the later use of dendritic cells infected with vaccinia virus. This procedure was able to lead to the stimulation of 7 independent cyclin D1 specific CTL lines and 2 independent mdm2 specific CTL lines, altogether 9 lines.

2) the combination of adherent cells infected with recombinant adenovirus for the initial stimulation of CTL *in vitro* followed by DCs infected with recombinant vaccinia virus. This combination induced potent mdm2 specific responses.

3) The use of EL4 cells transfected with mdm2 or FRI. This method of restimulation resulted in the generation of mdm2 specific and FRI specific CTL, but was found to be the least potent of the methods resulting in stimulation of self protein specific CTL. CTL lines which were set up from Balb/c mice immunised with recombinant VV FRI or VV mdm2 and restimulated with P1.HTR cells transfected with FRI or mdm2, never developed any significant specificity.

It is thought that several factors contributed to the superiority of some restimulation protocols. These factors include firstly, properties of the viral expression system used for *in vivo* and in some cases *in vitro* stimulation of CTL. Secondly, properties of the antigen presenting cells used for *in vitro* stimulation of CTL, including the ability of these APC to costimulate T cells and contribute to the creation of a cytokine environment favourable for generating and sustaining CTL responses.

Firstly, the properties of the viral expression system used for the *in vivo* immunisation and/or *in vitro* restimulation of CTL will have a profound influence of the outcome of the CTL response as detected in ^{51}Cr release assays *in vitro*. These expression systems

included vaccinia virus for *in vivo* immunisation and in some cases vaccinia virus and/or adenovirus for stimulation of CTL *in vitro*.

In the present system vaccinia virus expressing recombinant self protein was used for the *in vivo* stimulation of CTL to self proteins. The reasons for the choice of this expression system for *in vivo* immunisations were several. The virus has an excellent record for the *in vivo* and *in vitro* induction of CTL to exogenous proteins. The entire life cycle of the virus takes place in the cytosol. This contributes to the efficiency of the delivery of newly synthesised proteins to the class I pathway. There appears to be a positive correlation between the quantity of an individual protein within the cell and the amount of peptide epitopes generated from the protein (77). Increased protein production is consequently expected to contribute to an increased density of MHC/peptide complexes on the cell surface and an increase in the over all avidity of the interaction between self specific CTL and antigen presenting cell.

In addition to delivering exogenous proteins to the class I pathway, viral infection results in the production of local cytokines including IL-2, IFN-γ and IFN α/β (110). Several transgenic models have showed that local sustained production of IL-2 (168) or IFN-γ (394) can activate tolerant self specific T cells. Other cytokines including IL-6 and IL-1 are also known to be produced by splenocytes from vaccinia virus immunised mice (291). The local cytokine production might also produce a bystander effect which could contribute to the activation of self specific CTL (395; 396).

Despite the benefits gained by using recombinant vaccinia virus to induce CTL to self proteins, vaccinia virus does present potential problems. The main obstacle to the use of vaccinia virus as a vehicle is the possibility of inducing an overwhelming CTL response to vaccinia proteins which would reduce the response to less efficiently processed CTL epitopes. Peptides which bind equally well to class I and which are produced in similar quantities are known to be able to induce independent CTL population in the same mice when administered simultaneously as demonstrated by immunisation of mice with recombinant influenza virus expressing two different D^b restricted epitopes from the nucleoprotein of influenza virus (397). However, the avidity model for negative selection as well as the nature of the immunodominant self peptides identified in human cancer patients, point towards the existence of immunodominant self epitopes which are <u>not</u> efficiently processed and presented by class I *in vivo*. Consequently, such self epitopes might not compete well with efficiently presented vaccinia derived epitopes. The precursor frequency of self peptide specific CTL able to interact with antigen presenting cells with high avidity is likely to be low compared to the number of vaccinia virus specific precursors able to engage in high avidity interactions. As the size of the activated

$CD8^+$T cell pool is constant (398), CTL directed against less well presented self epitopes might be difficult to detect.
Indeed, restimulation *in vitro* with vaccinia virus infected dendritic cells resulted in a high degree of CTL activity which appeared to be directed against vaccinia virus epitopes in as much as cells infected with any recombinant vaccinia virus were lysed but uninfected cells were not. It cannot be excluded that some of the CTL response was directed against β-galactosidase also expressed by all the recombinant viruses and known to contain an $H\text{-}2^b$ presented CTL epitope (61; 399).

Apart from producing potent CTL epitopes, vaccinia virus (and adenovirus) might produce peptides able to antagonise self (or non-self) specific CTL responses and thereby prevent the expansion of certain self (or non-self) specific CTL. Antagonist peptides are non-stimulatory ligands which can compete with T cell agonists for T cell receptor occupancy and transduce a qualitatively different signal that results in lack of T cell stimulation (400; 401). Viruses are known to be able to produce antagonist peptides as a means of escaping T cell recognition (400).

For *in vitro* stimulation, vaccinia virus was mainly used to infect cell populations enriched for dendritic cells. Initial experiments using adenovirus infected dendritic cells as antigen presenting cells did not result in the generation of any self protein specific CTL. A wide variety of other APCs were infected with recombinant adenovirus and used as stimulator cells in T cell cultures involving responder cells from immunised mice. It was found that only adenovirus infection of adherent cells derived from spleens appeared to be able to stimulate efficiently a self protein specific CTL response. It is thought that the potency of this cell type to stimulate CTL responses is due to the ability of the recombinant adenovirus to productively infect this cell population consisting of adherent cells such as epithelial cells and macrophages. Autologous spleen cells infected with recombinant adenovirus have previously been used to restimulate successfully CTL derived from spleens of *in vivo* immunised mice (303). The authors did not identify the cell type(s) within the preparation acting as APC, neither did they present any data on how the spleen cells were treated prior to adenovirus infection and use as APCs in the T cell cultures.

The second reason for the efficiency of some protocols for restimulation of self specific CTL, would most likely be linked to molecular properties of the antigen presenting cells used. This would include levels of costimulatory molecules on the cell surface and the ability to secrete or induce the secretion of cytokines favouring a CTL response.

With respect to costimulation, it has been established that activation of T cells requires two distinct signals, for review see (102; 127). The first signal originates from the

ligation of the T cell receptor complex and its coreceptors (CD4 or CD8). The second signal is dependent on either soluble factors such as IL-2 or the interaction of cell surface molecules which provide important costimulatory signals. The interaction of CD28 on T cells with B7-1 and/or B7-2 on antigen presenting cells is particularly important. The primary effect of the CD28 mediated costimulation appears to be to promote late cell cycle progression by maximising IL-2 production and regulating programmed cell death.

B7-1 is expressed at low levels on DCs, macrophages and thymic epithelial cells. B7-1 is up-regulated later in immune responses than B7-2 and appears important in prolonging primary T cell responses or costimulating secondary T cell responses. B7-2 is constitutively expressed on DCs and macrophages and is up-regulated on activated B cells. This molecule appears to be the domininant costimulatory ligand for the induction of primary immune responses (102). The levels of B7-1, but particularly B7-2 are considerably up-regulated on murine splenic dendritic cells when placed in culture, presumably due to the influence of cytokines such as IL-1 or GM-CSF (402). The efficiency of cell populations enriched for dendritic cells and infected with vaccinia virus or adherent cells infected with adenovirus could partly be due to the ability to sustain the response of T cells activated *in vivo* or even reverse anergy of self specific T cells. It should be noted that IL-2 were added to all cultures after 6 days *in vitro* culture. If the role of the B7-CD28 interaction is indeed to maximise IL-2 production (102), this interaction would become particularly important when the exogenously added IL-2 was consumed/degraded. There are some controversial reports that B7-1 and B7-2 might stimulate different T helper responses with B7-2 providing a signal to naive T cells to secrete IL-4 (124), however, this effect is mainly thought to play a role during initial priming *in vivo* and is not thought to be important during secondary stimulation.

Surface levels of costimulatory molecules could explain why potent responder T cells from mice immunised with self protein *in vivo* and stimulated continuously *in vitro* with EL4 cells or transfected EL4 cells were difficult to generate. EL4 cells transfected with mdm2 or FRI could be used to stimulate mdm2 and FRI specific CTL, but high levels of non-specific lysis was equally observed. It has been reported previously that repeated stimulation with EL4 cells of spleen cells from mice immunised with B7 transfected EL4 cells resulted in cultures dominated by NK cells due to the lack of B7-1 expression on the EL4 cells used for restimulation despite the fact that B7-1 positive cells had been used for the *in vivo* immunisation (105). Indeed, results from our lab showed that EL4 cells do not express B7-1 (H.Stauss, personal communication). Lack of suitable costimulatory molecules could also account for the fact that Hela K^b cells infected with recombinant adenovirus were poor stimulators of CTL responses. Hela cells is an epithelial cell line capable of infection by adenovirus (300), so lack of production of the recombinant

protein is unlikely to account for the inefficiency of this cell line as stimulator of CTL specific for self proteins.

In addition to the B7-CD28 or B7-CTLA-4 interaction it cannot be excluded that additional uncharacterised costimulatory molecules might be important. In particular a novel receptor involved in T cell activation, the so-called SLAM molecule, has recently been identified (403). This molecule is expressed on memory T cells and immature thymocytes and is rapidly induced on naive T cells after activation. The engagement of SLAM has been found to stimulate human PBL and result in proliferation of activated T cells. It is of special interest with respect to CTL stimulation that the molecule also appears to induce Th0/ Th1 cytokine production profiles in antigen activated T cell clones (403). This could be important for CTL stimulation. In addition to molecules involved in positive signalling, the presence or absence of molecules involved in negative signalling, including interaction with the newly identified negative regulator of T cell activation CD43 (404), could also be important.

The production of cytokines by APCs appears to influence CTL activation and function, either directly or indirectly, via the generation of certain T helper cell subsets, for review see (113; 121). Antigen presenting cells can produce cytokines which influence the development of T helper cells into two different types, Th1 and Th2. Th1 helper cells secrete predominantly IFN-γ, IL-2 and TNF-β and support cellular immunity, whereas Th2 helper cells secrete predominantly IL-4, IL-5, IL-6 and IL-10 and provide mainly help for antibody production. IL-12 is known to influence CD4 T cells to become IFN–γ producers and consequently stimulate CTL responses (113). Macrophages, B cells and interdigitating dendritic cells from the spleen are known to produce IL-12 (113; 405). This could explain why cultures based on dendritic cells as APCs or adherent cells derived from a spleen stimulate CTL responses, although it appears that other cytokines including IL-1 and TNF–α are also needed.
The role of cytokines generated by the irradiated spleen cells used as feeder cells in the T cell cultures is difficult to assess. The spleen contains B cells, macrophages, dendritic cells, epithelial cells and T cells, which all can produce different cytokines. It is not unlikely that the combination of the cytokines produced by the feeder cells and the antigen presenting cells can produce effects which influence the magnitude and specificity of the CTL response in one direction or another.

Recently it has become clear that different $CD8^+$ CTL subsets secreting different cytokines also exist. The generation of these $CD8^+$ subsets is influenced by other cytokines. IL-4 could induce IL-4 and IL-5 production but block IL-2 and IL-6 production by CTL derived from rat splenocytes and even induce IFN-γ production if IFN-γ activity was neutralised (406). IL-4 can induce cytotoxic $CD8^+$ CTL to switch to a

$CD8^+$ cytotoxic phenotype or a $CD8^-$ non-cytotoxic phenotype producing IL-4, IL-5 and IL-10 but not IFN–γ (111). The main source of IL-4 *in vivo* is thought to be Th2 cells, mast cells and basophils. In the *in vitro* culture system described in the present work, IL-4 could perhaps be provided by the spleen cells used as APC. This may be important as CTL were occasionally found to loose lytic activity during long term culture. In these cases, FACS staining sometimes indicated that the cultures were dominated by $CD4^+$ positive cells. It is possible that $CD4^+$ cells stimulated by components in FCS or other unknown antigens would compete out $CD8^+$ cells in certain long term T cell cultures. *In vivo*, transfer experiments of equal numbers of $CD4^+$ and $CD8^+$ T cells to athymic rats have indicated that rapidly diving $CD4^+$ T cells easily outgrow $CD8^+$ cells (407).

From the preceding paragraphs, it should be clear how the *in vivo* immunisation and *in vitro* T cell culture conditions could influence the generation of self specific CTL. In most cases the use of certain conditions did indeed generate CTL responses of similar magnitude and specificity using different batches of mice. However, in some cases, the magnitude of the response varied even if exactly the same conditions were used. As is clear from Figure 4.1.1 and 4.1.2. it was possible to generate very potent cyclin D1 specific CTL responses. Cyclin D1 specificity was detected in 7 lines altogether. However, there was variability in the lysis of cells infected with control vaccinia virus. In some cases, lysis of cells infected with recombinant vaccinia virus expressing a control proteins was only 20% less than cells infected with recombinant vaccinia virus expressing cyclin D1. Variability in the response to immunisation with the same antigen has also been observed by other groups. Immunisation of DBA/2J mice with the murine mastocytoma P815 was found to induce CTL responses of variable strength and directed against different epitopes in different mice. Even within the same mouse, injection of the immunogen at two different sites resulted in recognition of different epitopes in the corresponding local lymph nodes (408). The differential responses were suggested to be due to a low precursor frequency of the CTL directed against the individual epitopes. The specificity of the CTL encountering the antigen first resulting in the earliest expansion would be random, but give this clone a numerical advantage before CTL specific for other epitopes can start proliferating

In summary, immunisation with recombinant vaccinia virus expressing the self proteins cyclin D1, mdm2, fibroblast growth factor receptor I or WT p53 showed that it was possible to stimulate several cyclin D1 and mdm2 specific CTL lines and one FRI specific CTL line. No CTL specific for WT p53 were detected. It is most likely that variability with respect to the magnitude and specificity of CTL responses was linked to the properties of the expression system used for *in vivo* and *in vitro* immunisation, as well as

the combinations of antigen presenting cells used for *in vitro* stimulation of CTL. Although it was not possible to identify the epitopes recognised by the CTL, the results support the possibilities of inducing CTL specific for peptides derived from self proteins. Further characterisation of such self specific CTL with respect to their ability to eliminate tumours *in vivo* while not causing any damage to normal tissue will be required. However, the results have clearly showed that CTL tolerance to self proteins is not absolute and that self specific CTL can be stimulated by *in vivo* priming with the self protein.

5. Peptide stimulation of autoreactive high avidity CTL which can recognise naturally processed self protein

Introduction

In vivo immunisation with whole self protein expressed in recombinant vaccinia virus had shown that it was possible to activate CTL to the self proteins cyclin D1 and mdm2, see chapter 4. The epitopes remained unidentified as it was not possible to establish long term lines. As a complement to the whole protein immunisation procedure, a peptide based approach was used to identify specific CTL epitopes in the self proteins cyclin D1, mdm2 and wild type p53.

Peptides bound to murine class I molecules *in vivo* have been shown to have particular amino acids in certain positions within the peptide. These amino acids are referred to as anchors and combination of anchor residues are referred to as motifs (31). Class I binding motifs have been used to predict CTL epitopes in foreign proteins including viral proteins, ovalbumin or murine tumour antigens (331; 409; 410). Systematic screening of protein sequences for class I binding motifs has been used to identify epitopes in several human tumour antigens including the self protein gp100 recognised by CTL from different melanoma patients (20) and the HPV encoded E7 protein recognised by CTL from cervical cancer patients (186). As class I binding motifs have been useful to identify class I restricted CTL epitopes, four selected self proteins were screened for class I binding motifs. Motif containing peptides were used for class I binding assays and CTL induction.

Class I molecules of the H-2^b haplotype were selected for the screening for class I restricted CTL epitopes. The identification of H-2^b (K^b or D^b) binding motifs was facilitated by the availability of the RMA-S cell line.
RMA-S cells are derived from the T cell lymphoma cell line RMA established from a C57BL/6N (K^bD^b) mouse. The cell line has low class I expression due to a lack of TAP2 protein in the cells (328). The level of class I molecules can be up-regulated upon incubation of the cells for 20-24 hours at 26°C, but the class I molecules on the surface are unstable at 37°C unless exogenous peptide is added (35; 36; 326; 327). The cell line therefore provides a useful tool for studying binding of peptides to the class I molecules K^b and D^b, found on RMA-S cells. In the murine system there is no similar convenient whole cell binding assay available for other class I molecules, except for L^d which can be up-regulated by incubation with L^d binding peptide (411).

5.1. Use of class I binding motifs to identify class I binding self peptides

Class I expression was checked on RMA-S cells as variations in the phenotype of the cell line might have occurred during long time culture and multiple passages. The level of class I molecules was quantified by FACS analysis using the K^b specific Y3 antibody and the D^b specific Hb27 antibody. Y3 binds to the α2 domain of K^b (83) and it only recognises K^b in association with β2 microglobulin (337; 338). The epitope recognised by Y3 is dependent on stabilisation of K^b by peptide but independent on which peptide is bound(83). The level of class I molecules measured with this antibody should therefore be a good measure of the number of peptide class I complexes on the cell surface, independent of the conformation individual peptides adopt within the class I molecule. Hb27 (28-14-8 S) binds to the α3 domain of D^b molecules as well as to L^d and D^q (336). There is no available information on whether the recognition of the epitope varies depending on the peptide bound, but it seems unlikely as the epitope recognised is outside the peptide binding groove of the class I molecule.

Upon temperature induction the number of K^b molecules reacting with antibody Y3 as measured by FACS analysis was found to reach 75-85% of the number on parental RMA cells reacting with the antibody. The number of D^b molecules reached 18-23% of the level found on RMA cells, see Figure 5.1.1 for a representative staining profile. The level of K^b on un-induced RMA-S cells was usually around 7% of the level found on RMA cells, and the level of D^b molecules was around 5% of the level found on RMA cells, see Figure 5.1.1 and (327). This means that temperature induction results in an approximately ten fold increase in the number of cell surface K^b molecules recognised by Y3 and a four fold increase in the number of cell surface D^b molecules recognised by Hb27. D^b molecules have been reported previously to be transported at lower levels to the cell surface of RMA- S cell and it has been suggested that this is due to a lower binding affinity of D^b for β_2m and/or fewer peptides available for D^b binding (337; 412).

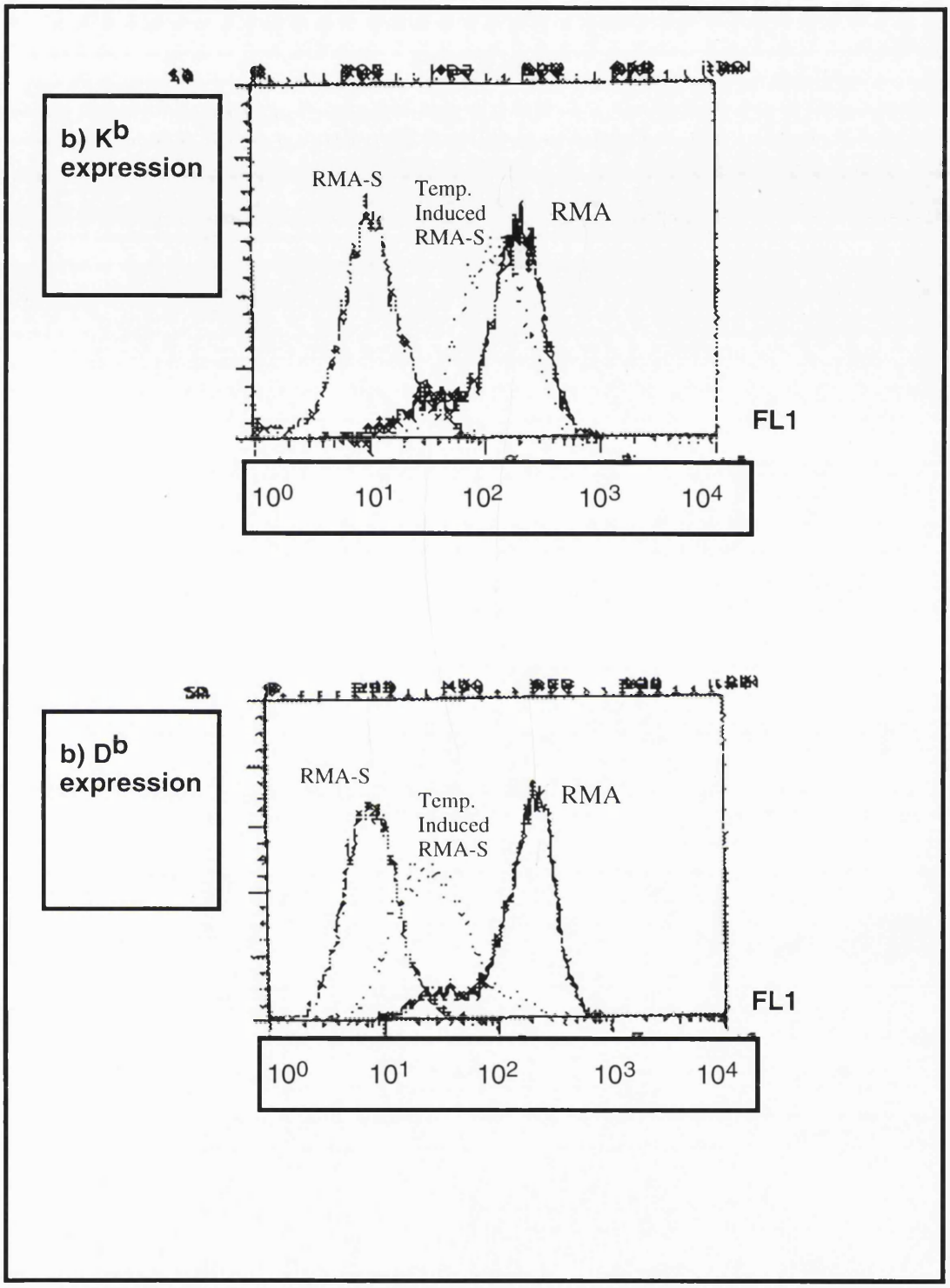


Figure 5.1.1.
MHC class I expression on RMA cells , RMA-S cells and temperature induced RMA-S cells.

RMA-S cells were temperature induced for 24 hours at 26·C and stained with K^b specific antibodies (Y3) or D^b specific antibodies (Hb27) and analysed by FACS analysis. The staining profiles of RMA cells and RMA-S which have not been temperature induced are shown for comparison.

The four self proteins cyclin D1, mdm2 , WT p53 and fibroblast growth factor receptor I (FRI) were screened for the presence of K^b and D^b binding motifs.

The motifs used for screening were based on the major anchors defined by (34) and were x x x x F/Y x x L/M/I/V for K^b binding peptides and x x x x N x x x M/I/L for D^b binding peptides.

Cyclin D1, mdm2, WT p53 and FRI were found to contain 5, 3, 8 and 27 K^b or D^b motifs. Due to the large number of potential FRI binding peptides, it was decided to focus only on the motif containing peptides from cyclin D1, mdm2 and p53 to identify peptides which could stabilise class I molecules and/or induce autoreactive CTL.

The 16 peptides derived from the cyclin D1, mdm2 and WT p53 sequences were synthesised together with two control peptides containing known class I epitopes, OVAp257 (331; 379; 413; 414) and SVp324 (SV9) (415; 416; 417). The peptide sequences are listed in Table 5.1.1. The peptides were tested for binding to K^b and D^b on temperature induced RMA-S cells using a whole cell binding assay. The mutant lymphoma cells were incubated for 24 hours at 26°C in medium containing 10% FCS. The cells were washed, distributed into 96 well plates and incubated for 2 hours at 37°C with 8 different concentrations of peptide spanning the range from 100 μM peptide to 10 pM peptide in ten fold dilution steps. FCS was present in the medium during the binding assay. Potential activity of proteases present in the FCS (350; 418; 419; 420) was minimised by heating the FCS containing medium for 10 minutes at 100°C as recommended by (350).

All the peptides which had been selected for the presence of class I binding motifs did stabilise the relevant class I molecule to some extent, Figure 5.1.2.a-d., although the concentrations required to induce half maximal stabilisation (MB_{50}) of class I molecules varied more than 1000 fold, see Table 5.1.1.

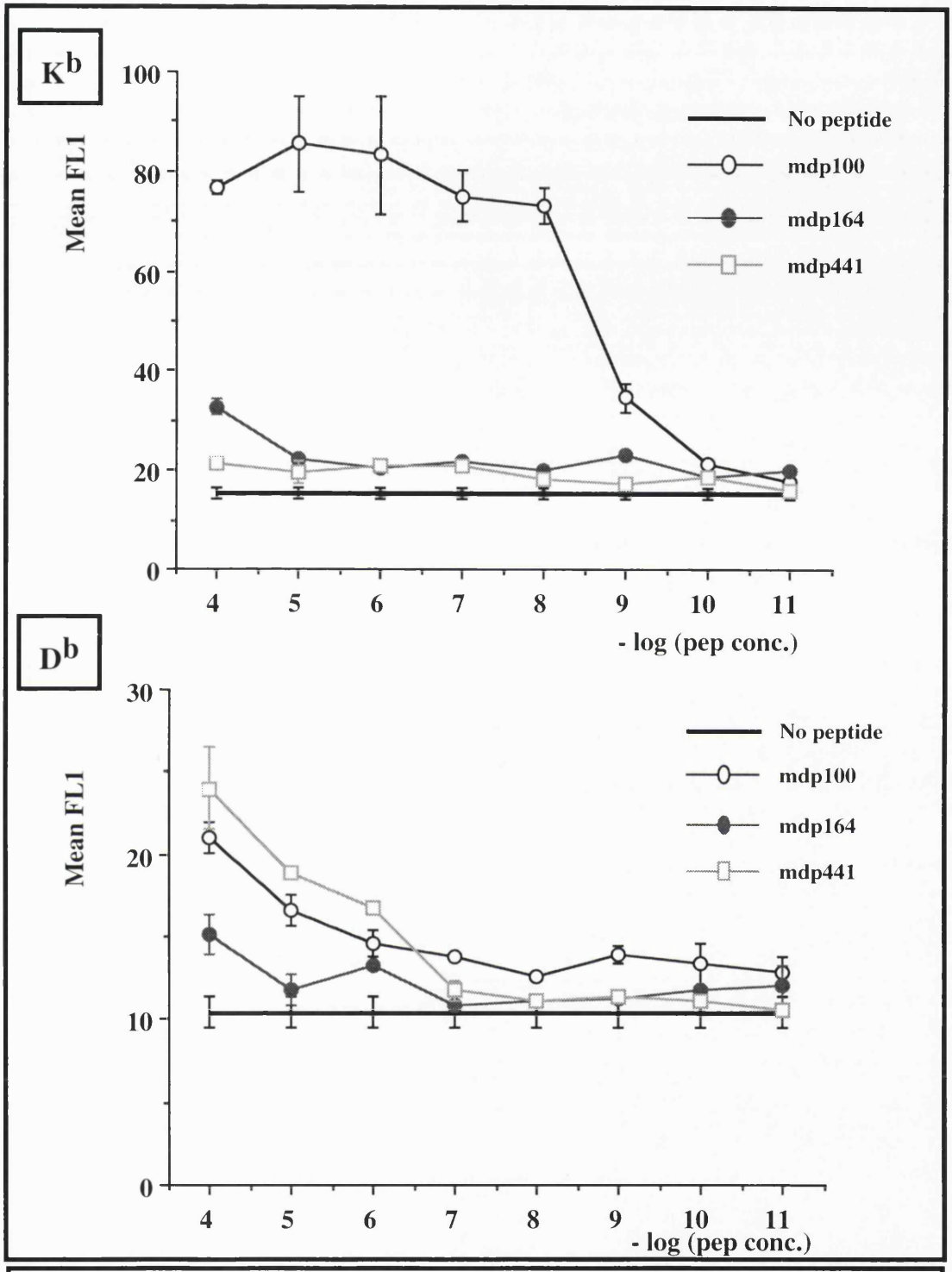


Figure 5.1.2.a.

Stabilisation of surface expression of murine class I molecules K^b or D^b by murine mdm2 peptides.

FACS analysis of class I expression on temperature induced RMA-S cells after incubation with different concentrations of mdm2 peptides containing K^b or D^b binding motifs.

The negative control (RMA-S cells with no added peptide) represents in each case the average of 4 measurements and the standard deviation is indicated.

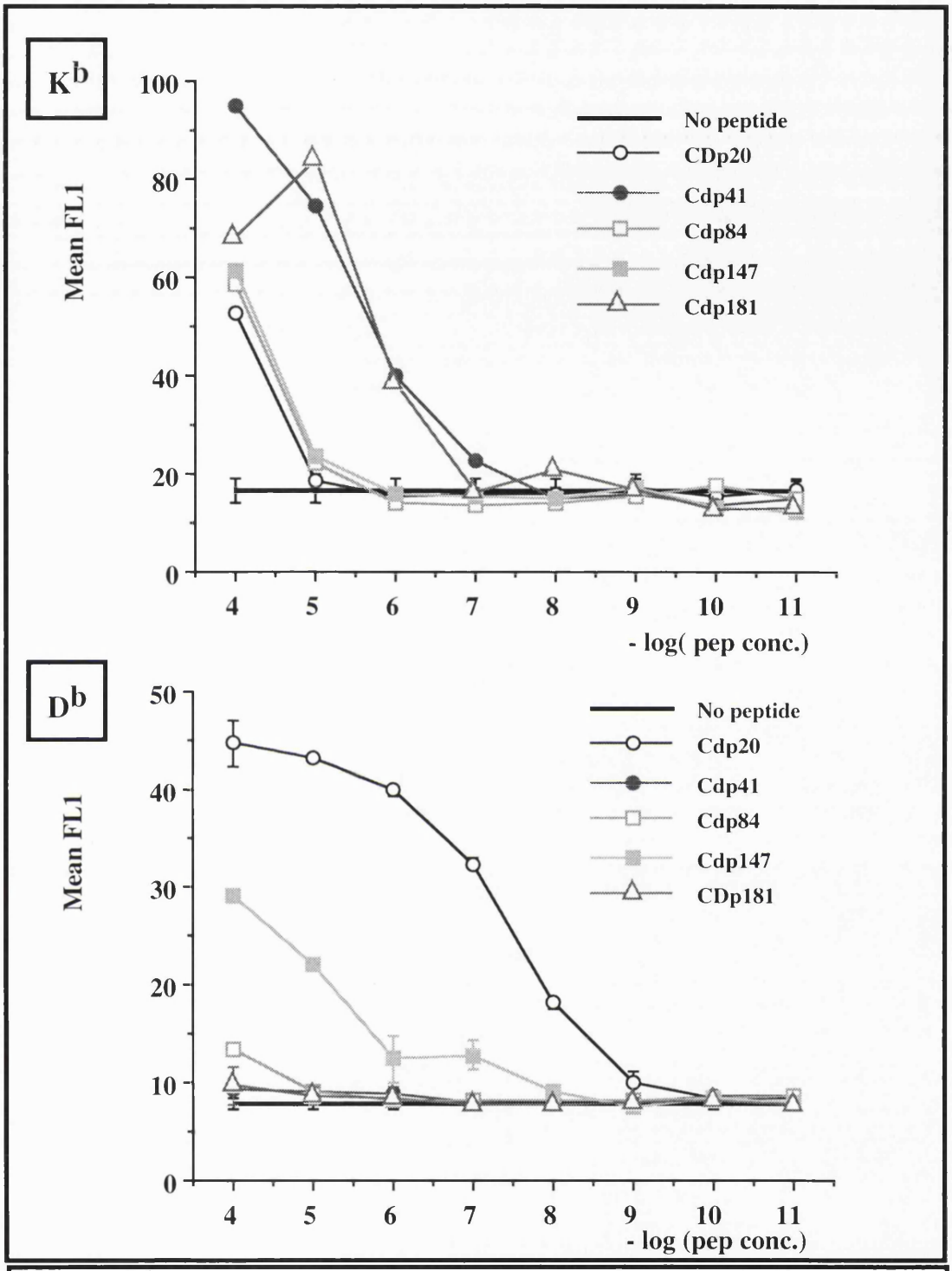


Figure 5.1.2.b.

Stabilisation of surface expression of murine class I molecules K^b or D^b by murine cyclin D1 peptides.

FACS analysis of class I expression on temperature induced RMA-S cells after incubation with different concentrations of cyclin D1 peptides containing K^b or D^b binding motifs.

The negative control (RMA-S cells with no added peptide) represents the average of 4 (K^b) or 6 (D^b) measurements and the standard deviation is shown.

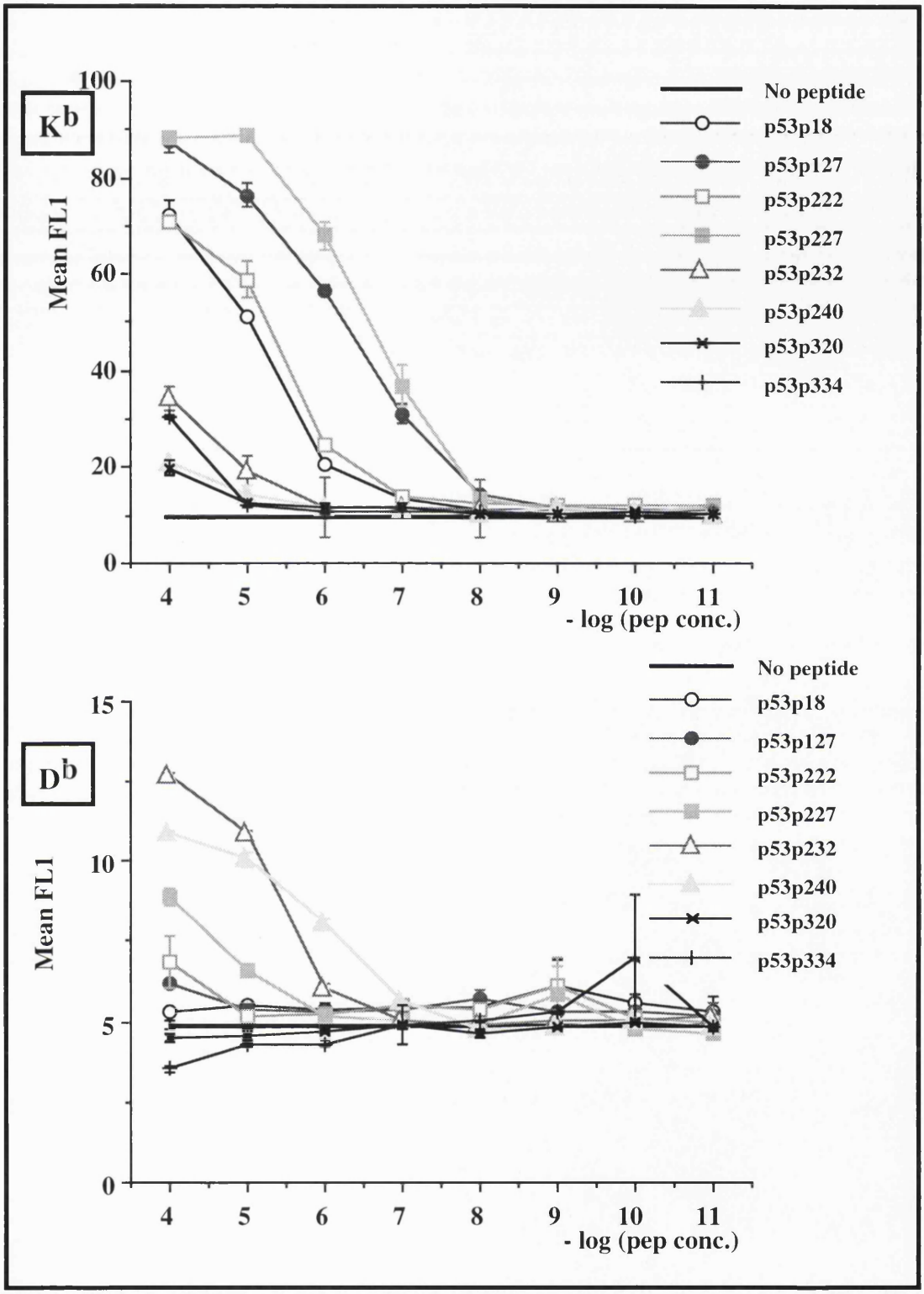


Figure 5.1.2.c.

Stabilisation of surface expression of murine class I molecules K^b or D^b by murine WT p53 peptides.

FACS analysis of class I expression on temperature induced RMA-S cells after incubation with different concentrations of WTp53 peptides containing K^b or D^b binding motifs.

The negative control (RMA-S cells with no added peptide) represents in each case the average of 4 measurements and the standard deviation is shown.

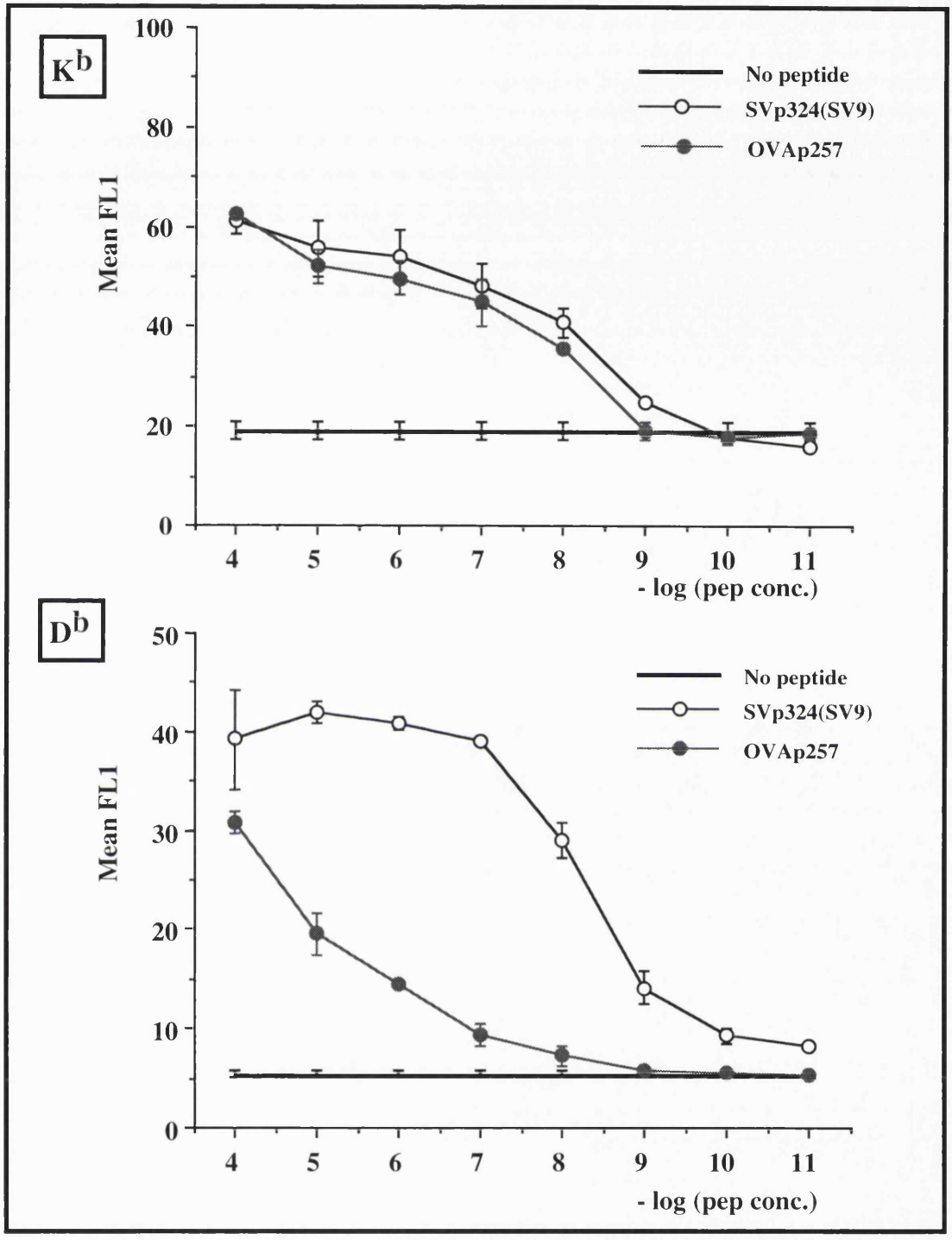


Figure 5.1.2.d.

Stabilisation of surface expression of murine class I molecules K^b or D^b by OVAp257 and SVp324 (SV9) peptides.
FACS analysis of class I expression on temperature induced RMA-S cells after incubation with different concentrations of ovalbumin peptide OVAp257 or Sendai virus peptide SVp324 (SV9).
The negative control (RMA-S with no added peptide) represents the average of 8 or 6 determinations respectively and the standard deviation is indicated.

Table 5.1.1.

Stabilisation of cell surface expression of murine K^b or D^b class I molecules by murine self peptides containing known K^b or D^b binding motifs.

The amino acids which serve as anchors are underlined and in bold.

Peptide	Derived from	Sequence	Motif	Concentration of peptide required to give half maximal stabilisation of class I molecules (MB_{50}).
mdp100	mdm2 aa 100-107	YAMI**Y**RN**L**	K^b	3 nM (K^b) 5 μM (D^b)
mdp164	mdm2 aa 164-172	ETEE**N**TDE**L**	D^b	20 μM (Kb) 50 μM (D^b)
mdp441	mdm2 aa 441-449	GRPK**N**GCI**V**	D^b	>100 μM (Kb) 5 μM (D^b)
cdp20	Cyclin D1 aa 20-28	TNLL**N**DRV**L**	D^b	50 nM (D^b) 50 μM (K^b)
cdp41	Cyclin D1 aa 41-48	SVSY**F**KC**V**	K^b	5 μM (K^b) >100 μM (D^b)
cdp84	Cyclin D1 aa 84-91	YLDR**F**LS**L**	K^b	40 μM (K^b) 50 μM (D^b)
cdp147	Cyclin D1 aa 147-155	KLKW**N**LAA**M**	D^b	5 μM (D^b) 50 μM (K^b)
cdp181	Cyclin D1 aa 181-188	HAQT**F**VA**L**	K^b	1 μM (K^b) >100 μM (D^b)
p53p18	WT p53 aa 18-25	SQET**F**SG**L**	K^b	5 μM (K^b) >100 μM (D^b)
p53p127	WT p53 aa 127-134	LNKL**F**CQ**L**	K^b	500 nM (K^b) 30 μM (D^b)
p53p222	WT p53 aa 222-229	AGSE**Y**TT**I**	K^b	3 μM (K^b) 50 μM (D^b)
p53p227	WT p53 aa 227-234	TTIH**Y**KY**M**	K^b	300 nM (K^b) 10 μM (D^b)
p53p232	WT p53 aa 232-240	KYMC**N**SSC**M**	D^b	20 μM (Kb) 4 μM (D^b)
p53p240	WT p53 aa 240-248	MGGM**N**RRP**I**	D^b	20 μM (Kb) 600 nM (D^b)
p53p320	WT p53 aa 320-327	LDGE**Y**FT**L**	K^b	50 μM (K^b) >100 μM (D^b)
p53p334	WT p53 aa 334-341	RFEM**F**RE**L**	K^b	40 μM (K^b) >100 μM (D^b)
OVAp257	Hen's egg white ovalbumin aa 257-264	SIIN**F**EK**L**	K^b	50 nM (K^b) 5 μM (D^b)
SV9 (SVp324)	Sendai Virus nucleoprotein aa 324-332	FAPG**NY**PA**L**	D^b/ K^b	8 nM (K^b) 7 nM (D^b)

The peptides were separated into four categories based on the concentration required to induce half maximal stabilisation of the class I molecule for which the peptide contained the anchor motif ("relevant class I molecule"). To estimate specificity, binding to class I molecules for which the peptide did not contain the anchor motif was also measured ("irrelevant class I molecule"). Stabilisation of irrelevant class I molecules was either weak (8 out of 16 peptides) or not measurable (6 out of 16 peptides). Only two out of 16 peptides bound to the irrelevant class I molecule with intermediate affinity (mdp100 and p53p227). For these peptides, the concentrations of peptide required to produce half maximal stabilisation of the relevant class I molecules was respectively 1700 times and 100 times lower than the concentrations of peptide required to produce half maximal stabilisation of the irrelevant class I molecules. The results are summarised in Table 5.1.2.

Table 5.1.2.

Summary of class I binding properties of motif containing peptides.		
Category	**Relevant class I molecule**	**Irrelevant class I molecule**
No stabilisation (100 μM peptide does not stabilise class I)	**0**	**6** mdp441, cdp41, cdp181, p53p18, p53p320, p53p334
Weak stabilisation (MB_{50} between 10 μM and 100 μM)	**4** mdp164, cdp84, p53p320, p53p334	**8** mdp164, cdp20, cdp84, cdp147, p53p127, p53p222, , p53p232, p53p240
Intermediate stabilisation (MB_{50} between 1 μM and 10 μM)	**6** mdp441, cdp41, cdp147, p53p18, p53p222, p53p232.	**2** mdp100,p53p227
Strong stabilisation (MB_{50} 1 μM or less)	**6** mdp100, cdp20, cdp181, p53p127, p53p227, p53p240	**0**

The results summarised in Table 5.1.1. and 5.1.2. show that class I binding motifs were valuable for predicting stabilisation of class I molecules by individual peptides. There was variability in the concentrations of peptide required to induce half maximal stabilisation of class I molecules, despite the presence of identical dominant anchor motifs within the peptides. Crystallographic studies and mutational analysis have indicated that amino acids at other positions than the anchor positions can contribute positively or negatively to the

stability of the binding of peptide to class I molecules. This includes variation at position 2 or 3 for K^b (421; 422; 423; 424) or position 3 or at the N- or C termini for D^b (32; 425). The best K^b binding peptide mdp100 did indeed contain a so-called submotif defined for K^b binding peptides, involving a small residue at P2 (A or G) and Y at position 5 (423), but this submotif was also present in the intermediate binding peptide p53p222.

So called non-dominant anchor residues identified by elution of naturally processed peptides were not included in the present screen for potential class I binding peptides. Such anchors are R, I, L, S and A at position 1, N at position 2, Y (strong) or P at position 3, R, D, E, K, T at position 4, T, I, E, S at position 6, N, Q, K at position 7 and I and V at position 8 for K^b. For D^b, A, N, I, F, P, S, T, V at position 1, M (strong), A, Q, D at position 2, I, L, P, V (all strong) and G at position 3, K, E, Q, V (all strong) or D or T at position 4, L or F (both strong) or A, Y, T, V, M, E, Q, H, I, K, P, S at position 6 , D, E, Q, V, T, Y at position 7 and F, H, K, S, Y at position 8 influence binding (31). The 16 self peptides under investigation were checked for the presence of any of the above mentioned subdominant anchors. It was found that the strongest binding peptides, mdp100, cdp20, cdp181, p53p127, p53p227 and p53p240 did not contain more subdominant anchor residues than the weaker binding peptides. On the basis of these findings and the results of others (425), it can be suggested that weak anchors are important but still too ill defined to be useful for prediction of the class I binding capacity of a particular peptide. As clearly demonstrated with substitutions of K^b binding ovalbumin peptides, the contribution of each residue to class I binding is not simply additive and instead is dependent on the sequence context (426). However, the dominant anchors were found to be important for predicting class I binding.

The fact that peptides which do not contain the correct anchor motif in some cases bind a class I molecule, although weakly, is not surprising. The presence of an anchor motif is not an absolute requirement for binding, as peptides lacking the correct consensus motif can bind to MHC molecules if added exogenously in large concentrations (335; 427).

In summary, anchor motifs identified by natural elution of peptides were found to be useful for identifying class I binding self peptides. The influence of amino acids other than the dominant anchor residues was evident, but no known submotifs were found to make a consistent positive or negative contribution to the class I binding of the limited number of self peptides investigated in this study.

5.2. Induction of CTL to self peptides

To identify CTL epitopes in cyclin D1, mdm2 and WT p53, peptides containing a class I binding motif were to be tested for their ability to induce CTL *in vitro* irrespective of their ability to stabilise class I molecules *in vitro*.
The strategy consisted of the following steps:
1) Definition of conditions for induction and analysis of CTL *in vitro* based on the use of a cell population enriched for dendritic cells as APCs.
2) Investigation of the ability of the 16 selected class I binding motif containing peptides to induce CTL *in vitro* using the conditions defined in 1).
3) Characterisation of CTL with respect to CD8 dependency and class I restriction.
4) Characterisation of CTL with respect to the recognition of endogenously processed self protein
5) Characterisation of CTL with respect to avidity for the target antigen.

5.2.1. A cell population enriched for dendritic cells and purified by a simple two step procedure efficiently primes CTL to self peptides *in vitro*

Cell populations enriched for dendritic cells have been used successfully with human and murine responder T cells for *in vitro* induction of peptide specific CTL able to recognise endogenously processed protein. Examples include the stimulation of murine CTL specific for adenovirus or Sendai virus proteins (428) or influenza virus nucleoprotein (429) and human CTL specific for human papilloma virus type 11 E7 protein (383) or HIV proteins (430; 431).

As the efficiency of dendritic cells for *in vitro* induction of CTL specific for foreign proteins is so well documented, it was decided to investigate the use of this antigen presenting cell type for CTL induction to self peptides. DCs can be purified from mouse spleens by a variety of methods giving populations of antigen presenting cells varying widely in DC content. Here, a simple two step purification method was used to enrich for dendritic cells as DCs are efficient in inducing CTL even when present at a low frequency in the total population of antigen presenting cells (432). The method chosen involved an overnight adherence step to remove macrophages followed by separation of low density dendritic cells from high density lymphocytes and red blood cells by centrifugation of the cells on 14.5% (w/v) metrizamide. The purified cells were analysed by flow cytometry using monoclonal antibodies specific for the α-chain of the dendritic cell surface integrin CD11c (antibody N418), the B cell surface glycoprotein B220 (antibody RA3-6B2), the macrophage surface protein Mac-1 (antibody M1/70.15) as well as antibodies specific for murine class II molecules (antibody Tib120) or B7.1 molecules (antibody 16-10A1), see Figure 5.2.1. The main contaminant was found to be B cells as judged from the presence of the B220 marker, whereas most macrophages were removed.

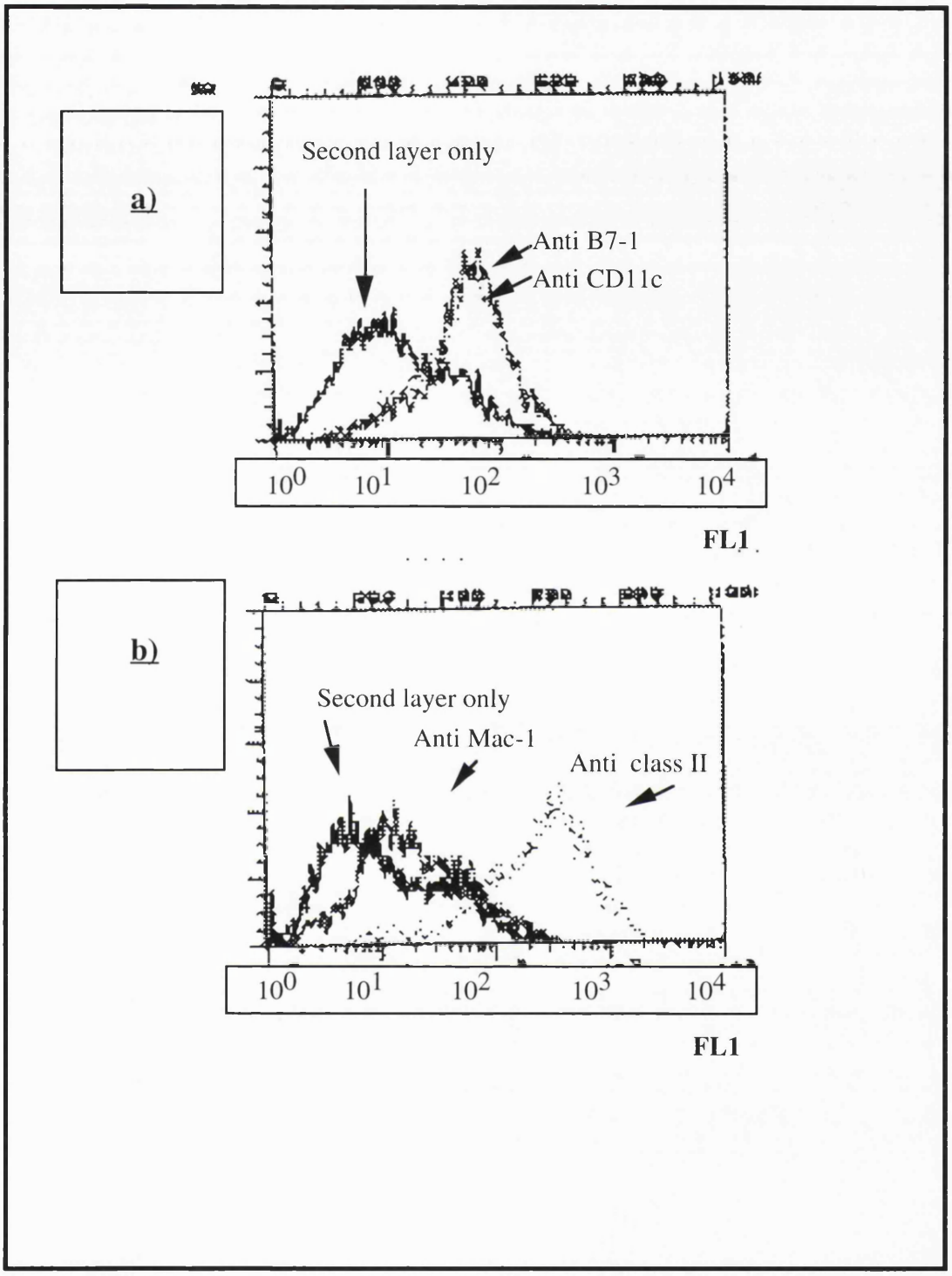


Figure 5.2.1., a and b.
FACS analysis of a mouse splenic non-adherent cell population purified by centrifugation on a metrizamide gradient.
Cells were stained for B7-1 or the DC integrin CD11c (a), or class II molecules or the macrophage surface molecule Mac-I (b). The second layer was goat anti hamster polyvalent immunoglobulins in (a) and goat anti rat polyvalent immunoglobulins in (b).

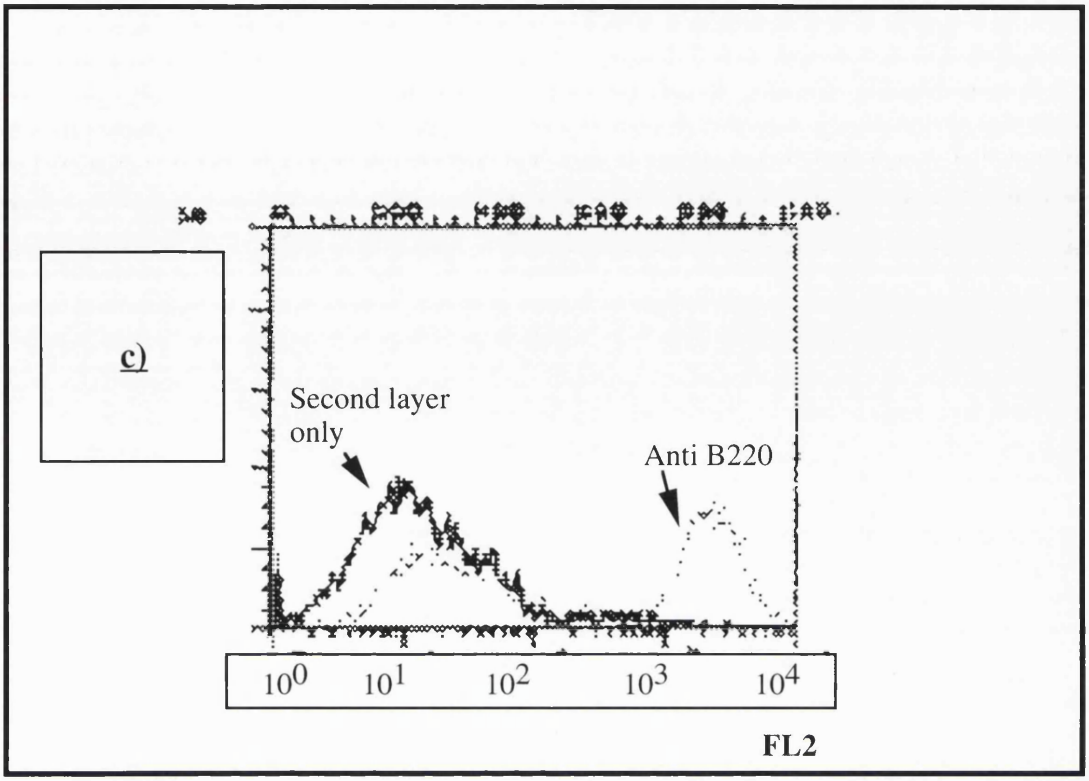


Figure 5.2.1, c.
FACS analysis of a mouse splenic non-adherent cell population purified by centrifugation on a metrizamide gradient, continued.
Cells were stained with biotinylated antibodies specific for the B cell surface molecule B220. The second layer was cytochrome conjugated streptavidin

In conclusion, the purified cell population was enriched for cells expressing high levels of class II, of a DC marker CD11c and B7-1. Few cells stained for the macrophage specific marker Mac-1. Contaminating B cells still remained. B cells do not express CD11c and high levels of B7-1 is only found on activated B cells. For simplicity, the cell population enriched for dendritic cells will be referred to as dendritic cells, DCs.

The ability of peptide pulsed DCs to induce CTL *in vitro* was tested. The strong class I binding mdm2 peptide, mdp100, was used as a test peptide. Purified DCs were pulsed with 100 μM mdp100 peptide for 4 hours as recommended by (428) and used to stimulate responder cells derived from a naive murine spleen. 100 nM peptide was additionally added to the cultures. Some cultures were set up with peptide alone and no DCs. After six days, four independent CTL lines were tested for lytic activity against target RMA-S cells coated with mdp100 peptide, Figure 5.2.2. The concentration of peptide required to obtain half maximal lysis of the target cells (ML_{50}) by day 6 in culture was approximately 1000 fold lower if CTL had been stimulated with DCs and peptide than if CTL had been stimulated with 100 nM peptide only. It was consequently clear that the DCs were extremely potent for CTL induction *in vitro*. It was decided to use DCs for all initial *in vitro* stimulation of CTL.
The cell lines labelled "a " and "b" in Figure 5.2.2. were stimulated in medium using two different batches of FCS. There was a ten fold difference in the peptide concentrations required to produce half maximal lysis of target cells by the T cell lines stimulated with batches a and b, illustrating the importance of the particular batch of FCS used for T cell work. Batch b was used for all the T cell work described in this thesis.

It is common to stimulate CTL on a weekly basis, but it was decided to stimulate CTL every 12-14 days to try to promote other characteristics than rapid proliferation and to prevent exhaustion of the T cells. Restimulation at day 12-14 will result in restimulation of resting T cells. Initial experiments showed that leaving the CTL for 14 days with no feeding had no deleterious impact on the lytic activity, Figure 5.2.3. CTL lines which had been fed once at day 6 with peptide pulsed dendritic cells and at day 20 with 100 nM peptide only, were as viable and lytic as cells which had been fed on a weekly basis, i.e. at days 6, 13 and 20. For this reason, peptide induced CTL lines were generated by initial restimulation of the CTL after 6-7 days in culture, followed by two weekly passages. It should be mentioned that the cell number retrieved at day 20 from T cell cultures which had been stimulated only at day 6, was usually equal to or less than the input cell number. At subsequent passages at day 34, 50 etc., cell numbers had increased since the previous passage of the cells. Low increase in cell numbers during early stages of T cell cultures have equally been observed with T cells on 10-12 day restimulation cycles (433).

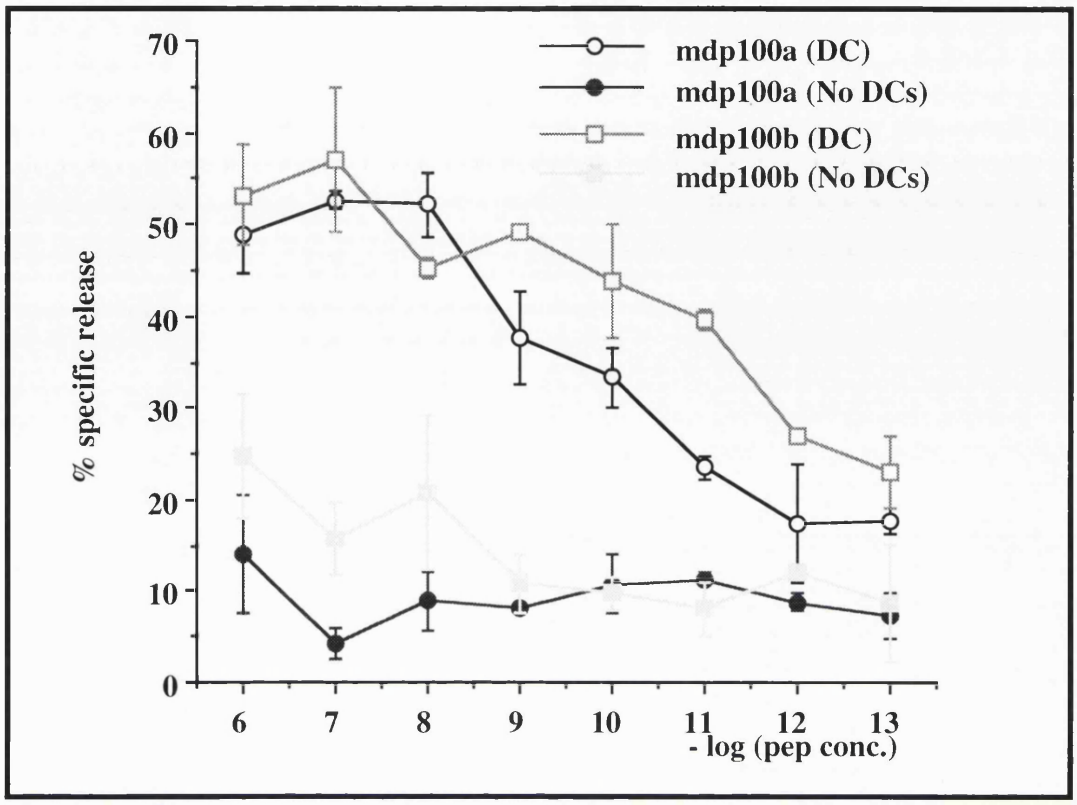


Figure 5.2.2.
Splenic dendritic cells are potent inducers of peptide specific CTL in vitro.
Responder T cell lines were derived from naive spleens and pulsed for 6 days in culture with
a) mdp100 pulsed DCs and 100 nM mdp100 peptide
(Line mdp100a (DC) and mdp100b (DC)) or
b)100 nM mdp100 peptide only
(Line mdp100a (No DCs) and mdp100b (No DCs))
The T cell lines were tested by ^{51}Cr release assay for recognition of temperature induced RMA-S cells coated with different concentrations of mdp100 peptide. Each point on the graphs represents the average of two values.

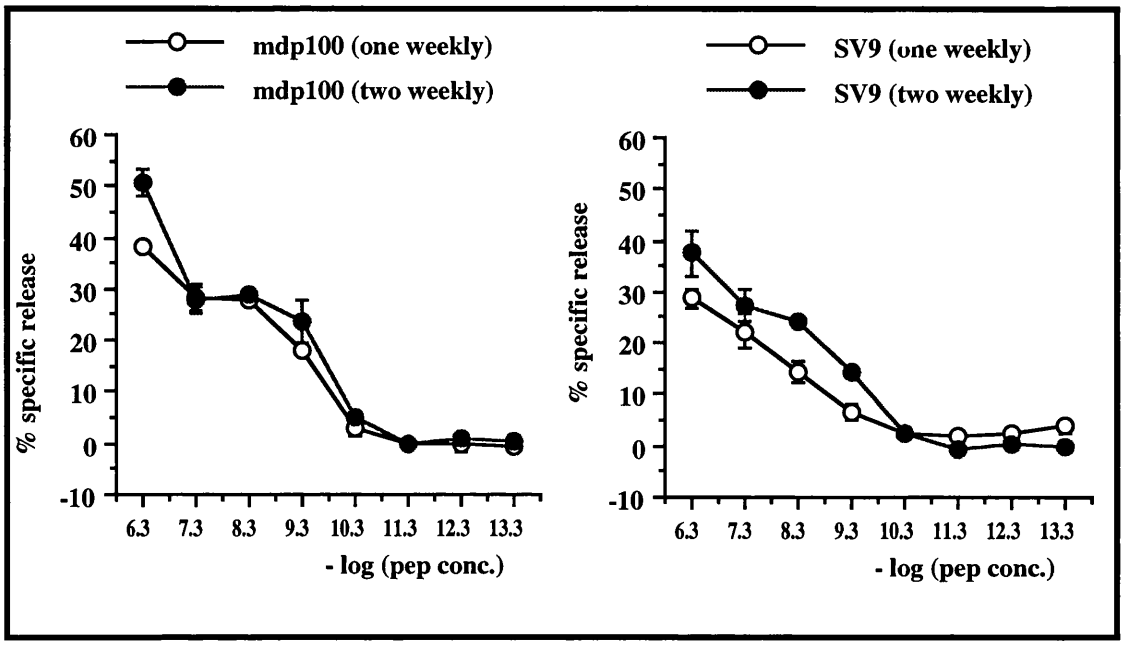


Figure 5.2.3
Peptide specific CTL are efficiently stimulated using a two weekly restimulation cycle.
T cell lines were set up with naive spleen cells as responders and purified DCs pulsed with mdp100 peptide or SV9 peptide as stimulators. The T cells were passed on day 6, day 13 and day 20 (one weekly) or on day 6 and day 20 (two weekly). The T cells were tested by ^{51} Cr release assay on day 26 for recognition of temperature induced RMA-S coated with different concentrations of peptide. Each point on the graph represents the average of two values.

It was desirable to use RMA-S cells as CTL targets in assays involving synthetic self peptide as it does not present most endogenous self peptides due to the lack of functional TAP2 protein. The use of the same cell line for peptide binding in CTL assays as well as class I binding assays would allow a comparison of the relative peptide concentrations required for class I stabilisation and CTL lysis thus providing information about the relative avidity of the peptide induced CTL (see later). However, there are reports that RMA-S cells have 3 to 5 fold less peptide receptive class I molecules on the cell surface than RMA cells supposedly due to a role of functional TAP molecules in the generation of peptide receptive class I molecules on the cell surface (434).

To test the relative ability of peptide coated RMA cells and RMA-S cells to serve as targets for peptide specific CTL, the known K^b epitope OVAp257 (used in the peptide binding experiment described by (434)) was used to prime peptide specific CTL from naive spleens. These CTL were tested for recognition of OVAp257 coated RMA or RMA-S cells, Figure 5.2.4. The concentration of peptide required to give half maximal lysis of peptide coated target cells by OVAp257 specific CTL was 80 pM when the antigen presenting cells were RMA-S cells and 500 pM when RMA cells were used as antigen presenting cells. A similar difference was also observed when mdp100 peptide specific CTL were tested for lysis of mdp100 coated target cells (not shown). Approximately 6 fold lower peptide concentration was thus required to produce half maximal lysis of peptide pulsed RMA-S than half maximal lysis of peptide pulsed RMA cells. This indicated that either a larger fraction of the input peptide was bound to RMA-S cells than to RMA cells despite RMA-S cells having less class I molecules on the cell surface than RMA cells (see section 5.1.) or RMA-S cells were overall better CTL targets. The latter seems less likely because RMA-S cells are RMA cells selected exclusively on the basis of low class I expression, although differences other than class I expression arising during cell culture cannot be excluded.

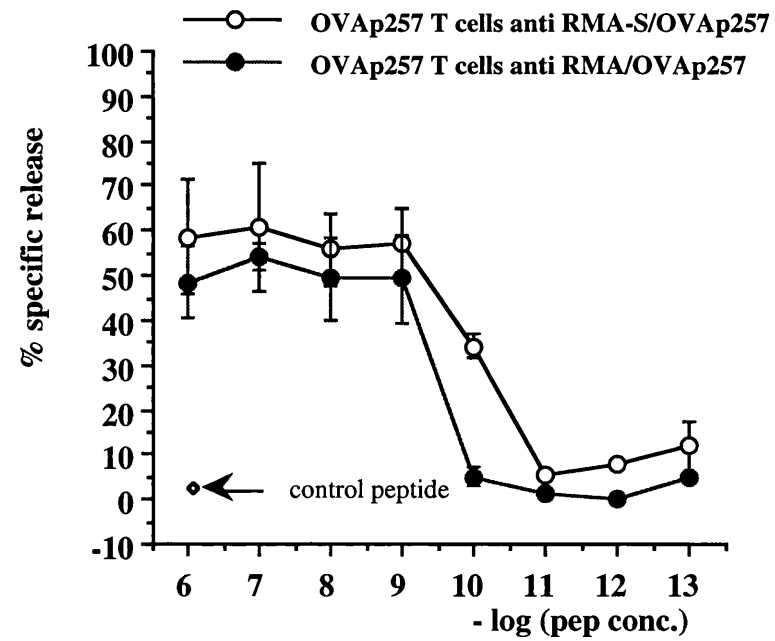


Figure 5.2.4.
Comparison of CTL lysis of peptide pulsed RMA cells or RMA-S cells. Responder T cells were derived from naive spleens stimulated with DCs pulsed with OVAp257 peptide at the first two restimulations and subsequently with100 nM OVAp257 peptide alone. The T cells were tested by ^{51}Cr release assay on day 52 in culture against RMA cells or temperature induced RMA-S cells coated with different concentrations of OVAp257 peptide.

5.2.2. Several class I binding self peptides stimulate self peptide specific CTL from naive spleens *in vitro*

The 16 selected self peptides (see section 5.1.) were tested for their ability to stimulate CTL from naive spleens *in vitro* using DCs as APCs. Naive spleens were stimulated for 6 days in culture with peptide pulsed dendritic cells and with 100 nM peptide added to the cultures. The CTL lines were tested for recognition of RMA-S cells pulsed with the peptide used to stimulate the CTL on day 6 in culture. It was found that by day 6, the peptides, mdp100, mdp441, cdp20, cdp41 and p53p232 did induce CTL. The T cell lines were restimulated with dendritic cells pulsed with peptide on day 6 or day 7 and tested again for lytic activity 6 days later. At this stage, peptides p53p227 and p53p240 had also stimulated peptide specific CTL, see Figure 5.2.5. The T cells were restimulated on day 20 by adding 100 nM peptide, but no peptide pulsed dendritic cells, to the cultures. No peptides other than the seven peptides already mentioned (mdp100, mdp441, cdp20, cdp41, p53p227, p53p232 or p53p240) gave rise consistently to peptide specific CTL even upon prolonged culture. Peptide p53p18 and p53p320 were found to give rise to peptide specific CTL when the T cell cultures were repeatedly restimulated with peptide pulsed dendritic cells, but these CTL were of low avidity and did not maintain their specificity. On one occasion (out of 6 attempts) CTL specific for peptide p53p127 were observed. These CTL could not be maintained as a stable line and were of low avidity. In contrast, numerous independent CTL lines were established specific for mdp100, mdp441, cdp20, cdp41, p53p227, p53p232 or p53p240. For some of the peptides more than 10 independent peptide specific CTL lines were established. Some of these lines were kept in culture up to 80 days.

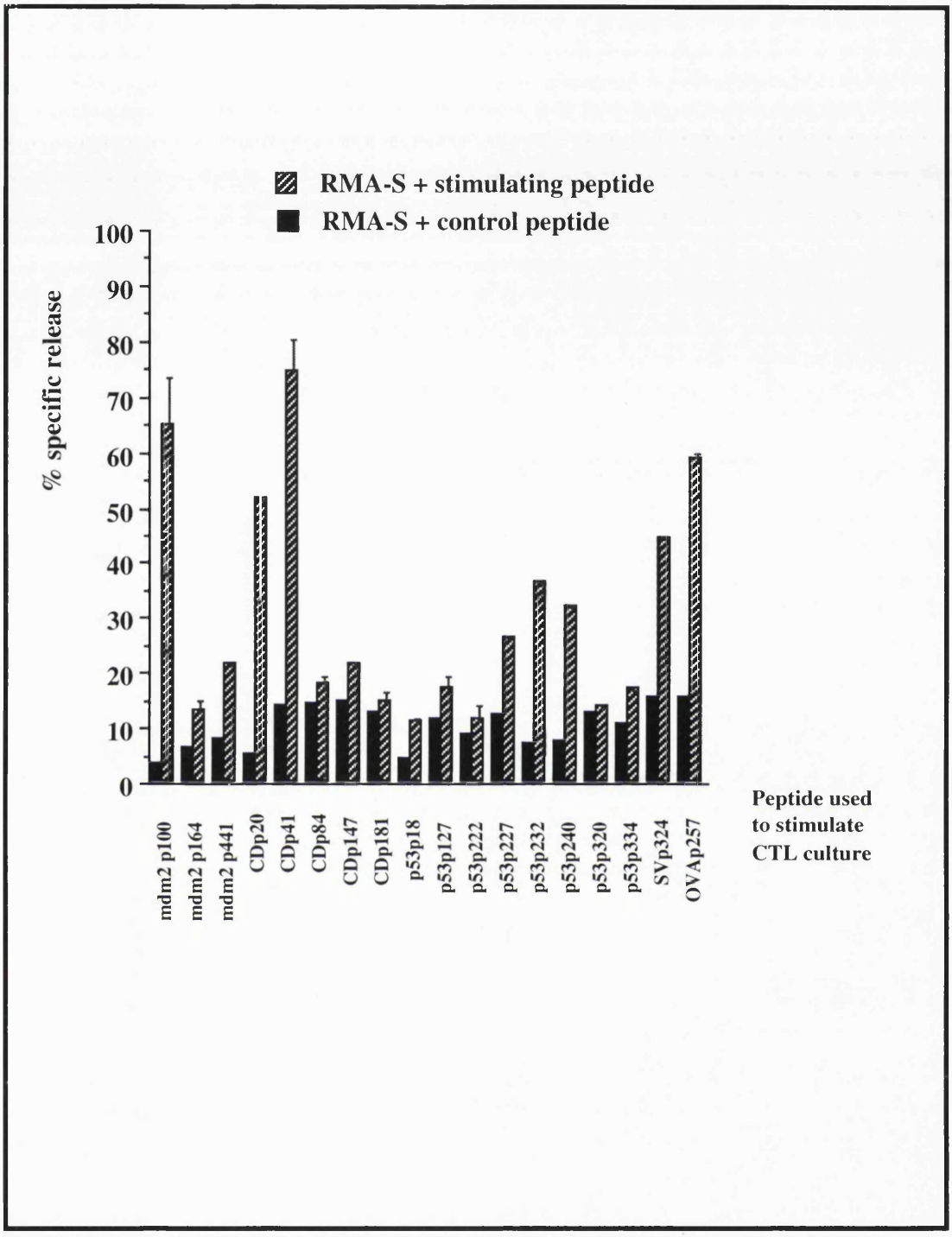


Figure 5.2.5.
CTL induction in vitro by self peptides selected on the basis of class I binding motifs.
CTL lines were generated from spleens of naive mice and stimulated in vitro with DCs pulsed with a self peptide or one of two control peptides, SVp324 or OVAp257. The CTL were restimulated on day 6 with peptide pulsed DCs. The CTL were tested on day 12 in a ^{51}Cr release assay against target RMA-S cells coated with the peptide used to stimulate the culture or with a control peptide.

Altogether it was found that only peptides which were classified as strong or intermediate class I binders (see Table 5.1.1) induced CTL. However, several strong or intermediate binders did not induce CTL. None of the weak or non-binding peptides induced CTL. The results are summarised in Table 5.2.1.

Table 5.2.1.

CTL induction by self peptides containing class I binding motifs		
Class I binding (see section 5.1.)	**Number of peptides which consistently induce CTL**	**Number of peptides which do not induce CTL**
Weak stabilisation of class I molecules (MB_{50}: between 10 μM and 100 μM)	**0**	**4** (mdp164, cdp84, p32p320, p53p334)
Intermediate stabilisation of class I molecules (MB_{50}: between 1 μM and 10 μM)	**3** (mdp441, cdp41, p53p232)	**3** (cdp147, p53p18, p53p222)
Strong stabilisation of class I molecules (MB_{50}: 1 μM or less)	**4** (mdp100, cdp20, p53p227, p53p240)	**2** (cdp181, p53p127)

5.3. Characterisation of self peptide specific CTL

5.3.1. CD8 dependency and class I restriction of self peptide specific CTL lines

It was investigated whether the specific lytic activity observed was dependent on CD8. The peptide specific CTL lines were tested for their CD8 dependency in ^{51}Cr release assays against peptide coated RMA-S cells on day 12/13 or day 26 in culture. The CTL were incubated with target cells at a high E/T ratio of 25:1 in the absence of blocking antibody or in the presence of different concentrations of CD4 or CD8 antibodies. All peptide specific CTL lines tested were found to be dependent on CD8 for their lytic function, as increasing concentrations of CD8 antibody blocked the effector function of the CTL. The results for two representative peptides as well as the known CTL epitope OVAp257 are shown in Figure 5.3.1. The data for the rest of the peptides are summarised in Table 5.3.1. Overall, the concentrations of CD8 specific antibodies required to block CTL lysis was within a similar range for all the self peptide specific CTL lines when these were tested at early stages in culture, day 13 or day 21. The concentrations required to block the CTL specific for the two exogenously derived CTL epitopes, OVAp257 and SVp324/SV9 were slightly higher than for the other self peptide specific CTL lines at early stages of culture. CD8 dependency has in many cases been found to correlate with the affinity of the CTL, with high affinity CTL being less dependent on CD8 than low affinity CTL (100; 161; 435). On this basis, the present results indicate that in early cultures the self specific CTL lines possess T cell receptors with low to intermediate affinities for their target MHC/peptide complexes. To address the issue in more details, titrations of CTL lines against the target antigens were performed with early culture effectors, supporting the hypothesis (see later).

Class I restriction of the peptide specific CTL was examined using P1.HTR cells transfected with murine K^b or murine D^b class I molecules and coated with peptide as targets in CTL assays. P1.HTR cells express endogenous L^d, D^d and K^d. The results for two CTL lines induced to an intermediate class I binder (mdp441) or to a strong class I binder (mdp100) as well as for CTL lines specific for control OVAp257 peptide upon 51 days in culture are shown, Figure 5.3.2. CTL lines specific for mdp100, cdp41, p53p232 or p53p240 were all restricted by the class I molecule binding with highest affinity to the peptide used for CTL induction irrespective of the length of time the peptide specific CTL lines had been in culture. Peptide specific CTL lines specific for mdp441, cdp20 and p53p227 did exhibit some lysis of P1.HTR transfected with the class I molecule they bound with lower affinity, but only when the CTL lines had been a long time in culture as shown in Figure 5.3.2. for peptide mdp441. OVAp257 CTL as well as SV9 specific CTL mostly recognised only P1.HTR K^b cells presenting the respective peptides although there were some exceptions which could either be due to non-specific lysis of P1.HTR cells or due to the fact that both OVAp257 and SV9 do indeed bind D^b.

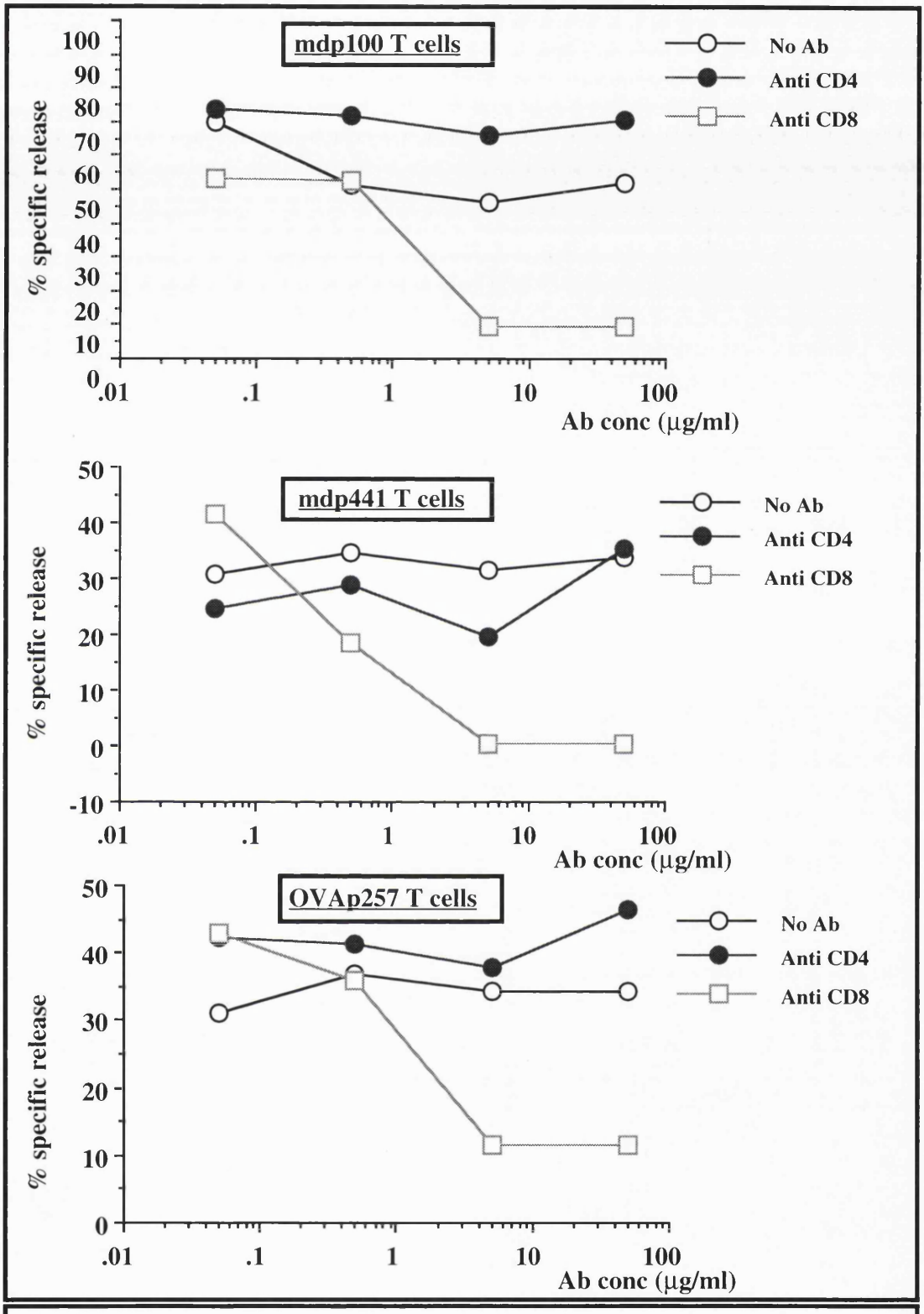


Figure 5.3.1.
The effector function of self peptide specific CTL is dependent on the CD8 molecule.

Peptide specific CTL lines were tested for lytic activity by ^{51}Cr release assay against RMA-S cells pulsed with the peptide used for stimulation of the CTL and in the presence of anti CD8 or anti CD4 antibodies.

Table 5.3.1.

Blocking of lytic activity of self peptide specific CTL with anti CD8 antibodies	
Peptide used to stimulate T cell line	**Concentration of anti CD8 Ab needed to reduce lysis to baseline levels**
mdp100 21 days in culture	1.5 μg /ml
mdp441 21 days in culture	0.75 μg/ml
cdp20 13 days in culture	1 μg/ml or less
cdp41 13 days in culture	3 μg/ml
p53p227 13 days in culture	3 μg/ml
p53p232 13 days in culture	3 μg/ml
p53p240 13 days in culture	1 μg/ml or less
SV9 13 days in culture	10 μg/ml
OVAp257 13 days in culture	5 μg/ml

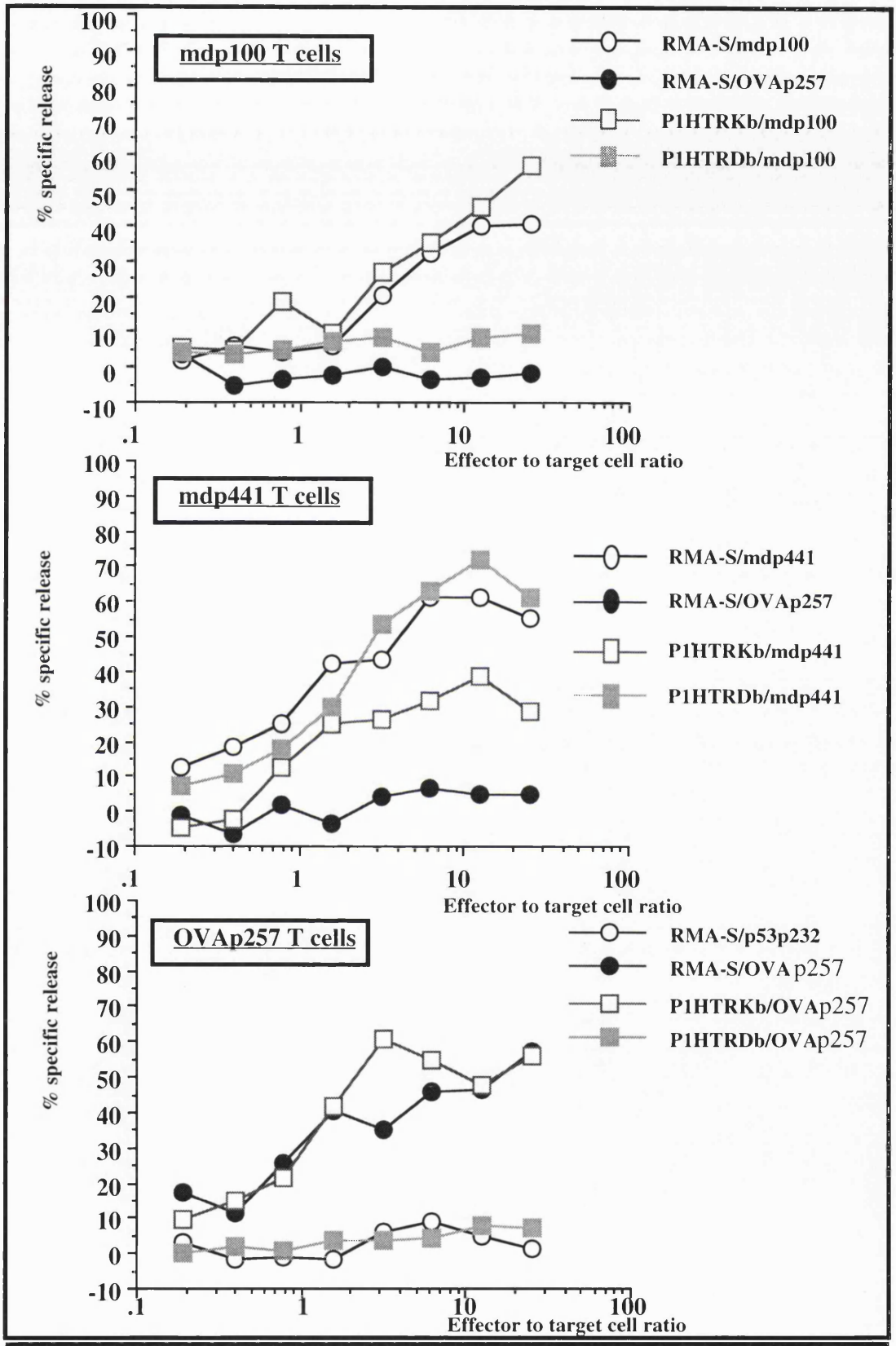


Figure 5.3.2.
Class I restriction of self peptide specific CTL.
CTL lines were tested for lytic activity by ^{51}Cr release assay against target cells coated with the peptide used for CTL stimulation in vitro or with a control peptide.

5.3.2. Recognition of endogenously processed self proteins by self peptide specific CTL

The main purpose of generating CTL specific for self peptides was to obtain T cells which could recognise endogenously processed self protein. The peptide specific CTL were examined at various time points for the recognition of endogenously processed protein in the form of target cells infected with a recombinant vaccinia virus expressing the protein, see Figure 5.3.3.a. and 5.3.3.b for two representative examples. It was found that only CTL specific for mdm2 peptide mdp441 peptide recognised endogenously processed mdm2. The mdp441 specific CTL did not recognise endogenously processed mdm2 at early stages in culture, but began to do so around day 40 to 56 in culture, depending on the line. None of the other peptides stimulated CTL which recognised endogenously processed protein upon long term culture, see Figure 5.3.3.c. and Figure 5.3.3.d.

The mdm2 p441 specific CTL line at day 40 in culture shown in Figure 5.3.3.b. lysed RMA-S cells pulsed with mdp441 and RMA cells infected with vaccinia virus mdm2. The T cells did not lyse target cells pulsed with control mdp100 peptide or RMA cells infected with control vaccinia virus p53 to a similar extent. RMA cells or EL4 cells were not lysed (not shown). 3 other mdp441 specific CTL lines developed into CTL which recognised uninfected EL4 cells and RMA cells in addition to RMA-S pulsed with mdp441 peptide but not with control peptide. One example is shown in Figure 5.3.4.a. EL4 cells and RMA cells are T cell lines and express endogenous mdm2, see Figure 5.3.4.b. RMA-S cells also express endogenous mdm2, Figure 5.3.4.b. However, due to the defect in TAP2 production in this cell line, RMA-S cells do not present endogenous self peptide on the cell surface and would consequently not be expected to present any mdm2 derived peptides. In conclusion, altogether four independent mdm2 p441 specific CTL lines were obtained which appeared to recognise endogenously processed protein. For one of the lines, the evidence for mdm2 recognition was direct in as much as vaccinia virus mdm2 infected target cells were recognised but not control vaccinia virus infected target cells. For the three other lines, the evidence remained indirect in as much as target cells expressing endogenous mdm2 were recognised whereas target cell expressing endogenous mdm2 but unable to present endogenous peptides were not recognised by these CTL unless the mdp441 peptides was added exogenously. It would have been desirable to examine whether these CTL recognised naturally class I presented peptides eluted from EL4 or RMA cells. However, these kind of experiments require many T cells. A feature of the mdp441 specific CTL was that they did not grow very rapidly. When the CTL had been in long term culture and tested at various time points there were never enough cells left for this kind of experiments.

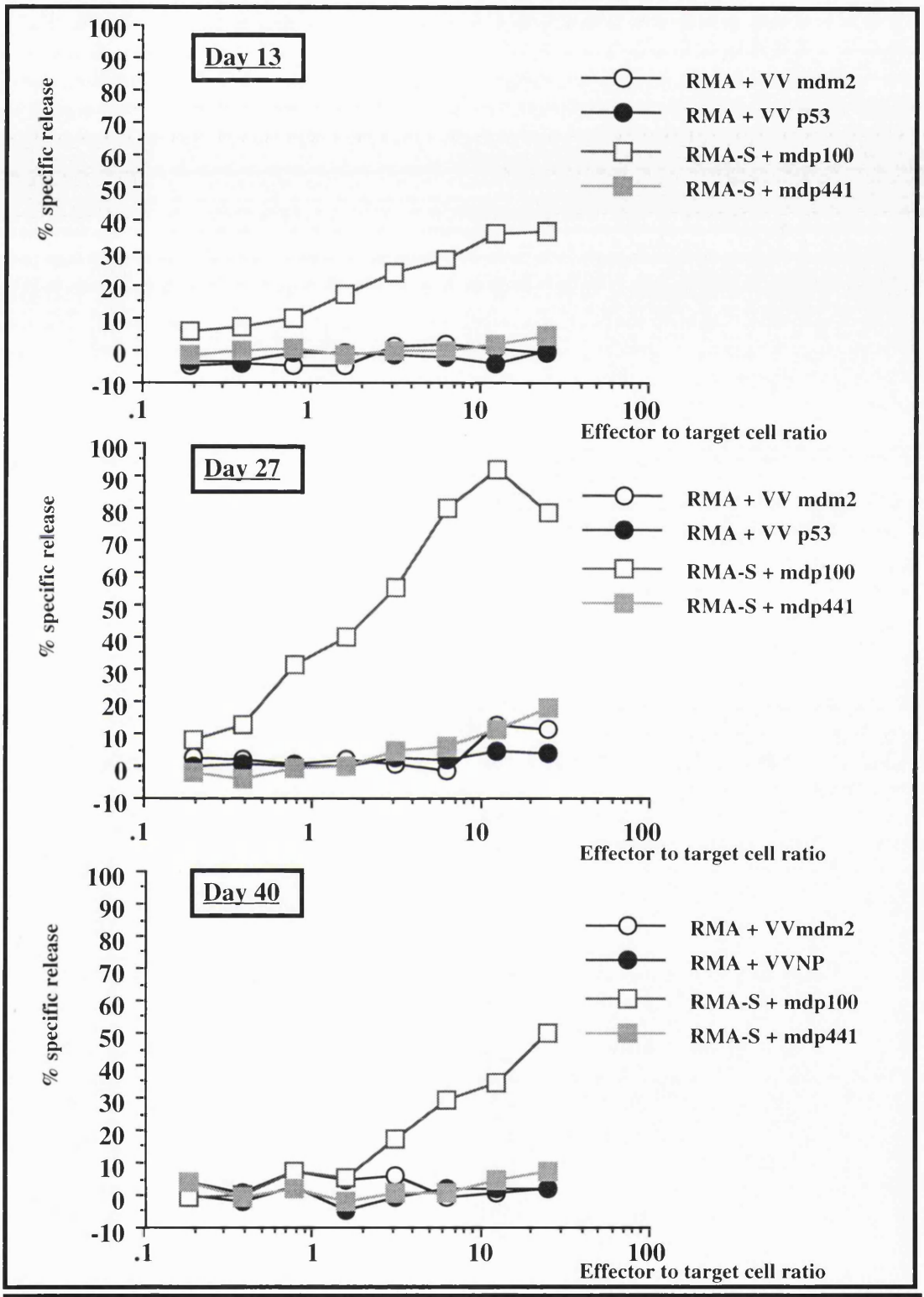


Figure 5.3.3.a

mdp100 peptide specific CTL do not lyse cells infected with recombinant vaccinia virus mdm2.

CTL lines were generated from naive splenocytes which were stimulated with mdp100 peptide in vitro.The lines were tested at various time points by ^{51}Cr release assay for recognintion of target cells infected with vaccinia virus mdm2 or control vaccinia virus.

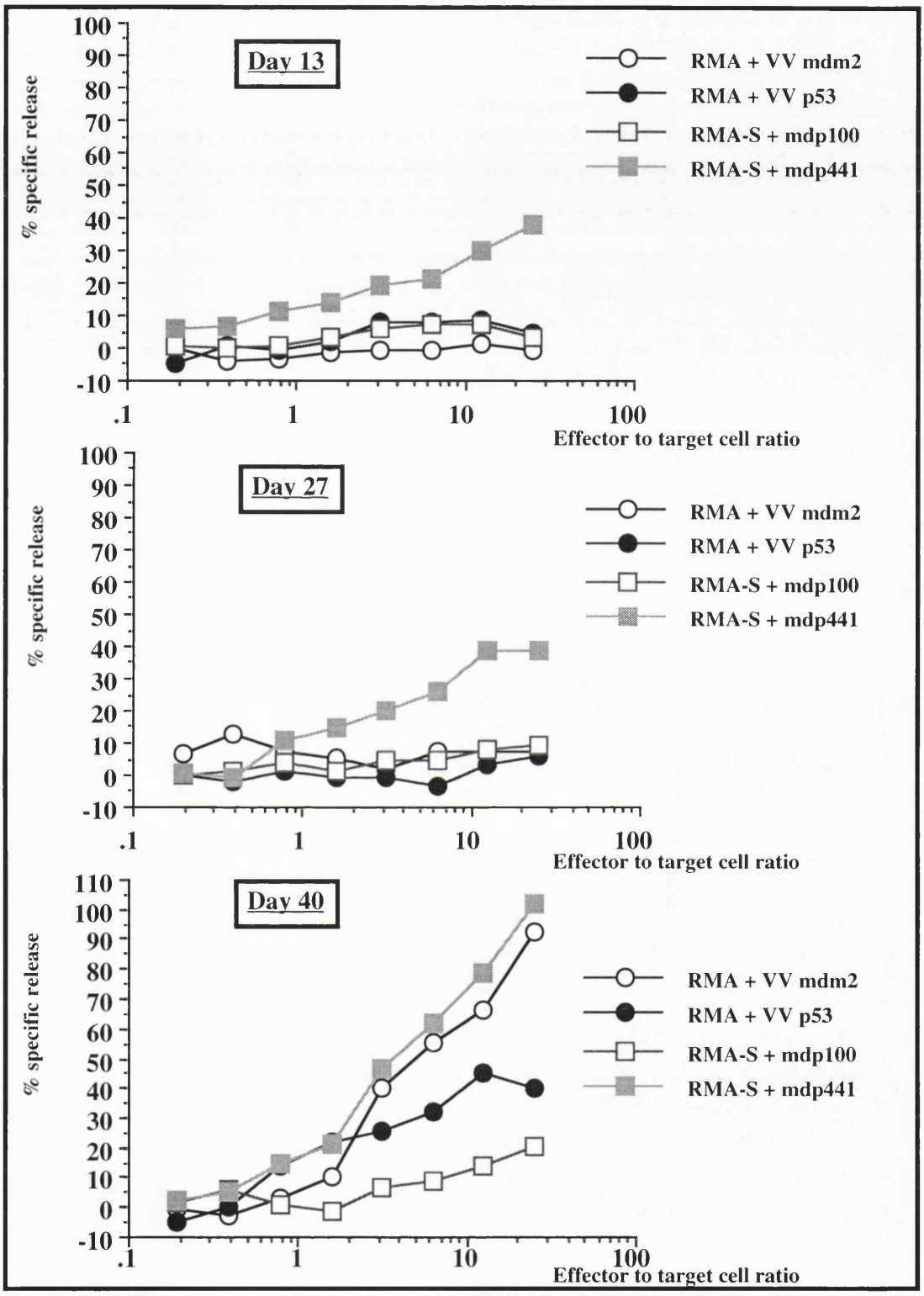


Figure 5.3.3.b.
Repeated stimulations of naive spleen cells with mdp441 peptide stimulate mdp441 specific CTL which lyse cells infected with vaccinia virus mdm2 specifically. Responder T cells were derived from naive splenocytes which were stimulated with mdm2p441 peptide in vitro. The cultures were tested at various time points by ^{51}Cr release assay for lysis of peptide pulsed target cells or target cells infected with recombinant vaccinia virus mdm2 or control recombinant vaccinia virus

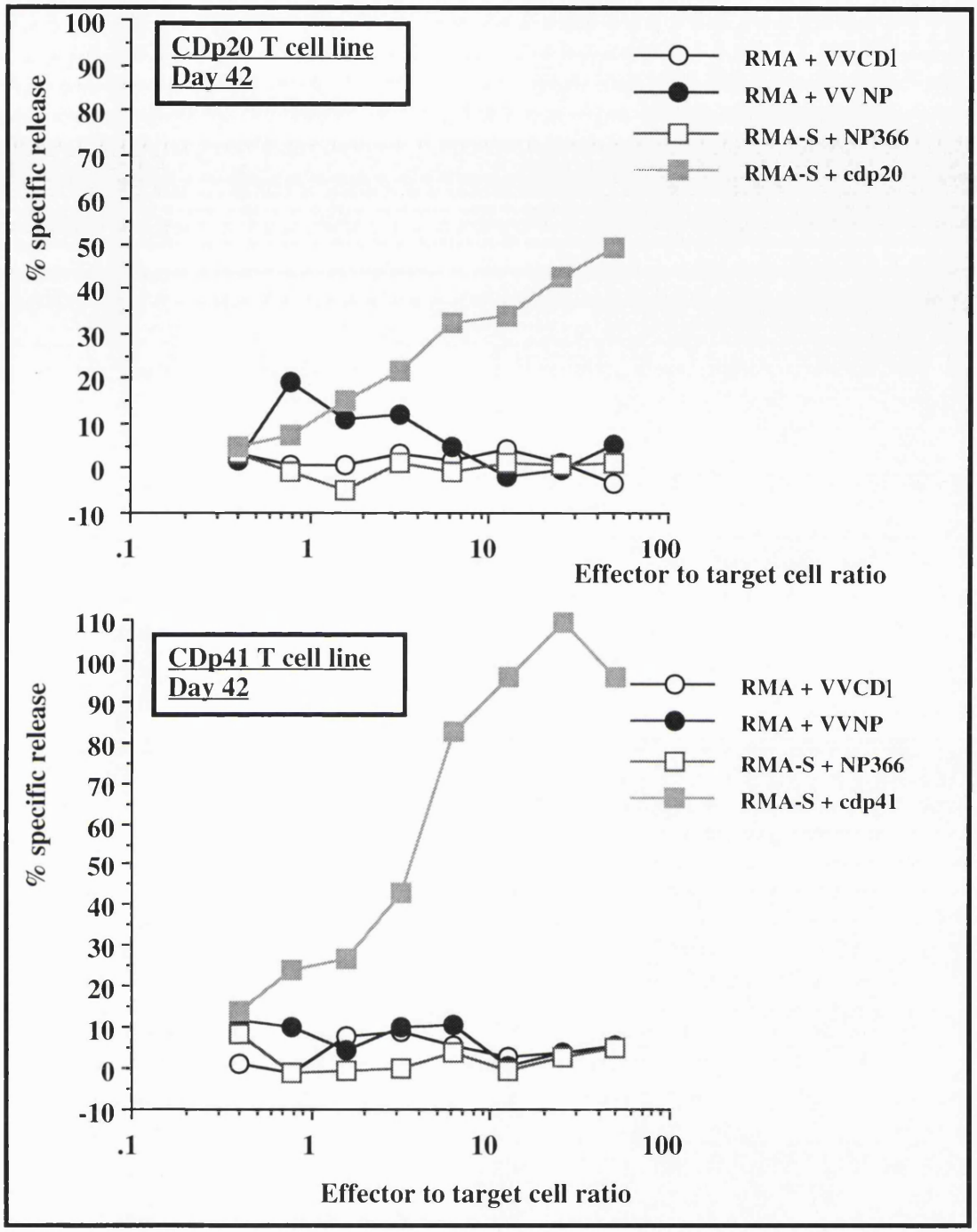


Figure 5.3.3.c.
CTL lines specific for cyclin D1 peptides cdp20 or cdp41 do not lyse cells infected with recombinant vaccinia virus cyclin D1.
CTL lines were generated from naive spleens which were stimulated with cyclin D1 peptide cdp20 or cyclin D1 peptide cdp41. The lines were tested at various time points by ^{51}Cr release assay for recognition of peptide pulsed target cells or target cells infected with recombinant vaccinia virus cyclin D1 or control recombinant vaccinia virus.

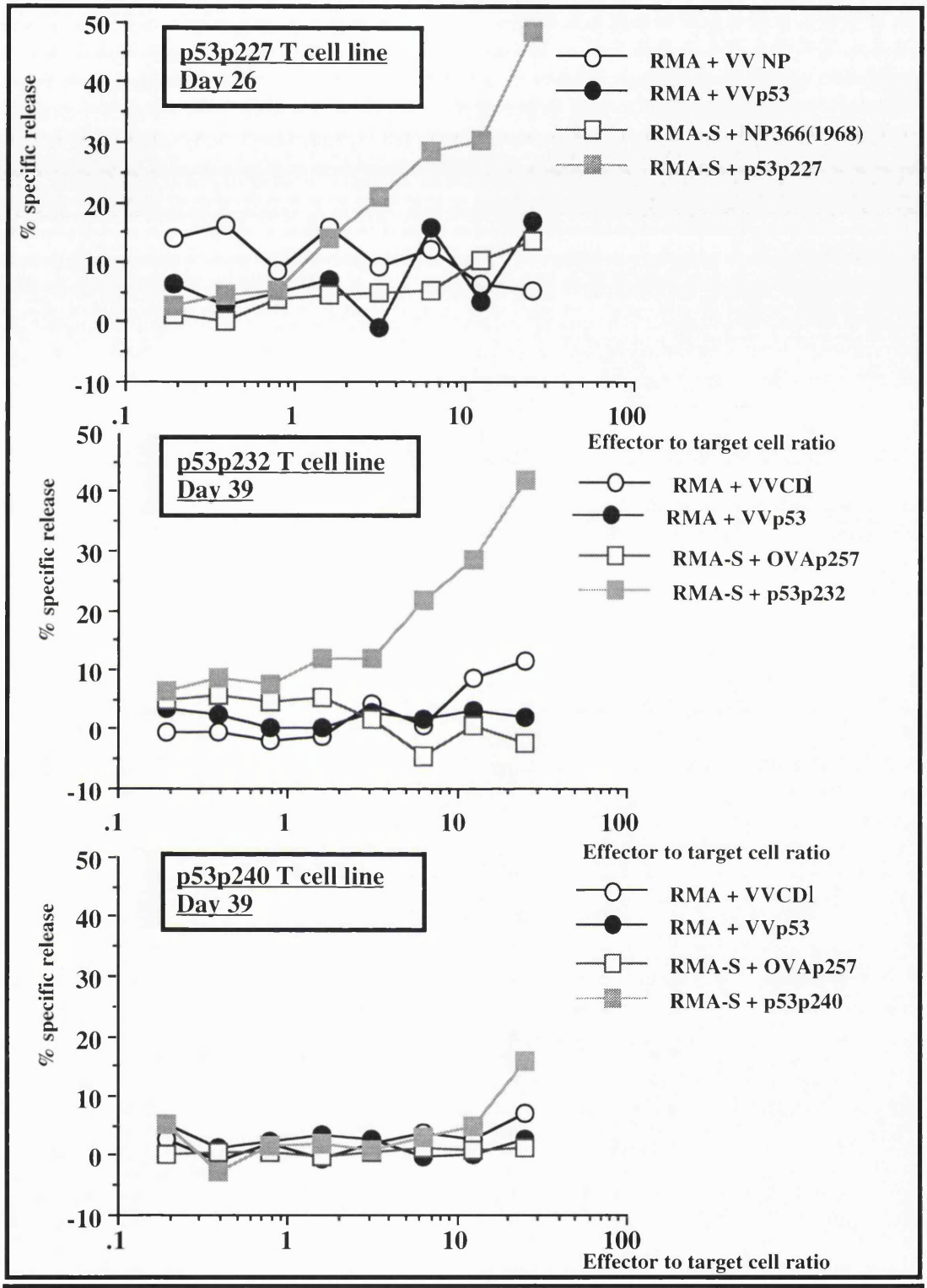


Figure 5.3.3.d.
CTL lines specific for p53 peptides p53p227, p53p232 or p53p240 do not lyse target cells infected with recombinant vaccinia virus p53.
CTL lines were generated from naive spleens which were stimulated in vitro with p53 peptides p53p227, p53p232 and p53p240 respectively. The lines were tested at various time points by ^{51}Cr release assay for recognition of peptide pulsed target cells or target cells infected with vaccinia virus p53 or control vaccinia virus.

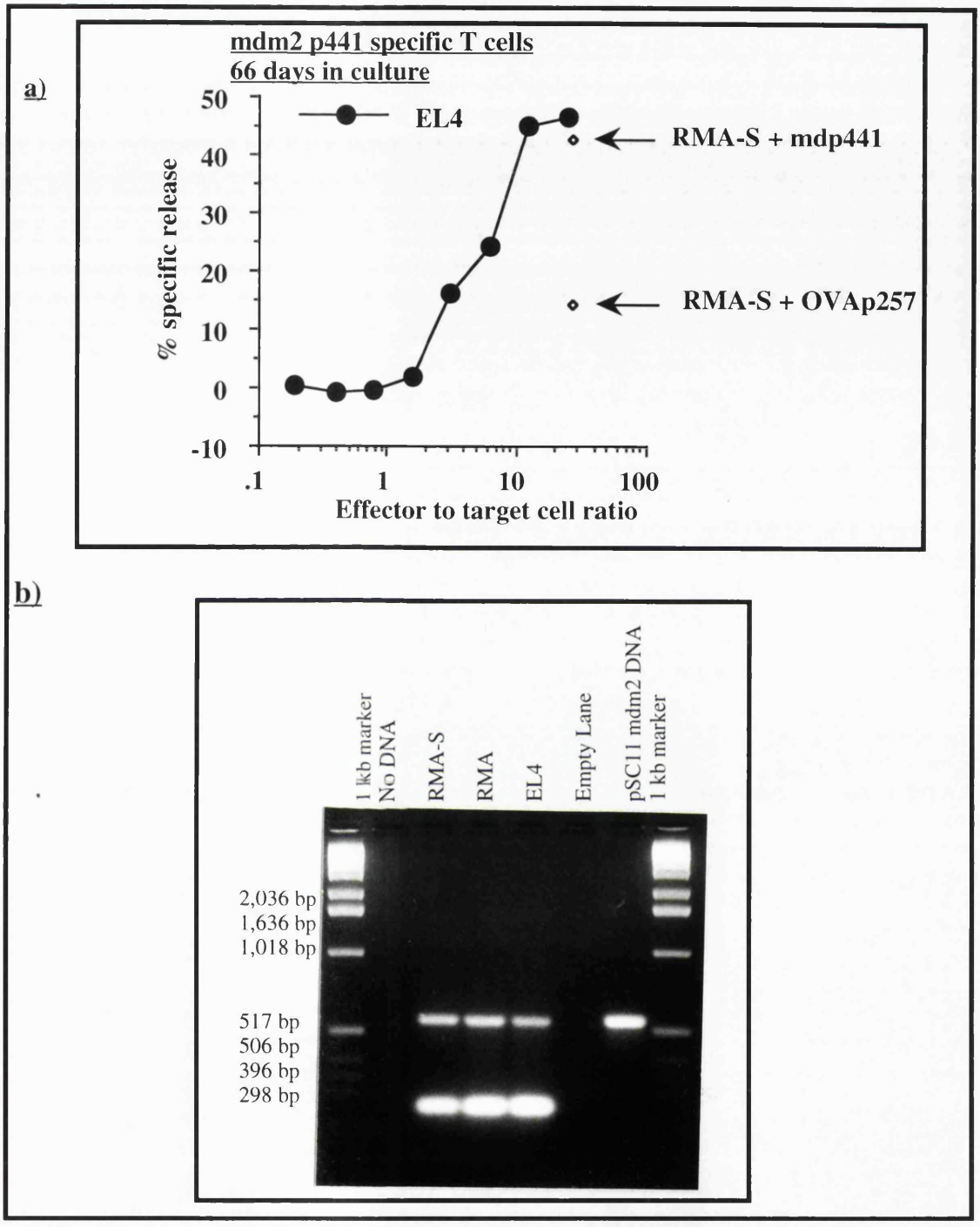


Figure 5.3.4.
Some mdm2p441 specific CTL lines lyse EL4 cells.
a) Responder T cells were derived from naive spleens and stimulated with peptide mdp441 in vitro and tested for recognition of EL4 cells or RMA-S cells pulsed with mdp441 peptide or control peptide.
b) Production of mdm2 mRNA in thymoma cell lines RMA-S, RMA and EL4. RT-PCR of total RNA from RMA-S cells, RMA cells and EL4 cells respectively using mdm2 specific and HPRT specific primers in the PCR reaction.
The amplified fragments are 251 bp long with the HPRT primers and 558 bp long with the mdm2 primers.

5.3.3. mdp441 specific T cells recognising endogenously processed protein are of high avidity

The work described in section 5.3.2. showed that one self peptide, mdp441, stimulated CTL which recognised processed protein, but only after the cells had been at least 39 days in culture. This was observed with altogether four independent specific CTL lines. Peptide specific CTL lines stimulated with the 6 other peptides known to induce CTL, did not recognise endogenously processed protein irrespective of the time the lines had been in culture. It was therefore investigated whether a relative measure of the avidity of the individual peptide specific CTL lines would give any information useful for the prediction of the behaviour of individual lines, Figure 5.3.5.a-j.

The peptide specific CTL were tested for recognition of target RMA-S cells coated with a range of different peptide concentrations varying from 1 μM to 100 fM. It was found that the titration profiles for four of the self peptide specific CTL lines, as well as for the CTL lines specific for the control peptide OVAp257 and SV9 (SVp324), did not change significantly with time up to 50 to 60 days in culture for some of the lines, Figure 5.3.5. a, d, e, g, h and i. However, 2 of the self peptides, mdp441 and p53p227 and too a lesser extent cdp20, induced CTL lines which recognised target RMA-S cells coated with very low concentrations of peptide when the lines had been in long term culture. The difference between the concentration of peptide giving half maximal lysis in early cultures (day 13) and later cultures (day 40 and in some case from around 50 days and onwards) was nearly 10^5 fold for p53p227 specific CTL and 10^6 -10^7 fold for mdp441.

The peptide concentrations giving half maximal lysis for individual peptide specific CTL lines were compared with the peptide concentration giving half maximal stabilisation of class I molecules on RMA-S cells to give a so-called "avidity score" for the CTL lines, Table 5.3.2. It was found that most of the peptide specific CTL lines recognised between 10-10^3 fold lower peptide concentration in CTL assays as compared to the concentrations of peptides found to stabilise class I molecules as measured by FACS analysis. This ratio remained constant. The ratio for the known CTL epitope OVAp257 was mostly around 10^3. This value is identical to the value obtained by other groups using similar cells (RMA-S cells) as CTL targets and for whole cell class I stabilisation assays (424).

Peptide mdp441 and p53p227 and to a lesser extent cdp20 stimulated long term CTL lines which obtained very high avidity scores. The high avidity mdp441 cell lines all recognised endogenously processed protein. It was only possible to obtain the results for the concentration required to induce half maximal lysis for two out of the four mdp441 specific CTL lines. The remaining two lines recognised the target peptide so efficiently that the titration curve had not started going down even at target peptide concentration of 100 fM concentration. These two lines as well as the mdp441 line which induced CTL

which recognised target cells with a ML_{50} value of 400 pM by day 66 all recognised RMA cells and/or EL4 cells just as well as the same cells infected with vaccinia virus mdm2, see section 5.3.2. and Figure 5.3.4. The mdp441 line, which appeared to be of high avidity already by day 39 with a ML_{50} value of 100 fM, was the only line able to discriminate between the endogenous levels of mdp441 present in RMA cells and the levels produced by superinfection of RMA cells with recombinant vaccinia virus expressing mdm2, see Figure 5.3.3.b.

P53p227 specific CTL lines were also of high avidity. These lines did not reproducibly recognise endogenously processed p53 as was the case with mdp441 specific CTL lines. It was only possible to maintain two p53p227 lines in culture for longer than 40 days. The other lines developed unexpectedly in that they recognised RMA cells infected with recombinant vaccinia virus as well as RMA cells but not RMA-S cells coated with p53p227 peptide.

The results were highly reproducible. In some cases as many as 9 different lines specific for the same peptide would give similar titration curves when the lines were tested after a similar time in culture, see Table 5.3.2.

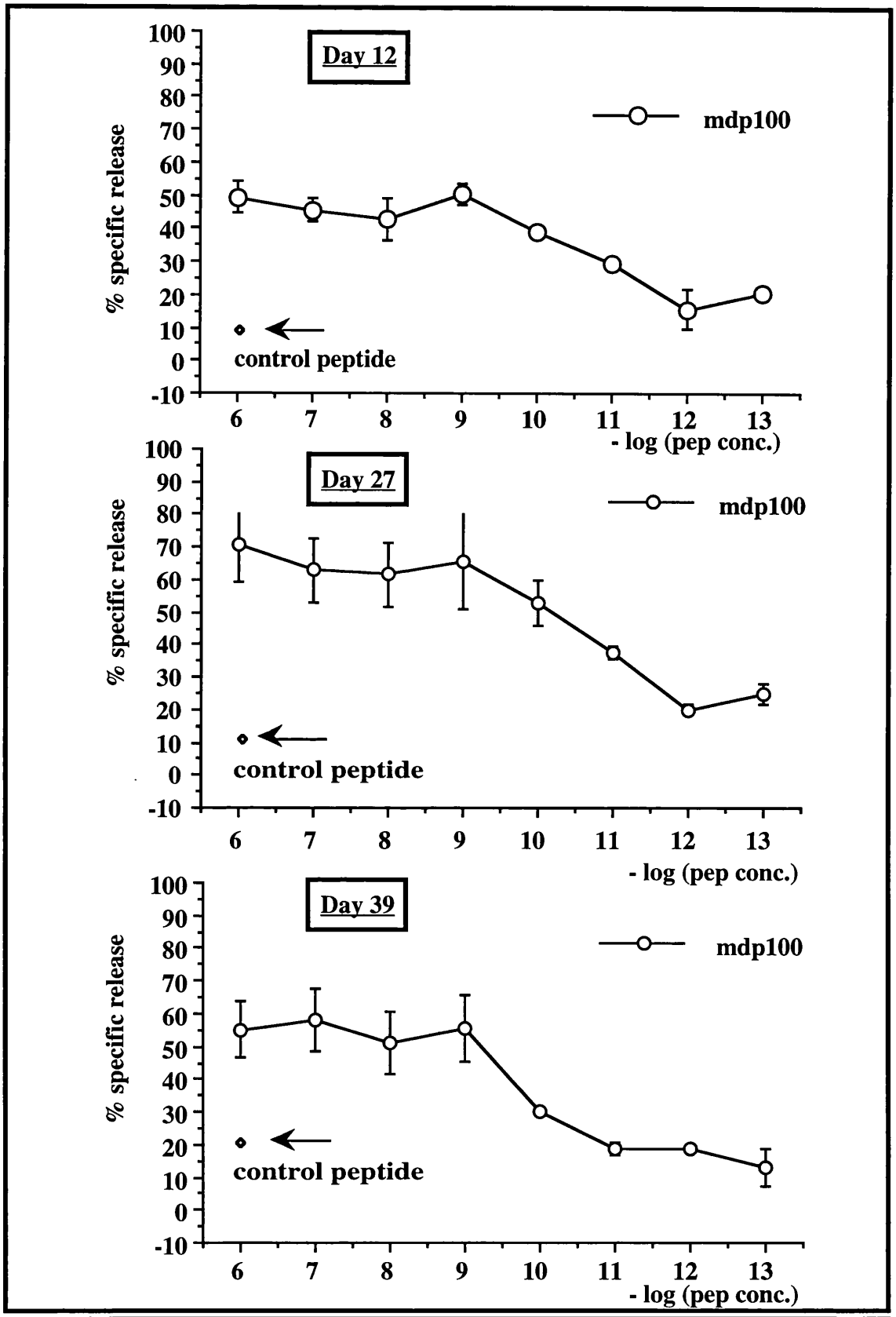


Figure 5.3.5.a.
Lytic activity of mdp100 peptide specific CTL line against RMA-S target cells coated with different concentrations of mdp100 peptide.
^{51}Cr release assays.

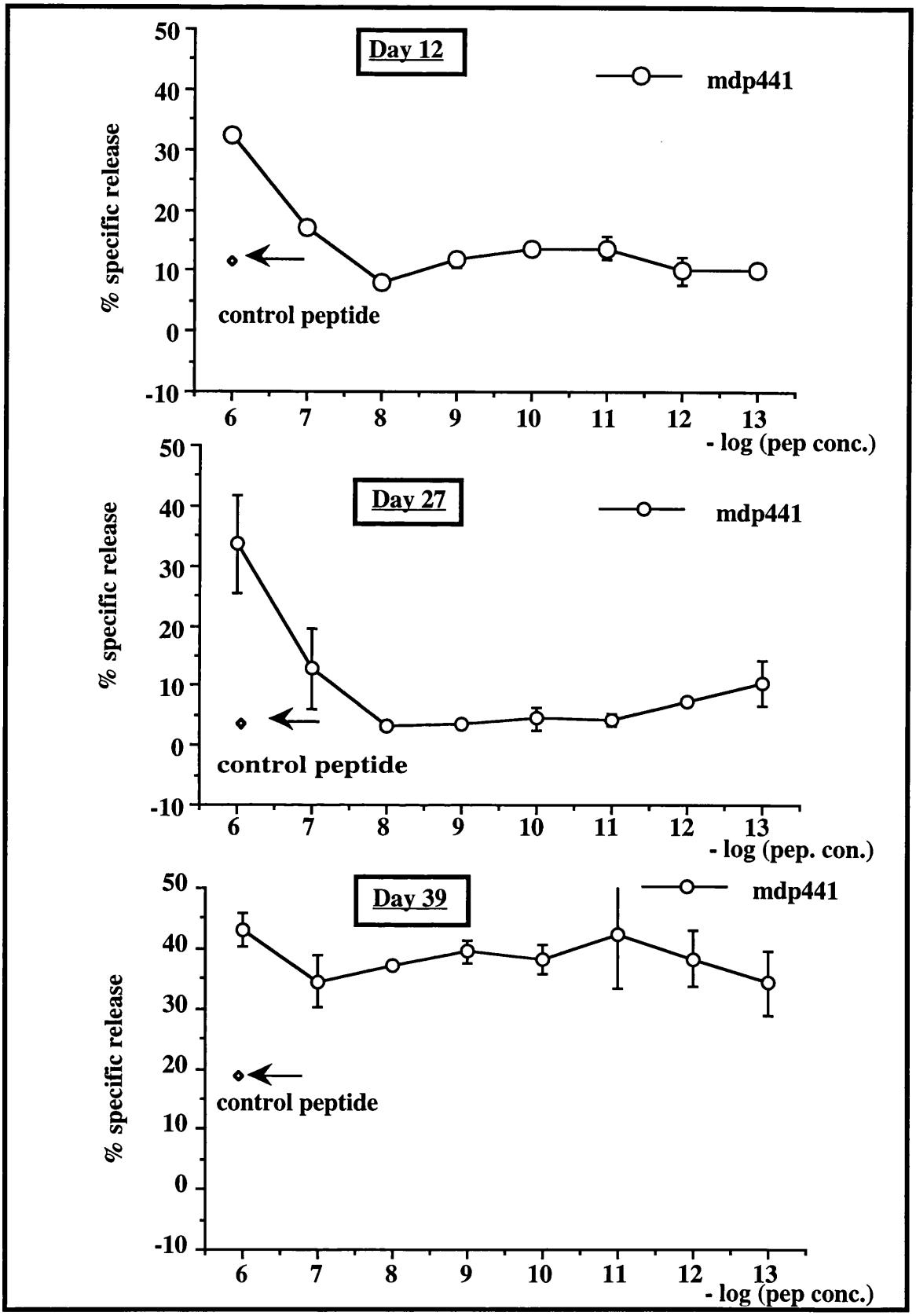


Figure 5.3.5.b.
Lytic activity of mdp441 peptide specific CTL line against RMA-S target cells coated with different concentrations of mdp441 peptide.
^{51}Cr release assays.

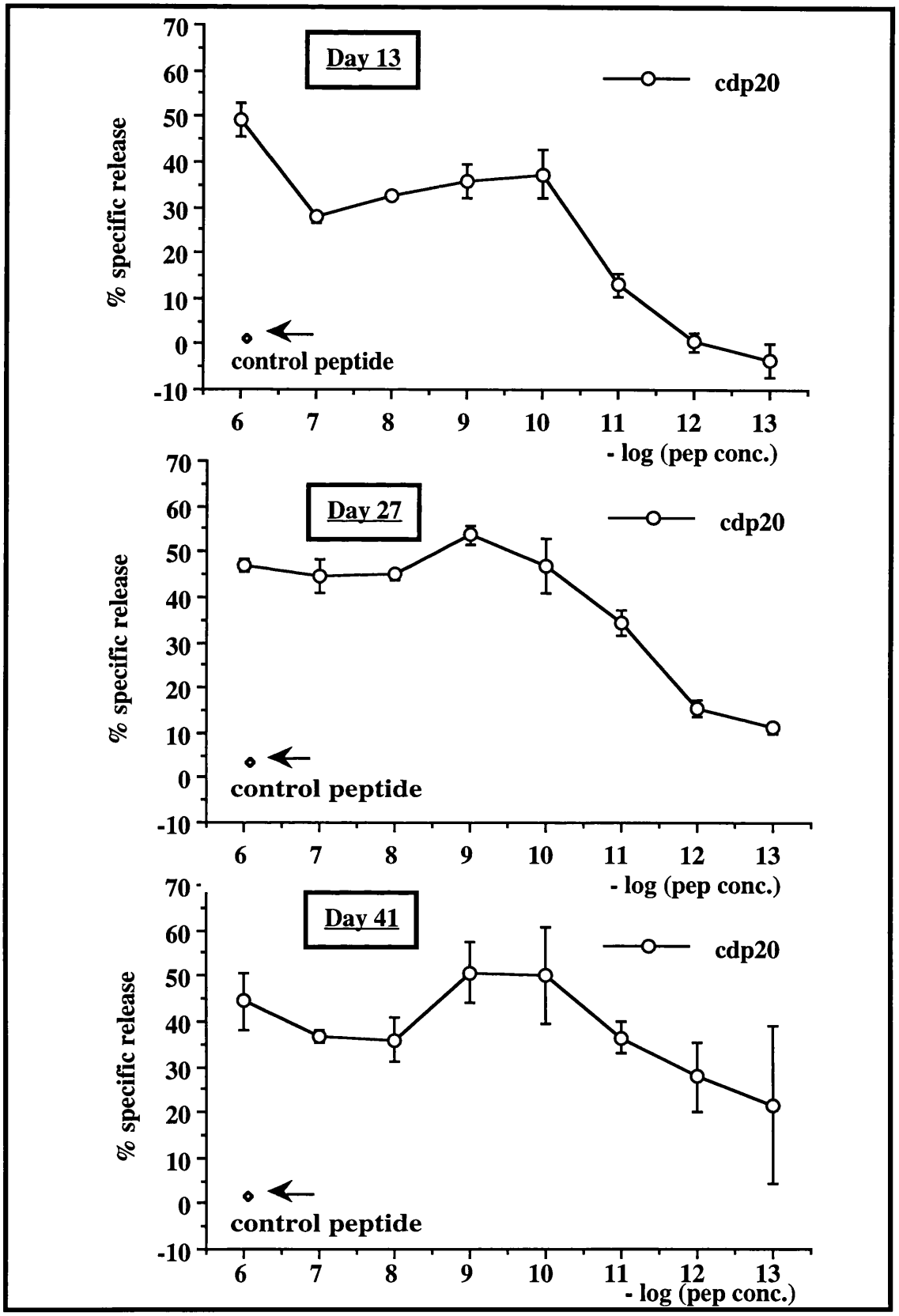


Figure 5.3.5.c.
Lytic activity of cdp20 peptide specific CTL line against RMA-S target cells coated with different concentrations of cdp20 .
^{51}Cr release assays.

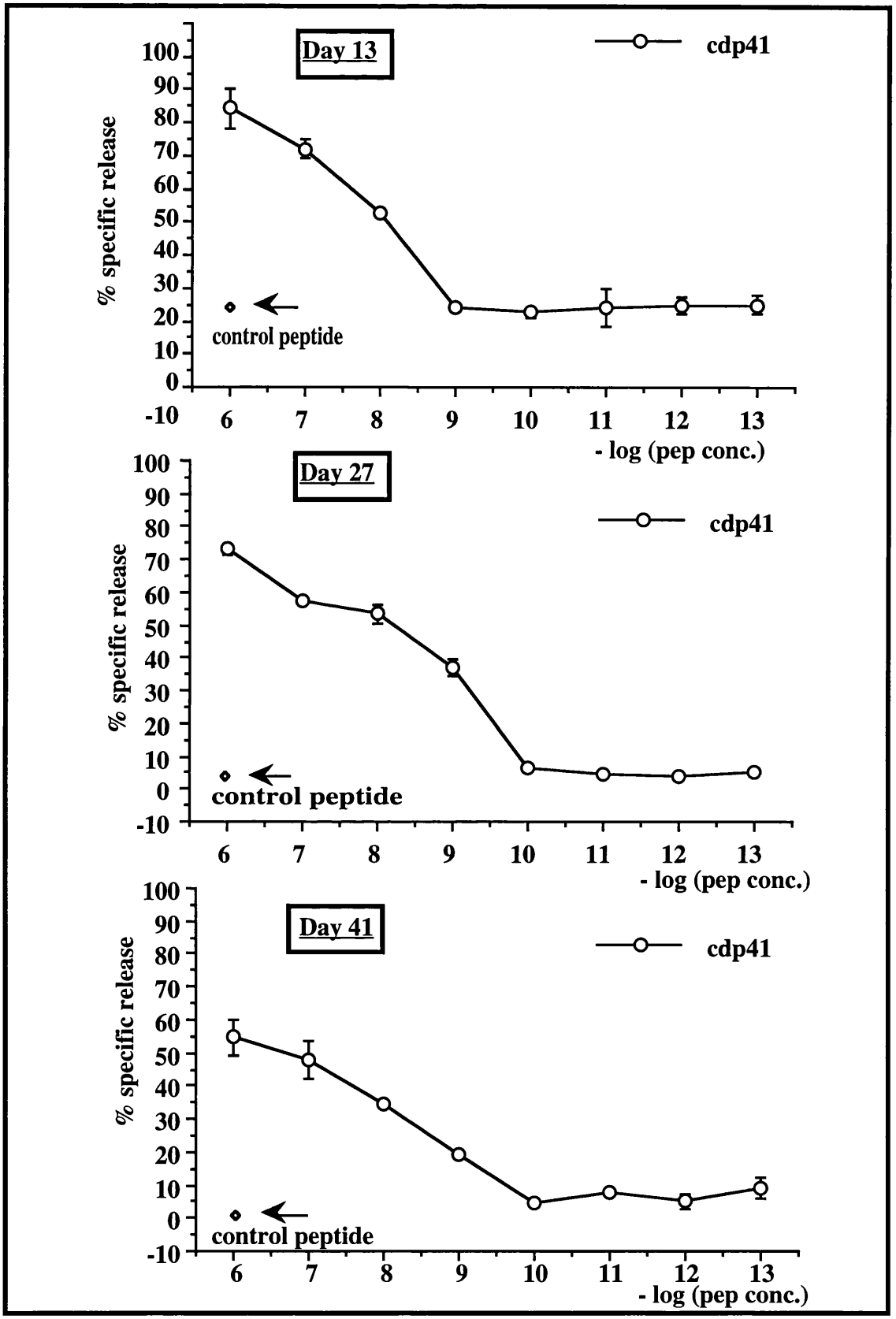


Figure 5.3.5.d.
Lytic activity of cdp41 peptide specific CTL line against RMA-S target cells coated with different concentrations of cdp41 peptide
^{51}Cr release assays.

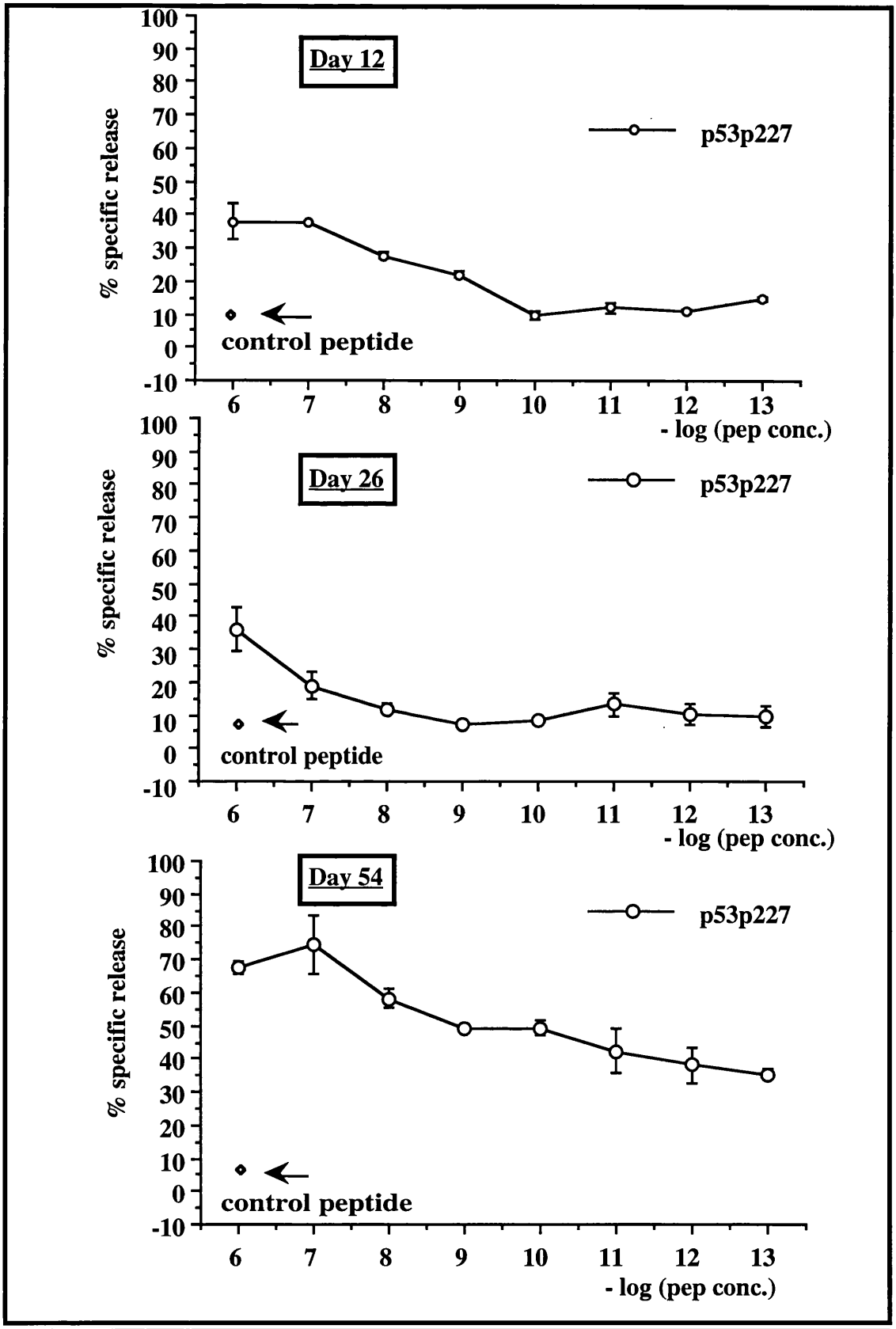


Figure 5.3.5.e.
Lytic activity of p53p227 peptide specific CTL lines against RMA-S cells coated with different concentrations of p53p227 peptide.
^{51}Cr release assays.

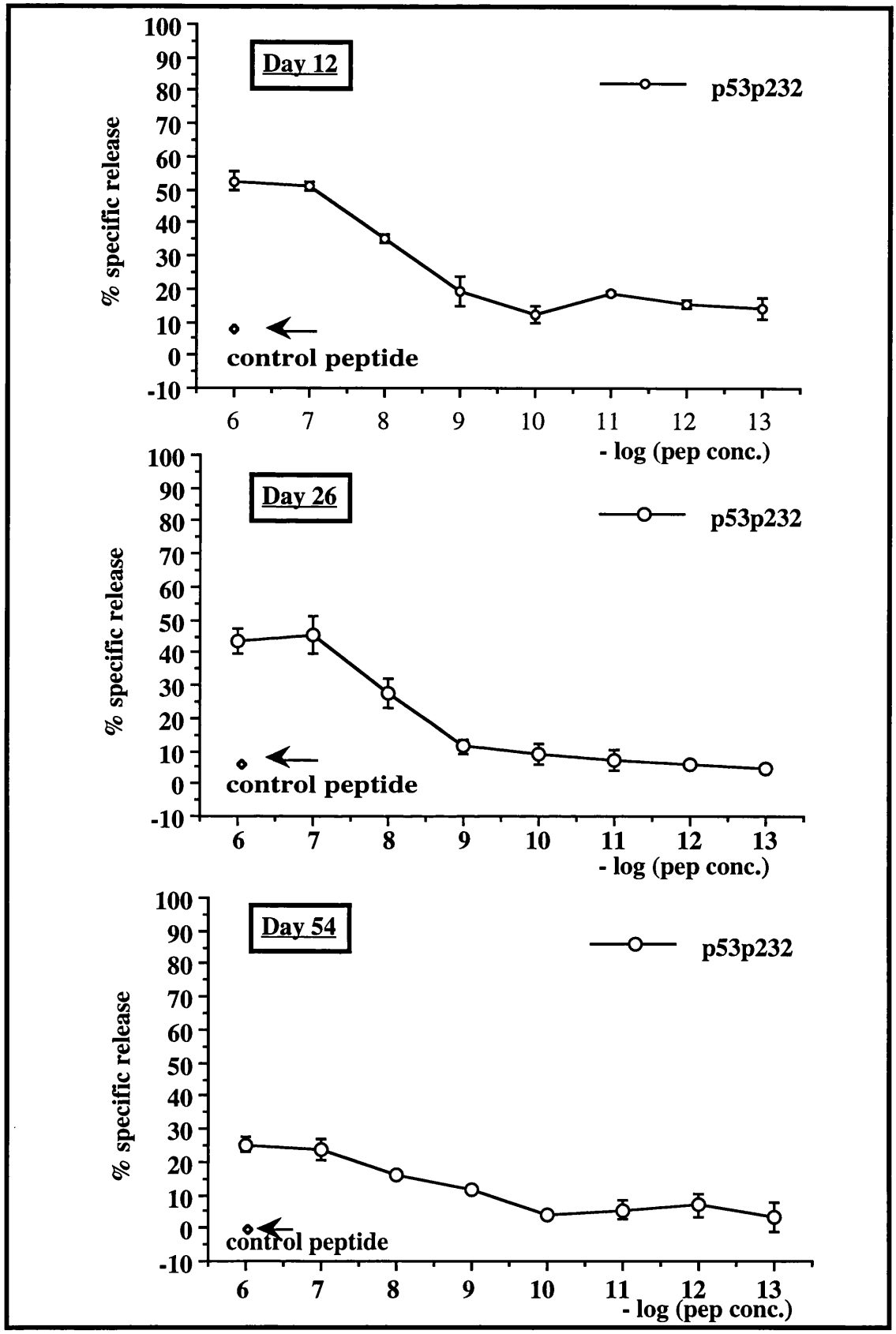


Figure 5.3.5.f.
Lytic activity of p53p232 peptide specific CTL lines against RMA-S cells coated with different concentrations of p53p232 peptide.
^{51}Cr release assays.

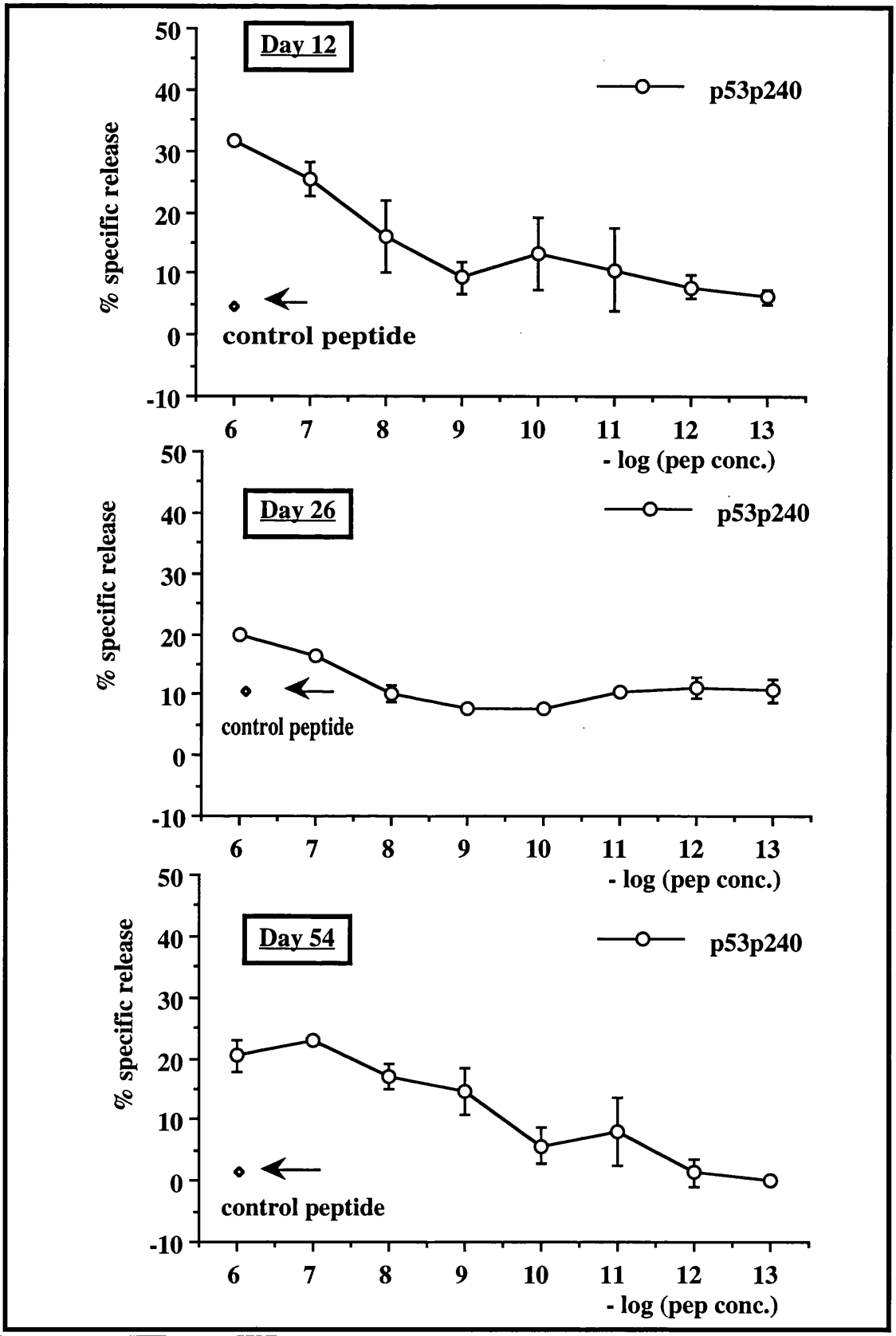


Figure 5.3.5.g.
Lytic activity of p53p240 peptide specific CTL lines against RMA-S cells coated with different concentrations of p53p240 peptide.
^{51}Cr release assays.

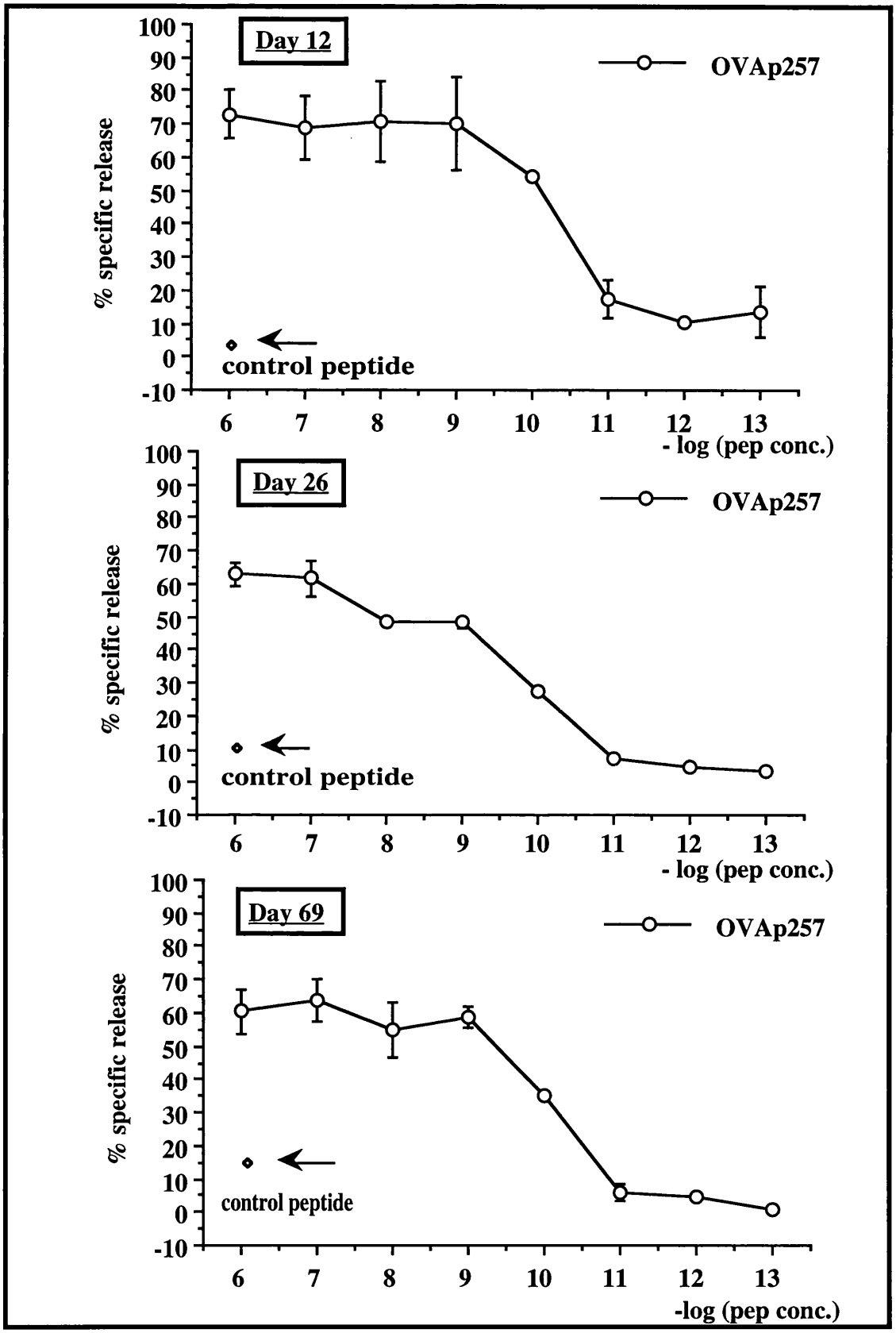


Figure 5.3.5.h
Lytic activity of OVAp257 peptide specific CTL lines against RMA-S cells coated with different concentrations of OVAp257 peptide.
^{51}Cr release assays.

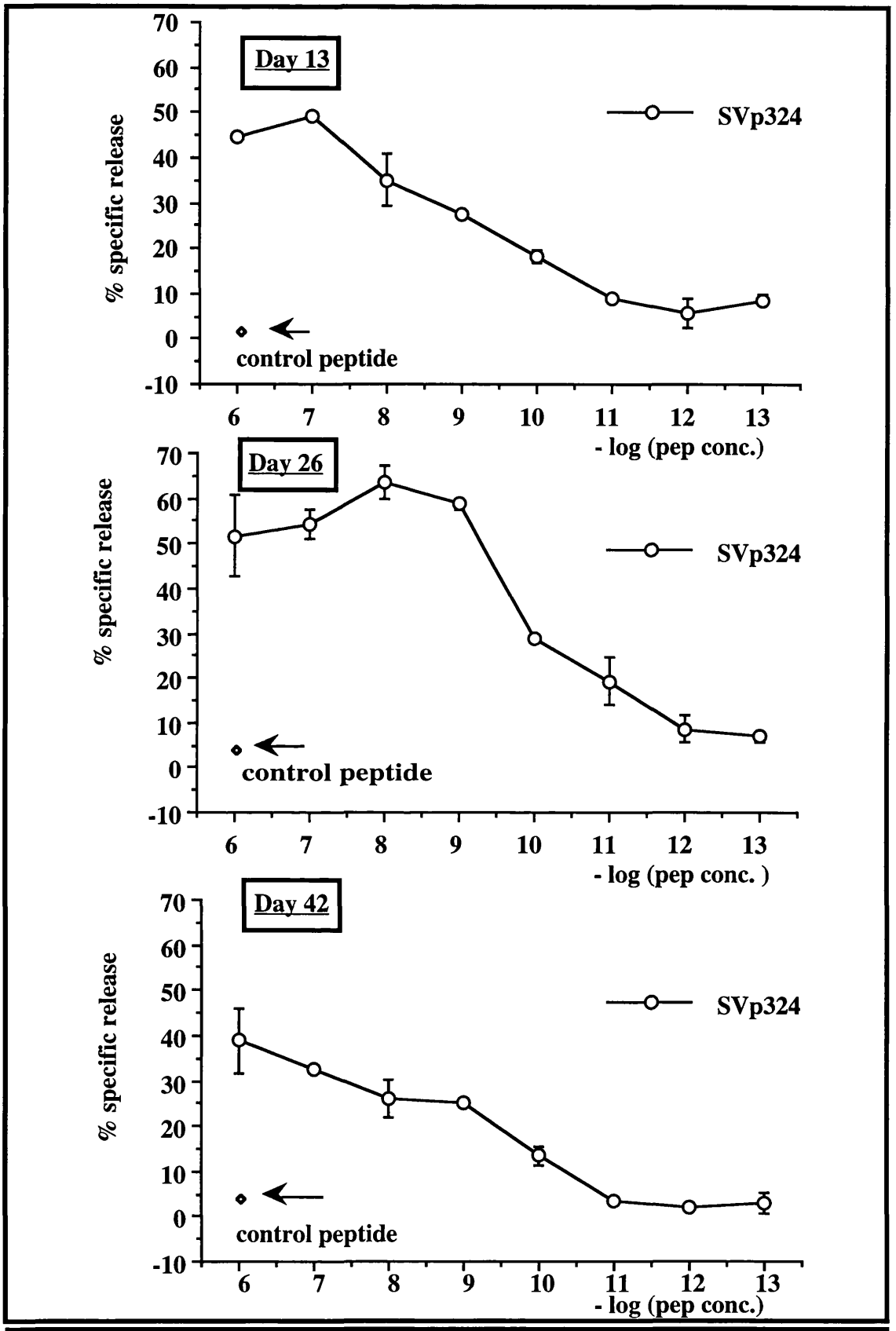


Figure 5.3.5.i.
Lytic activity of SVp324 (SV9) peptide specific CTL line against RMA-S cells coated with different concentrations of SVp324 peptide.
^{51}Cr release assays.

Table 5.3.2.

Avidity scores for peptide specific CTL lines stimulated with murine self peptides or control peptides			
Peptide	**Peptide concentration required to give 50% maximum class I stabilisation (MB_{50})**	**Peptide concentration required to obtain 50% maximum lysis in CTL assays (ML_{50})** The number of independent experiments/lines is shown in brackets	**MB_{50}/ML_{50}**
mdp100	3 nM (K^b)	day 12-13 (7 ex): 20-70 pM day 21-26 (3 ex): 20-100 pM day 39-55 (3 ex) : 20 -200 pM	day 12-13 4×10^1-2×10^2 day 21- 26 3×10^1-2×10^2 day 40-55 2×10^1- 2×10^2
mdp164	50 μM (D^b)	No CTL induction	-
mdp441	5 μM (D^b)	day 12-13 (9 ex): 50-400 nM day 21-27 (6 ex): 20 -400 nM day 39-88 a) 50 nM by day 41, then 400 pM by day 66 b) 100 fM by day 39 and on wards	day 12-13 1×10^1-1×10^2 day 21-27 1×10^1-3×10^2 d39-88 a) 1×10^2, day 41 then 1×10^4 b) 5×10^7
cdp20	50 nM (D^b)	day 12-14 (3 ex): 20-500 pM day 26-27 (2 ex): 5 -10 pM day 41-68 (3 ex): 500-700 fM	day 12-14 1×10^2-3×10^3 day 26-27 5×10^3-1×10^4 day 41-68 1×10^5
cdp41	5 μM (K^b)	day 12-14 (4 ex): 600 pM-10 nM day 26-27 (3 ex): 600 pM -6 nM day 40-68 (6 ex): 600 pM-10 nM	day 12-14 5×10^2-8×10^3 day 26-27 8×10^2-8×10^3 day 40-68 5×10^2-8×10^3
cdp84	40 μM (K^b)	No stable CTL lines	-
cdp147	5 μM (D^b)	No stable CTL lines	-
cdp181	1 μM (K^b)	No stable CTL lines	-

Table 5.2.3., continued.

Avidity scores for peptide specific CTL induced with murine self peptides or control CTL epitopes			
Peptide	**Peptide concentration required to give 50% maximum class I stabilisation (MB_{50})**	**Peptide concentration required to obtain 50% maximum lysis in CTL assays (ML_{50})** The number of independent experiments/lines is shown in brackets	**MB_{50}/ML_{50}**
p53p18	5 μM (K^b)	No stable CTL lines	-
p53p127	500 nM (K^b)	No stable CTL lines	-
p53p222	3 μM (K^b)	No stable CTL lines	-
p53p227	100 nM (K^b)	day 12-13 (3 ex): 3-100 nM day 26 (2 ex): 10-100 nM day 39-54 (2 ex): a) 20 nM, d42 then 1 pM b)<100 fM, day 39 on wards	day 12-13 1×10^0- 3×10^1 day 26 1×10^0-10^1 day 39-54 a) 5×10^0, day 42 then 1×10^5 b) 10^6, d39 onwards
p53p232	4 μM (D^b)	day 12-13 (3 ex): 7-100 nM day 26 (2 ex): 7-50 nM day 39- 54 (2 ex): 1-10 nM	day 12-13 4×10^1-6×10^2 day 26 8×10^1-6×10^2 day 39-54 4×10^2-4×10^3
p53p240	600 nM (D^b)	day 12-13 (3 ex): 4-30 nM day 26 (1 ex): 7- 70 nM day 42-54 (2 ex): 1-10 nM	day 12-13 2×10^1-2×10^2 day 26 8×10^0-8×10^1 day 42- 54 6×10^1-6×10^2
p53p320	50 μM (K^b)	No stable CTL lines	-
p53p334	40 μM (K^b)	No stable CTL lines	-
OVAp257	50 nM (K^b)	day 12-13 (4 ex): 50-100 pM day 26 (2 ex): 50-300 pM day 37-69 (5 ex): 50-100 pM	day 12-13 5×10^2-1×10^3 day 26 2×10^2-1×10^3 day 37- 69 5×10^2-1×10^3
SV9 (SVp324)	8 nM (K^b)	day 12-13 (6 ex): 70-500 pM day 26 (3 ex): 50-500 pM day 40-54 (3 ex): 100-500 pM	day 12-13 2×10^1-1×10^2 day 26 2×10^1-2×10^2 day 40-54 2×10^1-8×10^1

5.3.4. The concentration of peptide used to prime CTL does not affect the avidity of the resulting CTL population

All peptide specific CTL lines were induced using the same conditions. This included initial priming with peptide pulsed dendritic cells with 100 nM peptide added to the culture, followed by passage on day 6 with peptide pulsed dendritic cells and 100 nM peptide added to the culture and then finally two weekly restimulations using only 100 nM peptide in the cultures. This peptide concentration is below the plateau for maximum class I stabilisation for the mdp441 peptide and all other peptides except mdp100. mdp441 specific CTL cultures stimulated with 100 nM peptide (50 times lower concentration than the concentration required to induce half maximal stabilisation of class I molecules in bindings assays) got dominated by high avidity CTL able to recognise endogenously processed protein. It was of interest to investigate whether reducing the peptide concentration would induce high avidity mdp100 specific CTL recognising processed protein. The conditions used are listed in Table 5.3.3.

The concentration of free peptide added to the CTL cultures and used to pulse the DCs was proportionately lowered. As an example, CTL lines stimulated with 1 nM peptide instead of the usual 100 nM added to the culture would have been stimulated with dendritic cells pulsed with 1 μM instead of the usual 100 μM. The only exception was the mdm2 p441 line which was stimulated with 1 μM peptide added to the culture. This line was induced by dendritic cells pulsed with the usual peptide concentration, 100 μM.

3 different concentrations were used to stimulate mdp100 specific CTL and 2 different peptide concentrations were used to stimulate mdp441 specific CTL. It was found that altering the peptide concentration did not alter the avidity of the predominant CTL population.

Low concentrations of mdp100 peptide could still stimulate mdp100 specific CTL. These concentrations were 3-300 times lower than the concentration required to induce half maximal stabilisation of class I molecules in class I stabilisation assays. These mdp100 specific CTL did not grow well and the cultures were outgrown by large granular cells killing non-specifically and thought to be natural killer cells. This phenomenon was observed with two independent lines. This means that using low concentrations of peptide does not per se stimulate high avidity CTL. Stimulating mdp441 with higher concentrations of peptide resulted in the generation of mdp441 specific CTL which were of similar avidity as CTL lines stimulated with low concentrations of peptide.

Table 5.3.3.

Effect of different peptide concentrations used for CTL stimulation on the avidity of the resulting CTL

If two values for ML_{50} are indicated, results are from two different lines.

Peptide and conc. of peptide used to stimulate CTL	ML_{50} Day 5/6 in culture	ML_{50} Day 12/13 in culture	ML_{50} Day 26 in culture
mdp100 100 nM	20-70 pM	20 pM/30 pM	20 pM
mdp100 1 nM	1 nM	1 nM/ 1 nM	Non specific
mdp100 10 pM	70 nM	200 nM/ 30 nM	Non specific
mdp441 1 μM	N.D.	2 nM	100 nM
mdp441 100 nM	N.D.	400 nM	50 nM

5.3.5. Induction of mdp441 peptide specific CTL by *in vivo* immunisation with mdp441 peptide.

In vitro induction of mdm2 peptide mdp441 specific CTL had shown that it was possible to induce high avidity mdp441 specific CTL, which recognised endogenously processed protein. It was decided to try to immunise mice with mdp441 peptide to see whether it was possible to generate a high avidity response at earlier stages in culture. Different immunisation protocols were tested. Variations included the number of immunisations, adjuvant used, the time between last immunisation and removal spleen. Three different adjuvants were used: Incomplete Freund's and Muramyl dipeptide emulsified in an aqueous adjuvant or a lipid adjuvant.

Most of the immunisation protocols did not appear to stimulate mdp441 specific CTL *in vivo*. Sometimes the CTL responses observed from immunised mice were even significantly worse than with naive mice and cultures could exhibit non-specific lytic activity. It was originally found that immunisation of mice only once and with subsequent removal of the spleen one or three weeks later generated CTL of higher avidity than CTL derived from naive mice. When different adjuvants were tried, it was found that mice immunised at day -21 with peptide emulsified with muramyl dipeptide in aqueous adjuvant could induce CTL which recognised processed protein by day 7 in culture (not shown). These cells lost specificity rapidly and lysed neither mdp441 pulsed target cells nor vaccinia virus mdm2 infected target cell at later stages in culture. This was found to be a general phenomenon. Whenever an immunisation procedure stimulated a potent response in early culture, as judged by the lysis of peptide coated or vaccinia mdm2 infected targets, the CTL lost their activity upon prolonged *in vitro* culture and the cultures were outgrown by large granular cells unable to kill target cells specifically.

Discussion

In vivo immunisation with recombinant vaccinia virus expressing murine self proteins had shown that it was possible to stimulate CTL which could specifically lyse cells presenting endogenously processed self protein. To supplement the information gained from *in vivo* immunisation with whole protein, a peptide based approach was used to stimulate CTL able to recognise endogenously processed self protein. The purpose of the investigation was to gain insight in the mechanisms of tolerance and stimulation of autoreactive CTL and identify epitopes recognised by potential autoreactive CTL.

The work described in this chapter was divided into three main parts.
1) Screening of self proteins for class I binding motifs and analysis of class I binding by the motif containing peptides.
2) Use of motif containing peptides to induce self peptide specific CTL.
3) Characterisation of self peptide specific CTL.

Class I binding motifs were used to identify 16 self peptides from cyclin D1, mdm2 and WT p53. Of these, 7 consistently induced stable peptide specific CTL lines. One peptide, the mdm2 peptide mdp441, stimulated high avidity CTL which could recognise endogenously processed protein. This type of peptide might be useful for tumour immunotherapy.

Screening of self proteins for class I binding motifs and analysis of class I binding by the motif containing peptides.

The peptides were screened for binding using a whole cell binding assay based on peptide binding to temperature induced RMA-S cells. RMA-S cells were chosen for binding assays for several reasons.

The first reason for using RMA-S cells to quantify peptide binding to MHC class I molecules in a whole cell binding assay was that RMA-S cells contain only low levels of mostly empty class I molecules (35; 38). The level of class I molecules can be up-regulated by incubating the cells at 23-31°C with induction at 26°C for 24 hours recommended as the standard treatment (35). The whole cell peptide binding assay measures the level of class I molecules in a particular conformation identified by a set of class I specific antibodies. Class I molecules can adopt several conformations on the cell surface. They can exist in an improperly folded conformation (referred to as $\alpha1\alpha2$- by Townsend and colleagues (38)). The unfolded conformation is not recognised by the K^b specific antibody Y3, but by the D^b specific antibody Hb27 (28-14-8S)(38). The $\alpha1\alpha2$- form is in equilibrium with a $\alpha1\alpha2$ + form which is stabilised by the addition of peptide

and β_2 microglobulin. The $\alpha1\alpha2$ + form is recognised by Y3 which recognises K^b and by Hb27 which recognises D^b. D^b molecules (as well as L^d molecules) do not bind β_2 microglobulin well due to a polymorphism at position nine of the heavy chain (436), but the molecule can exist on the cell surface in a peptide receptive form without association with β_2 microglobulin (437).

Temperature induced RMA-S cells express class I molecules in the $\alpha1\alpha2$+ form at 26°C. These molecules are not stable at 37°C. If no class I binding peptide is added, the molecules will disappear from the cell surface. This means that the binding of the antibodies Y3 and Hb27 to temperature induced RMA-S is proportional to the amount of class I molecules in the $\alpha1\alpha2$+ conformation and this again is proportional to the amount of peptide bound to the class I molecules on the cell surface. Once the class I molecule has bound peptide, the conformation might change as a consequence of the peptide binding (82; 83). Importantly, the $\alpha1/\alpha2$ domain epitope on K^b recognised by Y3 antibody is independent of individual peptide specific conformational changes induced by different peptides (82; 83). It is thought that the binding of Hb27 to $\alpha3$ on D^b is also independent on the nature of the bound peptide as this is the case for other $\alpha3$ binding antibodies (82). This means that quantification of class I expression as a result of peptide binding is not dependent on the nature of the peptide, thereby making a comparison of the binding characteristics of the different peptides possible. Recent data indicate that the binding of exogenously added peptide occurs via binding of the peptide to class I heavy chain/ β_2 microglobulin heterodimers formed by the association of exogenous β_2 microglobulin with heavy chains devoid of both endogenous β_2 microglobulin and peptide (434).

Class I molecules on temperature induced RMA-S cells have been referred to as peptide receptive rather than empty, as it is unclear to what extent the molecules are truly empty (434). The amount of peptide receptive class I molecules is higher on RMA cells than on RMA-S cells presumably due to the role of peptides delivered by TAP molecules in shaping the conformation of the class I molecule (434). However, in the present work it was preferable to use RMA-S cells and not RMA cells to measure peptide binding to class I molecules. On RMA cells, high levels of class I molecules are already present on the cell surface of this cell line and peptide binding would have to be quantified by labelling of peptides such as by iodinating them. Modification of the peptides was not desirable as it is not be known to what extent chemical modifications would affect the binding characteristics of individual peptides (438).

The second reason for using RMA-S cells for bindings assays was that peptide pulsed RMA-S cells constitute an excellent target for self peptide specific CTL and it was desirable to use the same cell line for binding studies as for CTL assays. RMA-S cells do

not present endogenous peptides (35; 38) with the exception of peptides derived from Rauscher murine leukemia virus, the virus used to induce the parental RBL-5 tumour (439). Therefore there should be no background lysis due to endogenous self proteins present in the cell line itself.

Sixteen different peptides derived from the self proteins cyclin D1, mdm2 and WT p53 were tested for their binding to class I molecules on temperature induced RMA-S cells. Despite variability in the concentrations required to stabilise surface class I molecules, class I binding motifs were found to predict class I binding. Twelve peptides bound the class I molecule for which they contained the anchor motifs, at concentrations giving half maximal binding of 10 μM or less. Of these 12 peptides, only two, mdm2 peptide mdp100 and p53 peptide p53p227, bound an irrelevant class I molecule i.e. D^b of which the peptide did not contain the anchor residues, but the concentration required to reach half maximal stabilisation of the irrelevant class I molecules was 1700 and 100 times higher respectively than for the stabilisation of the relevant class I molecules.
The presence of secondary still ill defined anchor motifs or inhibitory peptides within the sequences are thought to account for the differences in class I binding between class I motif containing peptides. The best class I binding self peptide mdp100 did indeed contain submotifs consisting of a small amino acid such as alanine or glycine at position 2, combined with tyrosine at position 5 and leucine or isoleucine at position 8 (423), but this submotif was also found in the much weaker class I binder, p53 peptide p53p222. There was no clear connection between the peptide sequence and the class I binding for other motif containing peptides. For many of the peptides, a plateau for binding was not achieved at 100 μM peptide concentration and the mean fluorescence value observed at this concentration was used as maximum value. Only strong binders such as mdm2 peptide mdp100, cyclin D1 peptide cdp20 and positive control peptide OVAp257 and SVp324 reached a plateau when 100 μM peptide was added to the cells.

The whole cell binding assay does have certain limitations. Firstly, peptides can be taken up directly by cells and associate with newly synthesised class I molecules in the ER (38). In the present binding assay, no inhibitors of the transport from the endoplasmic reticulum such as Brefeldin A were added. Internalisation of peptide can be minimised by keeping the cells on ice during the binding assay (434) but this would prevent class I molecules not stabilised by added peptide from collapsing. Secondly, recycling of class I/peptide complexes can occur and favour the build up of high affinity complexes on the cell surface (440). Differences in on-off rates have been found to play a major role in determining the level of peptide bound to class I molecules on the cell surface with important implications for presentation to CTL. As an example, for two ovalbumin peptides, OVAp257 and OVAp55, a 40 fold difference in the half maximal binding

concentration in RMA-S whole cell binding assays was suggested to be accounted for by a 10 fold higher association rate and a 2 fold lower dissociation rate of OVAp257 compared with OVAp55 as measured by surface plasmon resonance studies with purified class I molecules (426). It can not be excluded that strong binding peptides with a low dissociation rate and high association rate would be favoured by both routes of presentation.

Use of motif containing peptides to induce self peptide specific CTL.

All 16 class I binding motif containing self peptides derived from cyclin D1, mdm2 and WT p53 were tested for their ability to stimulate primary CTL *in vitro*, irrespective of their ability to stabilise MHC class I molecules *in vitro*. A cell population enriched for dendritic cells (DCs) was used to stimulate primary peptide specific CTL *in vitro*.Work in other labs has shown that the use of professional APCs in combination with IL-2 for *in vitro* stimulation of CTL does not require T_h co-operation (441). Murine dendritic cells has been shown to be capable of priming murine peptide specific CTL *in vitro* (428; 429) and *in vivo* (442) and for being able to decrease or eliminate the need for CD4 helper T cells (118; 119). Powerful adhesion between the dendritic cell and the CTL precursor has been suggested to contribute to the remarkable efficiency of this cell type in CTL priming (428). DCs also have the ability to stimulate a CD4 Th1 response favourable for the development of CTL, both via direct interaction with class II molecules on CD4 T cells and subsequent production of Th1 promoting cytokines such as IL-12 (405; 432). Here, no effort to detect class II restricted helper epitopes was made and help for the CTL induction was provided by a combination of exogenously added IL-2 and the professional antigen presenting cells.

Cell populations enriched for dendritic cells were found to efficiently prime self peptide specific CTL derived from naive spleens. It was assumed without investigation that it was indeed the dendritic cells which were responsible for priming of the CTL. B cells are the main contaminating cell type in the APC preparation used. There is no evidence that this cell type is important for CTL priming to peptide antigens, although the role of B cells in T cell priming is controversial. B cells have been found to be inefficient in priming naive CD4 T cells (443) and some experiments indicate that B cells instead of stimulating naive T cells can even deliver a negative signal to them and induce tolerance (444). Experiments in B cell deficient mice have demonstrated that CTL priming can occur without the need for B cell help although B cells are able to activate T cell clones and primed T cells. In conclusion, lack of evidence that B cells are important for T cell priming, combined with overwhelming evidence of the role of DC, indicates that the potency of the purified antigen presenting population used in this study was due to the presence of dendritic cells.

Characterisation of self peptide specific CTL.

Analysis of the peptide specific CTL lines gave rise to three major findings:

1) The ability to bind to stabilise class I molecules is a prerequisite for CTL induction. Peptides which bound poorly to class I did not induce CTL. However, when binding to class I exceeded a certain threshold, peptides which differed by 1000 fold in the concentration required to induce half maximal stabilisation of class I molecules could induce CTL equally rapidly, by days 6 or 12/13 in culture.

2) Self peptide specific CTL lines had similar apparent avidities in early cultures as judged by the ratio of the concentrations of a given peptide able to induce half maximal stabilisation of class I molecules in binding assays and half maximal lysis by peptide specific CTL. Peptides which bound CTL efficiently, such as mdp100 (MB_{50} = 3 nM) and cdp20 (MB_{50} = 50 nM) would induce CTL which recognised target cells coated with lower concentrations of peptide than CTL induced to weaker binding peptides, such as cdp41 (MB_{50} = 5 μM) and p53p232 (MB_{50} = 4 μM) but when the avidity scores were compared they were found in most cases to be remarkably similar, around 10^{1}- 10^{3}.

3) However, 3 peptides induced CTL which did not conform to this pattern after culture for 5-6 weeks with 3 restimulations. These CTL recognised peptides at very low concentration. Two of these peptides were strong binders with MB_{50} values of 50 nM and 100 nM respectively. The CTL induced by these peptides did not recognise processed protein. One of the peptides was an intermediate binder, with a MB_{50} value of 5 μM. The CTL induced by this peptide did recognise endogenously processed proteins.

These findings must be interpreted in the light of understanding the interaction of the components constituting the trimolecular MHC-peptide-T cell receptor complex, general binding kinetics, as well as events underlying antigen presentation, T cell activation and thymic selection.

The first finding was that peptides which bound MHC class I weakly were not found to induce CTL.

This might be explained by the fact that even potent antigen presenting cells such as dendritic cells would not be able to compensate for the poor binding of peptides. The threshold required for activation of CTL would not be achieved.

The data confirms the findings of other groups, that irrespective whether the peptide contain binding motifs or not, a minimum level of peptide binding to MHC class I is required for the induction of CTL, although there is not always a strict correlation between the strength of the binding of peptides to class I and the ability to induce CTL (186; 435; 445; 446). The MHC class I molecule plays at least two roles in the stimulation of CTL. Firstly, the molecule presents the peptide epitope on the cell surface to the T cell receptor. Secondly, there is evidence that the class I molecule plays a role in the generation of endogenous peptide/MHC complexes. Cells devoid of particular class I molecules have been shown not to produce particular peptides known to be CTL epitopes presented by the class I molecule in question (447; 448; 449; 450).

The second major finding derived from analysis of self peptide specific CTL lines was that the overall avidity of CTL lines in early cultures as estimated by the ratio of the concentration required to induce half maximal binding in class I binding assays and to induce half maximal lysis by peptide specific CTL fell in a similar range for self peptide specific CTL in early cultures.

Avidity is a relative concept, here used to gain information about CTL by comparing the behaviour of different peptides with respect to their ability to bind to class I molecules and to be recognised in association with these class I molecules by primed peptide specific CTL. The amount of peptide bound will depend on the association and dissociation rates of the peptide from the MHC class I molecule i.e. the equilibrium constant K1 for the reaction: (MHC) + (peptide) = (MHC-peptide). In the CTL assay the concentration required to get half maximal lysis depends both on K1 as well as K2, the equilibrium constant for the reaction (MHC-peptide) + (TCR) = (MHC-peptide- TCR). It is assumed that interaction between the T cell and the peptide coated APC involves the sequential interaction of the antigenic peptide with the MHC molecule and the antigen peptide-MHC complex with the TCR and that the specific lysis in a CTL assay is a linear function of the proportion of activated T cells (451). Under these circumstances, a decrease of K1 results in an increase in the peptide concentration required to obtain 50% of the maximal response when the interaction of a TCR with the MHC peptide complex is considered (451). The value of the maximal response is not affected. This is what one appears to observe with many of the self peptides tested. Binding of the peptide to MHC class I seems to be the principal determining factor influencing the strength of the CTL responses to self peptides in early cultures. It would appear, that the contribution of the TCR interaction with the MHC-peptide complex is similar in early cultures when most of the peptides are tested. There seems to be some intermediate range of affinities of T cell receptors specific for self peptides which is predominant.

This hypothesis is supported by analysing the CD8 dependency of CTL lines in early cultures. The concentrations of CD8 antibody required to block lysis of peptide coated target cells were compared. It was found that all self peptide specific CTL lines were blocked by a concentration of CD8 antibody ranging from 0.75 µg/ml to 3 µg/ml at an effector to target ratio of 25:1. For comparison, studies using the LCMV NP as model antigen have shown that CTL lines derived from normal mice and recognising as little as 10^{-13} M peptide coated on target cells were blocked by 10 µg/ml CD8 antibodies whereas CTL lines derived from a NP transgenic mice recognising only 10^{-9} M peptide on target cells were blocked by 10 times less CD8 antibody, 1 µg/ml (161). The difference between the two T cell populations were suggested to be due to the deletion of high affinity T cell receptors specific for the target peptide and present in normal mice. Altogether work from several labs have indicated that there is an inverse correlation between the strength of the interaction of the T cell receptor with the MHC class I /peptide complex and the dependency of CD8 (100; 161; 435).

The third major finding resulting from the analysis of CTL lines induced by self peptides was, that three out of seven self peptides induced CTL which recognised target cells with increasing avidity with time in culture as judged from the concentrations of peptide required to induce half maximal lysis of peptide coated target cells in CTL assays. The avidity of the remaining four peptides did not change over time, neither did the avidity of the CTL lines specific for the known exogenously derived CTL epitopes OVAp257 or SVp324/SV9.

Only one of sixteen peptides induced high avidity CTL which recognised endogenously processed protein. The existence of high avidity self peptide specific CTL capable of recognising endogenously processed protein can be explained by considering the events underlying antigen processing and thymic selection.

On basis of present knowledge on T cell development (120; 148) self peptide specific CTL such as the peptide specific CTL recognising mdp100, mdp441, cdp20, cdp41, p53p227, p53p232 or p53p240 will have been positively selected in the thymus but escaped negative selection. As mentioned previously, it was originally thought that the same peptide ligand which could induce T cell activation and negative selection would mediate positive selection when presented at lower concentration (151). However, there is now evidence that there is a qualitative difference in the peptides which induce positive selection versus negative selection and activation. Peptides which act as agonists for T cell activation are found to be unable to induce positive selection, whereas variant peptides which act as antagonists for the same T cells can induce positive selection (154). Consequently, self peptide specific CTL existing in the periphery, including high affinity

self peptide specific CTL such as these against mdm2 peptide mdp441, will not have been selected by the peptide itself.

Negative selection occurs when a threshold number of T cell receptors on the T cell engages with high affinity with MHC class I/peptide complexes on the antigen presenting cell (120; 148; 452). The avidity of the interaction is a result of the affinity of the individual T cell receptor for individual MHC class I /peptide complexes, the overall density of peptide/MHC complexes on the cell surface as well as the interaction of other adhesive molecules on the surface of T cell and APC. This model explains why both low avidity self peptide specific T cells, such as mdp100 or cdp41 CTL, as well as high avidity self peptide specific CTL, such as mdp441 specific CTL, can be found in the periphery

The avidity model of negative selection predicts that T cells will escape negative selection if the affinity of the T cell receptor for the MHC/peptide complex is too low to trigger apoptosis of the immature thymocyte. As also mentioned in chapter 4, transgenic mice have been important for understanding the process of negative selection of class I as well as class II restricted T cells. Experiments using T cell receptor transgenic mice specific for the LCMV NP crossed with mice transgenic for LCMV NP showed that whereas high affinity T cells specific for the self protein were deleted, then low affinity T cells escaped deletion and got access to the periphery. This low affinity T cells were unable to recognise the endogenously processed protein (161). Similar conclusions were obtained using a class II restricted T cell receptor transgenic model involving injection of peptide analogues of the recognised antigen acetylcholine receptor peptide amino acid 1 to 9 (163). The ability of low affinity T cells to escape negative selection could explain why most of the self peptide specific CTL described in the present thesis interact with low to intermediate avidity with their target cells.

The avidity model also predicts that T cells can escape negative selection, irrespective of the affinity of the T cell receptor, if the peptide recognised by the T cell receptor is poorly presented in the thymus due to inefficient processing. These T cells will escape to the periphery, not do any damage to normal tissue presenting low levels of the epitope, but be activated if appropriate stimulus is provided (160). If a peptide such as mdm2 peptide mdp441 is poorly presented in the thymus, the number of class I MHC molecules presenting the peptide might not be sufficiently high to trigger the negative selection of T cells bearing receptors recognising the MHC class I/mdp441 complex with high affinity.
A similar model explains the existence of high avidity CTL specific for self peptides in humans such as the gp100 aa 280-288 specific CTL demonstrated in melanoma patients (144). CTL from five different patients recognising a HLA-A2.1 presented peptide were found to recognise the peptide at concentrations 10^4 to 10^5 times lower than the

concentrations found to give 50% maximal inhibition of binding in a competitive binding assay. A naturally presented peptide from MART-1/Melan A amino acid 32-40 was equally found to be only an intermediate good class I binder but able to stimulate high avidity CTL from melanoma patients (143). The peptide could induce half maximal stabilisation of class I molecules on T2 cells at 200 nM in a whole cell binding assay analogous to the one used in the present work, and half maximal lysis of peptide coated T2 cell at a concentration of 10 pM, indicating that the T cells could recognise a 2 x 10^4 lower concentration than could be measured in the binding assay (143).

In one out of four cases, a mdp441 line was found to distinguish clearly between quantitative differences in the level of mdm2 produced in the antigen presenting cell. Three other lines were not able to make that distinction. However, the status of mdm2 in these cell lines is not known. Mdm2 is clearly being produced in all these cell lines, but the level might be higher than in normal tissue. It would have been interesting to transfer mdm2 p441 T cells to normal mice and investigate whether the T cells would cause any damage to normal tissue. However, due to the number of T cells required for such an experiment, it was not feasible. It would be of major interest to investigate that issue in future experiments.

The mdp441 specific CTL always grew rather slowly and a sufficient number of CTL was never obtained to be able to confirm by elution of naturally presented peptides that the endogenously recognised peptide was indeed peptide mdp441. It cannot be excluded that the mdp441 specific CTL recognised some other mdm2 peptide not included in the screening or a peptide from a protein specifically induced by mdm2. There are several reports of stimulation of class II restricted T cells by peptides which have very little sequence similarity with the peptide used to activate the T cells in the first place (91; 453; 454). Similar cross recognition is less commonly reported for class I restricted CTL, although there are some examples, including the recognition of allo antigen by HLA-B8 restricted EBV peptide specific CTL (455).

It was found that high avidity mdp441 specific CTL recognised cells producing endogenously processed mdm2 protein. Work using other antigens including MCMV immediate early protein p89 peptide aa 168-176 or the tum$^-$ antigen P91A$^-$ peptide aa 12-24 has similarly shown that high avidity is a requirement for recognition of endogenously processed protein (100). Altogether, this indicates that the level of endogenously derived peptide presented on the cell surface is low compared to the peptide concentrations (1-100 μM) commonly used to pulse target antigen presenting cells. It might be argued that restimulation of T cells with high concentrations of peptide would promote the generation of low avidity T cells and low concentrations, high avidity T cells. This was not found to be the case for the peptides tested, mdp100 and mdp441. Work by others has also shown

that restimulation of CTL lines with high (1-6 μM) concentrations of peptide could still generate high affinity CTL (100).

Peptide p53p227 stimulated high avidity CTL lines after several weeks in culture. These CTL did not recognise endogenously processed protein. It seems likely that these epitopes are not generated by endogenous processing as might be the case for some of the peptides recognised by the low to intermediate avidity CTL. CTL induction by non-physiological peptides has been demonstrated previously and would presumably play no role in an vivo response to the whole antigen (456).

All the CTL work involving peptide specific CTL involved bulk cell lines and not clones. This was deliberate, as it was considered to be more relevant to study whole populations of CTL, than individual clones. *In vitro* cloning selects for particular growth properties of the CTL so that individual clones may be unrepresentative of the responding population. For this reason, several independent CTL lines were established for each individual peptide. The behaviour of the different lines generated with an individual peptide was very similar (see Table 5.2.3.) allowing for general conclusions to be drawn about the properties of the peptide specific CTL. Mdp441 specific CTL did not grow very rapidly. Attempts were made to obtain clones so that the avidity of individual clones could be compared with bulk cell lines. The clones obtained grew well, but were not lytic. In contrast CTL clones obtained with control OVAp257 peptide were lytic.

Immunisation with vaccinia virus mdm2 had previously been found to stimulate mdm2 specific CTL (see chapter 4). The magnitude of the response was highly dependent on the conditions used for restimulation of the CTL lines and not found to be directed against the mdp441 epitope. However, *in vitro* induction of CTL with a selected mdp441 peptide stimulated CTL which were able to recognise cells presenting endogenously processed mdm2 *in vivo*. There are three major explanations for this phenomenon.

Firstly, *in vitro* induction of CTL with mdp441 peptide showed that repeated restimulations were usually required to achieve high avidity mdp441 specific CTL recognising endogenously processed protein *in vivo*. This indicates that the precursor frequency of mdp441 specific high avidity CTL is low. In contrast, the precursor frequency of vaccinia virus specific CTL is very high (285). The stimulation provided by recombinant vaccinia virus might not have been sufficient to activate the low frequency mdp441 specific CTL present in the CTL repertoire.

Secondly, production of other epitope(s) derived from mdm2 or vaccinia virus may be favoured *in vivo* as it is possible to induce CTL recognising cells infected with recombinant vaccinia virus mdm2 but not recognising mdp441 peptide pulsed cells. In the

experiments described here, only peptides containing binding motifs were used for screening of CTL epitopes. Binding motifs have been found to be valuable in predicting CTL epitopes in foreign proteins including viral or tumour antigens and ovalbumin (331; 409; 410). However, the epitope recognised by mdm2 specific CTL induced by *in vivo* immunisation with VV mdm2 was not a binding motif containing peptide and there are other examples of CTL epitopes which do not contain class I binding motifs (286; 457). For this reason binding motifs should not be used exclusively to identify epitopes, but as here to supplement other methods such as immunisation with the whole protein. If the peptide(s) recognised by mdm2 specific CTL derived from *in vivo* immunisation with VV mdm2 binds better to class I and/or is more efficiently processed, then the peptide would be expected to be immunodominant (57; 426). It has previously been observed that peptides which can stimulate peptide specific CTL recognising endogenously processed protein *in vitro* might not be recognised by CTL induced by *in vivo* immunisation with the whole protein (458).

It was attempted to induce mdp441 specific CTL by *in vivo* immunisation with different adjuvants. The protocols used resulted in the generation of mdp441 specific lines which either lost specificity after short term culture or which did not have any specificity. The first type of results could be due to activation induced anergy or apoptosis of peptide specific CTL and the second type of results could be due to lack of sufficient help provided by *in vivo* immunisation. Immunisation with minigenes expressing CTL epitopes have in several cases been shown to be efficient in inducing CTL responses to poorly presented CTL epitopes such as the tumour antigen P815 aa 35-43 peptide (459) or the Influenza virus nucleoprotein aa 147-155 peptide (460). Epitopes have been shown to be presented with and without preceding ER signal sequences and the potency of individual constructs seem to depend of the epitope under study (460). Immunisation with such minigenes expressing the mdm2 p441 epitope would be interesting for future studies.

In summary, screening for class I binding motifs in the murine self proteins cyclin D1, mdm2 and WT p53 allowed for the identification of seven class I binding peptides which could induce CTL *in vitro*. One of these peptides, mdm2 peptide mdp441, induced CTL which could recognise endogenously processed mdm2 protein. The approach used has demonstrated the feasibility of the identification of low frequency high avidity CTL, which could allow for the identification of similar self protein derived peptide antigens in humans. Immunodominant epitopes from tumour self proteins and recognised by several different patients have been identified with examples including a peptide from the normal self protein MART-1 identified by TILs from 9 out of 10 melanoma patients (142), a peptide from the normal self protein gp100 recognised by 5 out of 5 CTL lines derived

from peripheral blood from five different melanoma patients (144) or a peptide from the normal self protein her2/neu recognised by 7 out of 7 TILs from breast and ovarian cancer patients (189). For clinical use, a number of different epitopes would most likely have to be identified and used to stimulate patients' CTL as CTL may recognise distinct epitopes in different patients (20) and in some cases even within the same patient (85). The CTL response may also change from recognition of one epitope to another over time.

6. Conclusion

The use of normal self proteins as targets for tumour specific cytotoxic T lymphocytes (CTL) requires first of all the presence of CTL precursors which can be stimulated to recognise and eliminate cells expressing the self protein in question. This study has used two approaches to investigate whether CTL precursors specific for four selected self proteins exist and can be activated. The four proteins are all expressed in normal tissue and found to be expressed at high levels in several forms of human cancer. One approach used *in vivo* immunisation of mice with a whole self protein expressed in such away that it would get access to the class I processing pathway. The second approach used *in vivo* or *in vitro* immunisation with peptides derived from the sequence of the self proteins and selected on the basis of class I binding motifs.

The first approach showed that it was possible to reproducibly induce CTL to cyclin D1 and mdm2 by *in vivo* immunisation with recombinant vaccinia virus expressing the protein. The second approach showed that *in vitro* priming and stimulation of CTL with synthetic peptides selected on basis of class I binding motifs, could identify one peptide from mdm2, mdp441, which could stimulate CTL recognising endogenously processed mdm2. The mdp441 peptide bound to MHC class I molecules with intermediate efficiency relative to other murine self peptides and known natural CTL epitopes derived from murine non-self proteins. The mdp441 peptide was not recognised by CTL induced by *in vivo* priming with recombinant vaccinia virus mdm2.

The findings indicate firstly that CTL precursors specific for self proteins can be activated *in vivo* or *in vitro*, secondly that there is more than one epitope derived from mdm2 which is naturally presented and able to be recognised by mdm2 specific CTL and finally that the method of CTL priming is crucial for determining which CTL epitopes are predominantly recognised by the primed CTL population.

In future experiments, it will firstly be important to determine the nature of the cyclin D1 and mdm2 epitopes recognised by cyclin D1 and mdm2 specific CTL primed *in vivo* with recombinant vaccinia virus. It will also be important to confirm that the mdp441 epitope is generated by *in vivo* processing of mdm2. The identification of the epitopes favoured by *in vivo* immunisation could be used to obtain information of the mechanisms underlying the preferential recognition of these epitopes and not epitopes such as mdm2 peptide mdp441.

Secondly, future studies should use knock-out mice lacking expression of cyclin D1, mdm2 or p53 to investigate the effects of these proteins on the T cell repertoire. Knock-out mice should be used to investigate whether the presence of a particular self protein is responsible for the predominance of self peptide specific CTL unable to recognise endogenously processed self protein such as mdp100, p53p232 and cdp41 specific CTL and the lack of generation of reproducible CTL responses to other self peptides such as p53p222 and cdp147. Cyclin D1 knock-out mice are viable and spleens from these animals have kindly been donated by Dr. V. Fantl, ICRF. Preliminary studies have already been undertaken to investigate whether cyclin D1 peptide specific CTL lines generated from splenocytes from these knock-out mice are of higher avidity than CTL lines derived from normal mice. The results so far are not clear and complicated by a shortage of mice. When the cyclin D1 knock-out colonies have expanded, these mice will hopefully be used to induce more cyclin D1 specific CTL. In addition, p53 knock-out and p53 and mdm2 double-knock-out mice are viable. It is planned to use these mice for CTL induction to p53 and mdm2 respectively.

Thirdly, it will be important to investigate the behaviour of mdm2 and cyclin D1 self specific CTL *in vivo*. The ideal target epitope for self peptide specific CTL used in immunotherapy will need to fulfil two criteria: 1) be generated in the tumour by endogenous processing of the self protein and 2) not stimulate attack on normal tissue expressing lower levels of the protein. The mdm2 or cyclin D1 epitopes recognised by CTL stimulated by *in vivo* immunisation might be more abundantly presented than subdominant epitopes such as mdp441. This can both be an advantage and disadvantage. It will be necessary to 1) investigate the ability of the different activated CTL populations to eradicate tumours *in vivo* and 2) carefully investigate the ability of the different activated CTL populations to react with normal tissue *in vivo*. Both issues can be investigated by adoptive transfer of CTL to nude mice. With respect to mdm2 and cyclin D1 specific CTL, the generated EL4 transfectants expressing mdm2 as well as the untransfected EL4 cells overexpressing cyclin D1 would be useful as tumour models.

Finally, it will be important to determine the precursor frequency of mdm2 or cyclin D1 specific CTL generated by *in vivo* immunisation as well as the precursor frequency of mdp441 specific CTL generated by *in vitro* immunisation. The precursor frequency of high avidity CTL specific for mdp441 able to recognise endogenously processed mdm2, appeared to be low, since several restimulations were necessary in order to detect those CTL. This could explain why CTL precursors specific for other mdm2 epitope(s) are preferentially activated by *in vivo* immunisation with recombinant vaccinia virus mdm2.

The present work was based on a murine model. In humans, CTL based immunotherapy using self proteins (or parts there of) as target antigens can be undertaken by either active

or passive immunisation. For both approaches, the ability to prime CTL specific for self proteins is fundamental.

Active immunisation can proceed either via a peptide based approach or by presenting the whole protein to the immune system. At present there are several such immunotherapy trials planned or already underway in humans. The peptide based approach offers the advantage of safety and the ability to stimulate large number of CTL of known specificity. A human peptide epitope equivalent to the murine mdp441 epitope is a subdominant epitope in the sense that CTL are not detected by *in vivo* immunisation with the whole protein but only by *in vitro* (or *in vivo*) immunisation with the peptide. Such subdominant epitopes have been found to be able to stimulate peptide specific CTL to lyse tumour cells expressing the whole protein from which the peptide was derived, as demonstrated in a murine model using HPV 16 peptide E7 49-57 to eradicate tumours overexpressing E7 (458). In the work described in this thesis, priming *in vivo* with mdm2 stimulated CTL which were not able to recognise the mdp441 epitope. Similarly, CTL derived from *in vivo* priming with a cell line expressing high levels of E7 were also unable to recognise the E7 49-57 peptide (458).

Priming of CTL to subdominant epitopes *in vivo* would require efficient presentation of the epitope *in vivo*. This could be achieved either by *in vivo* priming with autologous antigen presenting cells pulsed with peptide or with recombinant virus expressing the epitope but not the rest of the protein. Immunisation with recombinant virus producing peptide expressed by a minigene inserted in the virus has also shown promising results in stimulating CTL and/or causing regression of established murine tumours expressing a large number of different antigens (459; 460; 461).

Single peptides naturally presented by tumour cells have been shown to be recognised by CTL by a large number of cancer patient (142; 189). However, the CTL response of different individuals as well as within a single individual have in several cases been found to be diverse and directed against several different epitopes (20; 462). In addition the epitopes recognised within an individual protein have been found to change over time (212). The diversity and evolution of the CTL response might limit the advantage of using peptides for active immunisation. Instead whole protein can be used and is at the moment actively being used in clinical trials. Stimulation of CTL with whole protein increases the number of epitopes potentially able to be recognised by $CD8^+$ CTL and might equally provide $CD4^+$ helper T cell epitopes. Help from $CD4^+$ T cells is required for activation of $CD8^+$ CTL specific for some but not all epitopes, depending on the overall avidity of the interaction between CTL and antigen presenting cell (117; 463; 464; 465).

In the present work, recombinant vaccinia virus was used to prime CTL responses *in vivo* to self proteins. In humans, attenuated vaccinia viruses such as the Ankara (466) or Wyeth strains (288) can be used for *in vivo* immunisation. Preliminary trials at the National Cancer Institute (NCI) Naval Oncology Branch in patients with advanced metastatic cancer of recombinant vaccinia virus expressing carcinoembryonic antigen, CEA, have shown that patients were able to mount a CEA specific T cell response (288). Trials using the same construct in gastrointestinal cancer patients with minimal disease are currently under way. However, pre-existing immunity due to smallpox vaccination might limit the benefits of such vaccines (467). In mice, new viral or bacterial vectors expressing defined tumour antigens such as recombinant Listeria monocytogenes have been found to be able to cause regression of established macroscopic tumours in an antigen specific $CD8^+$ and $CD4^+$ T-cell-dependent manner (468) and will be interesting to explore in humans.

Concerns about the safety of viral or bacterial expression systems in humans, could be reduced by using alternative ways of delivering tumour proteins. Important advances in the development of methods for delivery of proteins to the class I antigen presenting pathway have recently been made. Such advances include immunisation with whole protein coupled to different carriers such as Iron oxide beads (469) and immunisation with DNA (9; 470). Several patients at the National Cancer institute (NCI) have been treated with direct injection of DNA encoding allogeneic HLA-B7 or a combination of HLA-B7 with β_2m in tumour cells in situ and shown specific immune responses to the tumour (melanoma, renal carcinoma or colorectal carcinoma) (471; 472). Preparations of heat shock proteins with bound peptide derived from intracellular proteins have also been found to prime CTL efficiently (48; 473; 474). Preparations of heat shock protein hsp96 derived from human autologous tumour cells are now in phase II clinical trials in both Europe and the U.S.(475). Immunisation of cancer patients could also be done by isolation of heat shock protein peptide complexes from allogeneic human cell lines and would offer the advantage of providing a large number of epitopes or epitope precursors not selected for HLA specificities (473).

Finally, active immunisation with genetically engineered autologous tumour cells has shown great promise in mice. As many tumour cells *in vivo* are unable to stimulate CTL despite the expression of antigens recognisable by CTL, the most relevant genetic manipulations of cancer cells are those which lead to subsequent protection against wild type tumour. Tumour cells transfected with a variety of cytokines have been shown to confer such protection (9). The effect of tumour cells expressing IL-2 and GM-CSF, is due to the involvement of $CD8^+$ T cells, whereas the mechanisms involved in the protection conferred by tumour cells expressing other cytokines such as IFN-γ and TNF-

α is not quite clear (476). Several gene therapy trials in humans based on the use of tumour cells genetically engineered to express cytokines such as IL-2, TNF-α , IFN-γ or GM-CSF have been approved by the US recombinant DNA advisory committee and in some cases initiated. These trials involve several different cancers such as brain cancer, breast cancer, colon cancer, malignant melanoma, small cell lung cancer or renal cancer (471). So far, it has been reported that neuroblastoma patients treated with tumour cells engineered to express IL-2 in a retroviral construct have shown specific as well as non-specific immune responses to the tumour (472).

The second approach used for immunotherapy of tumours is passive immunotherapy. In this case, CTL from patients are stimulated *in vitro* with autologous tumour cells or antigen in the form of cell lines expressing an identified tumour antigen or peptide epitope. CTL can either be derived from peripheral blood or from tumour masses, so-called tumour infiltrating lymphocytes (TILs). $CD8^+$ CTL derived from adoptively transferred TILs found to cause tumour regression in four melanoma patients were found to recognise specifically the melanoma self protein gp100 (19; 20). It is unknown what role gp100 plays in tumour development. The work presented in this thesis has shown that it is possible to select self proteins known to play a role in the transformation of murine and human cells and use these proteins to induce CTL. CTL directed against non-mutated oncogenes such as some of the proteins selected in the present work would be clinically more interesting than CTL directed against some random protein expressed in the tumour and possibly dispensable for the persistence of the tumour cell. At the Memorial Sloan-Kettering Cancer Center, New York, five patients with malignant lymphomas of B cell origin were treated with PBMC from HLA matched donors and the lymphomas were found to disappear. It was suggested that EBV specific CTL were responsible for tumour cell regression (477). Expression of EBV proteins is linked to the development of B cell lymphomas (185).

At NCI, Surgery Branch, tumour infiltrating lymphocytes recognising unidentified melanoma antigens have been grown *in vitro* with IL-2 and transferred back to melanoma patients, where response rates of up to 38% have been observed (18). To improve responses, trials are currently being carried out with tumour infiltrating lymphocytes transduced with the TNF-α gene. However, T lymphocytes are difficult to transduce with retroviral vectors currently used to deliver cytokine genes. At present, it appears that genetic modification of tumour cells for use in active immunotherapy is more promising (471).

Altogether, recent advances in gene technology as well as increased understanding of the mechanisms underlying the assembly and synthesis of class I peptide complexes for presentation to class I restricted CTL are encouraging for the use of active and passive

immunotherapy. The work presented in this thesis has demonstrated the ability of self proteins or peptides from self proteins to stimulate self peptide specific CTL. The existence of such self peptide specific CTL would argue for the use of normal self proteins as targets for tumour specific CTL. The main obstacle to overcome will be to avoid autoreactivity and lysis of surrounding normal tissue expressing the self protein. In a murine system, adoptively transferred T cells specific for the Freund Leukemia virus envelope protein (env) were able to eradicate a tumour expressing high levels of the env protein without causing autoimmune damage of surrounding tissue expressing lower levels of protein (203). In humans, adoptive transfer of T lymphocytes with known antigen specificity has only recently begun. Transfer of tumour infiltrating lymphocytes containing T cells specific for the self protein gp100 was able to cause regression of metastatic melanoma overexpressing gp100 without causing any damage to normal melanocytes or the eye expressing lower levels of gp100 (19). These preliminary results contribute to the conclusion reached through the work presented in this thesis that normal self proteins are suitable targets for tumour specific cytotoxic T lymphocytes.

Appendix I : Commonly used buffers and stock solutions.

All buffers used were prepared with water purified by a Millipore Reverse Osmosis system and then polished to give water at < 10 MΩ.Cm.

1 M HEPES, pH 7.0

Solution 1: 1 M Hepes (acidic)
238.3 g Hepes in 1 liter H_2O. Filtered through 0.2 µm filters
Solution 2: 1 M Hepes (basic)
260.3 g Hepes (sodium salt) in 1 liter H_2O. Filtered through 0.2 µm filters

1 volume solution 2 was mixed with 4 volumes solution 1. pH was checked and adjusted if necessary with either solution 1 or solution 2. The final solution was filtered through 0.2 µm filters.

PBSA, pH 7.2

8 g/ l (137 mM) NaCl
0.25 g/l (3 mM) KCl
1.43 g/l (10 mM) Na_2HPO_4
0.25 g /l (2 mM) KH_2PO_4

PBSB

5.336 g/l (48 mM) $CaCl_2$

PBSC

4 g/l (42 mM) $MgCl_2$

Complete PBS

400 ml PBSA
10 ml PBSB
10 ml PBSC

1 M Tris-HCl

121.1 g Tris base was dissolved in 800 ml H_2O. Concentrated HCl was used to adjust pH to the desired value. As a general guideline, 42 ml HCl was used to obtain pH 8.0, 60 ml HCl was used to obtain pH 7.6 and 70 ml HCl was used to obtain pH 7.4. The volume was adjusted to 1 liter with H_2O and the solution was sterilised by autoclaving.

1 M EDTA, pH 8.0

186.1 g ethylenediaminetetraacetate·H_2O was mixed with 800 ml H_2O under vigorous stirring. pH was adjusted to 8.0 with approximately 20 g NaOH pellets. The volume was adjusted to 1 liter and the solution was sterilised by atuoclaving.

TE buffer

10 mM Tris-HCl, pH 8.0
1 mM EDTA, pH 8.0
For 100 ml:
1.0 ml Tris-HCl, pH 8.0
0.2 ml 0.5 M EDTA, pH 8.0
H_2O up to 100 ml
Sterilisation by autoclaving.

STE buffer

10 mM Tris-HCl, pH 8.0
100 mM NaCl
1 mM EDTA , pH 8.0
For 100 ml:
1.0 ml Tris-HCl, pH 8.0
2.0 ml 5 M NaCl
0.2 ml 0.5 M EDTA, pH 8.0
H_2O up to 100 ml
Sterilisation by autoclaving.

3 M Sodium acetate, pH 5.3

408.1 g sodium acetate·$3H_2O$ was dissolved in 800 ml of H_2O. pH was adjusted to 5.3 with glacial acetic acid. The volume was adjusted to 1 liter with H_2O and the solution was sterilised by autoclaving.

20 x SSC

(3 M NaCl, 300 mM sodium citrate, pH 7.0)

175.3 g NaCl and 88.2 g of sodium citrate were dissolved in 800 ml H_2O. pH was adjusted to 7.0 with a few drops of 10 N NaOH. The volume was adjusted to 1 liter and the solution was sterilised by autoclaving.

10% (w/v) SDS

100 g SDS was dissolved in 900 ml H_2O over night. pH was adjusted to 7.2 by adding a few drops of concentrated HCl. The volume was adjusted to 1 liter with H_2O. The solution was not autoclaved.

Table I

Stock solutions of tissue culture reagents	
Reagent	**Procedure used to prepare and store stock solutions.**
BrdU	2.5 mg/ml in PBS Filtered through 0.2 μm. Stored in aliquots at + 4°C protected from light.
G418	200 mg/ml powder in PBSA. (the active concentration is 50% of the powder concentration i.e. 100 mg/ml) Filtered through 0.2 μm. Stored in aliquots at -20°C.
β-mercaptoethanol	5×10^{-3} M in IMDM medium. Filtered through 0.2 μm. Stored in aliquots at -20°C.
Mitomycin C	1 mg/ml in PBS Filtered through 0.2 μm. Stored protected from light at +4°C for 1-2 weeks maximum.
Transferrin	10 mg/ml in H_2O. Filtered through 0.2 μm. Stored in aliquots at -20°C.
X-Gal	150 mg/ml in DMSO. Made up immediately prior to use in a glass bijou protected from light. Filtration not necessary.

Appendix II: List of reagents

Reagent	Supplier	Catalogue number
Bulk chemicals		
Boric Acid	Laboratory Supplies	B/3800/53
$CaCl_2 \cdot 2H_2O$	BDH	10070
Deoxycholic acid, Sodium salt	Sigma	D5670
EDTA	BDH	10093 5V
Formaldehyde Anal^R	BDH	10113 6C
Formamide	Fissons	F/1551/7
Glucose	Sigma	G8270
HCl (1.18 specific gravity)	Fisons	H/1200/PB17
Hepes	Sigma	H9136
Hepes, sodium salt	Sigma	H0763
Hydrogen peroxide, 30% (w/w) sol	Sigma	H6520
18-hydroxyquinoline	Sigma	H-6878
KAc	Sigma	P-1147
KCl	BDH	10148 4C
KH_2PO_4	BDH	10203
$MgCl_2 \cdot 6\ H_2O$	BDH	0149 4V
$MgSO_4 \cdot 7H_2O$		
MOPS	Sigma	M-1254
NaAc	Fisons Analytics	S/2120/53
$NaHCO_3$	Sigma	S-4019
Na_2HPO_4	BDH	10249 4C
NaOH	BDH	10252 4X
NH_4Cl	BDH	10017
SDS	BDH	44244 4H
Trifluoroacetic acid	BDH	14083
Trizma Base	Sigma	T-1503
Restriction enzymes		
BamHI (20,000 u/ml)	Pharmacia	27-0868-04
EcoRI (25,000 u/ml)	Pharmacia	27-0854-04
Eco72I (12,000 u/ml)	Promega	R6981
HindIII (18,000 u/ml)	Pharmacia	27-0860-02
NotI (20,000 u/ml)	Pharmacia	27-0976-01
PstI (15,000 u/ml)	Pharmacia	27-0886-03

Hybond N membranes	Amersham	RPN203N
Lambda DNA HindIII DNA ladder	Gibco BRL	5612SA
Mineral oil	Sigma	M5904
Nick colums Sephadex G50	Pharmacia	17-0855-01
N-Lauroylsarcosine	Sigma	L-9150
Nonidet P-40	Sigma	N0896
One-Phor-All buffer PLUS	Pharmacia	27-0901
pd(N)6 (random hexamers)	Pharmacia	27-2166-01
Phenol (ultra pure)	Gibco BRL	540-5509UA
Protogel (30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide)	National Diagnostics	EC 890
Qiagen plasmid maxi kit	Quiagen Gmbh	12162
RNA ladder (0.24-9.5 kb)	Gibco BRL	15620-016
Salmon sperm DNA	Sigma	D1626
Taq reaction buffer	Advanced Biotechn.	0242
Tetracycline hydrocloric acid	Sigma	T-3383
Total RNA isolation reagent	Advanced Biotechn.	AB-0303

Tissue culture and immunological reagents

Agarose type VII (low melting point)	Sigma	A-9046
Arklone	ICI	MDL 3232/1
β–mercaptoethanol (tissue culture tested)	Sigma	M7522
5-bromo-2-deoxyuridine	Sigma	B-5002
Crystal violet	BDH	42553
Dimethyl sulfoxide	BDH	10323
Geniticin sulphate (G418)	Gibco BRL	0666-1811
IL-2 (human recombinant) 10,000 u/ml	Boehringer	1011 456
Incomplete Freund's Adjuvant	Bacto	0639-60-6
INF-γ (mouse recombinant) 200,000 u/ml	Genzyme	MG-IFN
Metrizamide, grade I	Sigma	M3383
Mitomycin C	Sigma	M-0303
$NaHCO_3$ (Hybri-max cell culture tested)	Sigma	S4019
Protein A Sepharose CL-4B	Pharmacia	17-0780-01
Rabbit serum (normal)	DAKO	X902
Trypan blue (0.4% solution)	Sigma	T 8154
X-Gal	Gibco BRL	5520 UC

Plastic ware

25 cm^2 tissue culture flasks	Falcon	3013
75 cm^2 tissue culture flasks	Falcon	3024
175 cm^2 tissue culture flasks	Falcon	3028
6 well tissue culture treated culture plates	Falcon	3046
24 well tissue culture treated culture plates	Falcon	3047
96 well, U-bottom, tissue culture treated culture plates	Falcon	3077
96 well, U-bottom, non-tissue culture treated plates	Falcon	3910
Polypropylene tubes, 50 ml	Falcon	2070
Universal containers, 30 ml	Sterilin	128 A
Bijou containers, 7 ml	Sterilin	129 A

References

1. **Roitt, I.** (1994) "*Essential Immunology*". 8th ed., Blackwell Scientific Publications, Oxford, UK.

2. **Kuby, J.** (1994) "*Immunology*". 2nd ed., W.H. Freeman and Company, New York.

3. **Kärre, K.** (1991) *"MHC gene control of the natural killer system at the level of the target and the host."* Semin Cancer Biol 2 : 295-309.

4. **Janeway, C.A.** (1992) *"The immune system evolved to discriminate infectious nonself from noninfectious self."* Immunol Today 13 : 13-17.

5. **Roth, C., C. Rochlitz, and P. Kourilsky.** (1994) *"Immune responses against tumours."* Adv Immunol 57 : 281-351.

6. **Melief, C.J.M.** (1992) *"Tumor eradication by adoptive transfer of cytotoxic T lymphocytes."* Adv Cancer Res 58 : 143-175.

7. **Kedar, E. and E. Klein.** (1992) *"Cancer immunotherapy: are the results discouraging?Can they be improved?"* Adv Cancer Res 59 : 245-322.

8. **Greenberg, P.D.** (1991) *"Adoptive T cell therapy of tumours: mechanisms operative in the recognition and elimination of tumor cells."* Adv Immunol 49 : 281-355.

9. **Dranoff, G. and R.C. Mulligan.** (1995) *"Gene transfer as cancer therapy."* Adv Immunol 58 : 417-454.

10. **Cavallo, F., A. Martin-Fontecha, M. Bellone, S. Heltai, E. Gatti, P. Tornaghi, M. Freschi, G. Forni, P. Dellabona, and G. Casoratti.** (1995) *"Co-expression of B7-1 and ICAM-1 on tumors is required for rejection and the establishment of a memory response."* Eur J Immunol 25 : 1154-1162.

11. **Leibson, P.J.** (1995) *"MHC-recognising receptors: they're not just for T cells any more."* Immunity 3 : 5-8.

12. **Hank, J.A., M.R. Albertini, J. Schiller, and P.M. Sondel.** (1993) *"Activation of multiple effector mechanisms to enhance tumor immunotherapy."* J Immunother 14 : 329-335.

13. **Hara, I., Y. Takechi, and A.N. Houghton.** (1995) *"Implicating a role for immune recognition of self in tumour rejection: passive immunisation against the brown locus."* J Exp Med 182 : 1609-1614.

14. **Goldenberg, D.M., S.M. Larson, R.A. Reisfeld, and J. Schlom.** (1995) *"Targeting cancer with radiolabeled antibodies."* Immunol Today 16 : 261-264.

15. **Houghton, A.N., S. Vijaysaradhi, B. Bouchard, C. Naftzger, I. Hara, and P.B. Chapman.** (1992) *"Recognition of autoantigens by patients with melanoma."* Ann NY Acad Sci 690 : 59-68.

16. **Fleuren, G.J., A. Gorter, P.J.K. Kuppen, S. Litvinov, and S.O. Warnaar.** (1995) *"Tumor heterogeneity and immunotherapy of cancer."* Immunol Rev 145 : 91-121.

17. **Van den Eynde, B. and V.G. Brichard.** (1995) *"New tumor antigens recognized by T cells."* Curr Opin Immunol 7 : 674-681.

18. **Hwu, P. and S.A. Rosenberg.** (1995)*"The use of gene modified tumor-infiltrating lymphocytes for cancer therapy."* Ann NY Acad Sci 716 : 188-199.

19. **Kawakami, Y., S. Eliyahu, C. H. Delgado, P. F. Robbins, K. Sakaguchi, E. Appella, J. R. Yannelli, G. J. Adema, T. Miki, and S. A. Rosenberg.** (1994)*"Identification of a human-melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in-vivo tumor rejection."* Proc Natl Acad Sci USA 91 : 6458-6462.

20. **Kawakami, Y., S. Eliyahu, C. Jennings, K. Sakaguchi, X.Q. Kang, S. Southwood, P.F. Robbins, A. Sette, E. Appella, and S.A. Rosenberg.** (1995)*"Recognition of multiple epitopes in the human-melanoma antigen gp100 by tumor-infiltrating T-lymphocytes associated with in-vivo tumor-regression."* J Immunol 154 : 3961-3968.

21. **Zinkernagel, R.M. and P.C. Doherty.** (1979)*"MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T cell restriction-specificity, function, and responsiveness."* Adv Immunol 27 : 1-51.

22. **Townsend, A.R.M., J. Rothbard, F.M. Gotch, G. Bahadur, D. Wraith, and A.J. McMichael.** (1986)*"The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides."* Cell 44 : 959-968.

23. **Björkman, P.J. and P. Parham.** (1990)*"Structure, function, and diversity of class I major histocompatibility complex molecules."* Annu Rev Biochem 59 : 253-288.

24. **Pullen, J.K., R.M. Horton, Z. Cai, and L.R. Pease.** (1992)*"Structural diversity of the classical H-2 genes: K,D, and L."* J Immunol 148 : 953-967.

25. **Engelhard, V.H.** (1994)*"Structure of peptides associated with MHC Class I molecules."* Curr Opin Immunol 6 : 13-23.

26. **Björkman, P.J., M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, and D.C. Wiley.** (1987)*"Structure of the human class I histocompatibility antigen, HLA-A2."* Nature 329 : 506-12.

27. **Madden, D.R.** (1995)*"The three-dimensional structure of peptide-MHC complexes."* Annu Rev Immunol 13 : 587-622.

28. **van Bleek, G.M. and S.G. Nathenson.** (1990)*"Isolation of an endogenously processed immunodominant viral peptide from the class I H-2K^b molecule."* Nature 348 : 213-216.

29. **Hunt, D.F., R.A. Henderson, J. Shabanowitz, K. Sakaguchi, H. Michel, N. Sevilir, A.L. Cox, E. Appella, and V.H. Engelhard.** (1992)*"Characterization of peptides bound to class I MHC Molecule HLA-A2.1 by mass spectrophotometry."* Science 255 : 1261-1263.

30. **Henderson, R.A., H. Michel, K. Sakaguchi, J. Shabanowitz, E. Appella, D.F. Hunt, and V.H. Engelhard.** (1992)*"HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation."* Science 255 : 1264-1266.

31. **Falk, K., O. Rötzschke, S. Stevanovic, G. Jung, and H.G. Rammensee.** (1991)*"Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules."* Nature 351 : 290-296.

32. **Young, A.C.M., W. Zhang, J.C. Sacchettini, and S.G. Nathenson.** (1994) *"The three-dimensional structure of H-2D^b at 2.4 Å resolution: implications for antigen-determinant selection."* Cell 76 : 39-50.

33. **Kubo, R.T., A. Sette, H.M. Grey, E. Appella, K. Sakaguchi, N.Z. Zhu, D. Arnott, N. Sherman, J. Shabanowitz, H. Michel, W.M. Bodnar, T.A. Davis, and D.F. Hunt.** (1994) *"Definition of specific peptide motifs for 4 major HLA-A alleles."* J Immunol 152 : 3913-3924.

34. **Falk, K. and O. Rötzschke.** (1993) *"Consensus motifs and peptide ligands of MHC class I molecules."* Semin Immunol 5 : 81-94.

35. **Ljunggren, H-G., N.J. Stam, C. Öhlen, J.J. Neefjes, P. Höglund, M-T. Heemels, J. Bastin, T.N.M. Schumacher, A. Townsend, K. Kärre, and H. Ploegh.** (1990) *"Empty MHC class I molecules come out in the cold."* Nature 346 : 476-480.

36. **Schumacher, T.N., M.-T. Heemels, J.J. Neefjes, W.M. Kast, C.J.M. Melief, and H.L. Ploegh.** (1990) *"Direct binding of peptide to empty MHC class I molecules on intact cells and in vitro."* Cell 62 : 563-567.

37. **Elliott, T., V. Cerundolo, J. Elvin, and A. Townsend.** (1991) *"Peptide-induced conformational change of the class I heavy chain."* Nature 351 : 402-406.

38. **Townsend, A., T. Elliott, V. Cerundolo, L. Foster, B. Barber, and A. Tse.** (1990) *"Assembly of MHC Class I molecules analyzed in vitro."* Cell 62 : 285-295.

39. **Germain, R.N.** (1995) *"The biochemistry and cell biology of antigen presentation by MHC class-I and class-II molecules - implications for development of combination vaccines."* Ann NY Acad Sci 754 : 114-125.

40. **Carreno, B.M., J.C. Solheim, M. Harris, I. Stroynowski, J.M. Connolly, and T.H. Hansen.** (1995) *"TAP associates with a unique class I conformation, whereas calnexin associates with multiple class I forms in mouse and man."* J Immunol 155 : 4726-4733.

41. **Scott, J.E. and J.R. Dawson.** (1995) *"MHC Class I expression and transport in a calnexin deficient cell line."* J Immunol 155 : 143-148.

42. **Roche, P.A.** (1995) *"HLA-DM: an in-vivo facilitator of MHC Class II peptide loading."* Immunity 3 : 259-262.

43. **Martinez-Kinader, B., G.B. Lipford, H. Wagner, and K. Heeg.** (1995) *"Sensitization of MHC class I-restricted T-cells to exogenous proteins - evidence for an alternative class I-restricted antigen presentation pathway."* Immunology 86 : 287-295.

44. **Harding, C.V. and R. Song.** (1994) *"Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class I MHC molecules."* J Immunol 153 : 4925-4933.

45. **Kovacsovics-Bankowski, M. and K.L. Rock.** (1994) *"Presentation of exogenous antigens by macrophages: analysis of major histocompatibility complex class I and II presentation and regulation by cytokines."* Eur J Immunol 24 : 2421-2428.

46. **Kovacsovics-Bankowski, M. and K.L. Rock.** (1995) *"A phagosome to cytosol pathway for exogenous antigen presented on MHC Class I molecules."* Science 267 : 243-245.

47. **Reis e Sousa, C. and R.N. Germain.** (1995)*"Major histocompatibility complex class I presentation of peptides derived from soluble exogenous antigen by a subset of cells engaged in phagocytosis."* J Exp Med 182 : 841-851.

48. **Suto, R. and P.K. Srivastava.** (1995)*"A mechanism for the specific immunogenicity of heat-shock protein-chaperoned peptides."* Science 269 : 1585-1588.

49. **Goldberg, A. and K. Rock.** (1992)*"Proteolysis, proteasomes and antigen presentation."* Nature 357 : 375-379.

50. **Goldberg, A.L.** (1995)*"Functions of the proteasome: the lysis at the end of the tunnel."* Science 268 : 522-523.

51. **Akiyama, K., K. Yokota, S. Kagawa, N. Shimbara, T. Tamura, H. Akioka, H.G. Nothwang, C. Noda, K. Tanaka, and A. Ichihara.** (1994)*"cDNA cloning and interferon γ down regulation of proteasomal subunits X and Y."* Science 265 : 1231-1234.

52. **Gaczynska, M., K.L. Rock, T. Spies, and A. Goldberg.** (1994)*"Peptidase activities of proteasomes are differentially regulated by the major histocompatibility complex- encoded genes for LMP2 and LMP7."* Proc Natl Acad Sci USA 91 : 9213-9217.

53. **Vinitsky, A., C. Cardozo, L. Sepp-Lorenzio, C. Michaud, and M. Orlowski.** (1995)*"Inhibition of the proteolytic activity of the multicatalytic proteinase complex (proteasome) by substrate-related peptidyl aldehydes."* J Biol Chem 269 : 29860-29866.

54. **Rock, K.L., C. Gramm, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang, and A.L. Goldberg.** (1994)*"Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules."* Cell 78 : 761-771.

55. **Fenteany, G., R.F. Standaert, W.S. Lane, S. Choi, E.J. Corey, and S.L. Schreiber.** (1995)*"Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin."* Science 268 : 726-731.

56. **Eggers, M., B. Boes-Fabian, T. Ruppert, P.-M. Kloetzel, and U.H. Koszinowski.** (1995)*"The cleavage preference of the proteasome governs the yield of antigenic peptides."* J Exp Med 182 : 1865-1870.

57. **Niedermann, G., S. Butz, H.G. Ihlenfeldt, R. Grimm, M. Lucchiari, H. Hoschützky, G. Jung, B. Maier, and K. Eichmann.** (1995)*"Contribution of proteasome-mediated proteolysis to the hierarchy of epitopes presented by major histocompatibility complex class I molecules."* Immunity 2 : 289-299.

58. **Fehling, H.J., W. Swat, C. Laplace, R. Kuhn, K. Rajewsky, U. Muller, and H. von Boehmer.** (1994)*"MHC Class I expression in mice lacking the proteasome subunit LMP-7."* Science 265 : 1234-1237.

59. **van Kaer, L., P.G. Ashton-Rickardt, M. Eichelberger, M. Gaczynska, K. Nagashima, K.L. Rock, A.L. Goldberg, P.C. Doherty, and S. Tonegawa.** (1994)*"Altered peptidase and viral-specific T cell responses in LMP2 mutant mice."* Immunity 1 : 533-541.

60. **Yewdell, J., C. Lapham, I. Bacik, T. Spies, and J. Bennink.** (1994)*"MHC-encoded proteasome subunits LMP2 and LMP7 are not required for efficient antigen presentation."* J Immunol 152 : 1163-1170.

61. **Grant, E.P., M.T Michalek, A.L. Goldberg, and K.L. Rock.** (1995)*"Rate of antigen degradation by the ubuiquitin-proteasome pathway influences MHC Class I presentation."* J Immunol 155 : 3750-3758.

62. **Shepherd, J.C., T.N.M. Schumacher, P.G. Ashton-Rickardt, S. Imaeda, H.L. Ploegh, C.A. Janeway, and S. Tonegawa.** (1993)*"TAP1-Dependent peptide translocation in vitro is ATP dependent and peptide selective."* Cell 74 : 577-584.

63. **Androlewicz, M.J., B. Ortmann, P.M. Vanendert, T. Spies, and P. Cresswell.** (1994)*"Characteristics of peptide and major histocompatibility complex class-I beta(2)-microglobulin binding to the transporters associated with antigen-processing (tap1 and tap2)."* Proc Natl Acad Sci USA 91 : 12716-12720.

64. **Ortmann, B., M.J. Androlewicz, and P. Cresswell.** (1994)*"MHC class I/β2 microglobulin complexes associate with TAP transporters before peptide binding."* Nature 368 : 864-867.

65. **Suh, W.-K., M.F. Cohen-Doyle, K. Fruh, K. Wang, P.A. Peterson, and D.B. Williams.** (1994)*"Interaction of MHC Class I molecules with the transporter associated with antigen processing."* Science 264 : 1322-1326.

66. **Grandea, A.G., M.J. Androlewicz, R.S. Athwal, D.E. Geraghty, and T. Spies.** (1995)*"Dependence of peptide binding by MHC class-I molecules on their interaction with tap."* Science 270 : 105-108.

67. **Androlewicz, M.J. and P. Cresswell.** (1994)*"Human transporters associated with antigen processing possess a promiscuous peptide-binding site."* Immunity 1 : 7-14.

68. **van Endert, P.M., D. Riganelli, G. Greco, K. Fleischhauer, J. Sidney, A. Sette, and J.-F. Bach.** (1995)*"The peptide-binding motif for the human transporter associated with antigen processing."* J Exp Med 182 : 1883-1895.

69. **Momburg, F., J. Roelse, J.C. Howard, G.W. Butcher, G.J. Hämmerling, and J.J. Neefjes.** (1994)*"Selectivity of MHC encoded peptide transporters from human, mouse and rat."* Nature 367 : 648-651.

70. **Schumacher, T.N.M., D.V. Kantesairia, M.-T. Heemels, P.G. Ashton-Rickardt, J.C. Shepherd, K. Fruh, Y. Yang, P.A. Peterson, S. Tonegawa, and H.L. Ploegh.** (1994)*"Peptide length and sequence specificity of the mouse TAP1/TAP2 transporter."* J Exp Med 179 : 533-540.

71. **Neisig, A., J. Roelse, A.J.A.M. Sijts, F. Ossendorp, M.C.W. Feltkamp, W.M. Kast, C.J.M. Melief, and J.J. Neefjes.** (1995)*"Major differences in transporter associated with antigen presentation (TAP)-dependent translocation of MHC-class I-presentable peptides and the effect of flanking sequences."* J Immunol 154 : 1273-1279.

72. **Roelse, J., M. Grommé, F. Momburg, G. Hämmerling, and J. Neefjes.** (1994)*"Trimming of TAP-translocated peptides in the endoplasmatic reticulum and in the cytosol during recycling."* J Exp Med 180 : 1591-1597.

73. **Elliott, T., A. Willis, V. Cerundolo, and A. Townsend.** (1995)*"Processing of major histocompatibility class I-restricted antigens in the endoplasmic reticulum."* J Exp Med 181 : 1481-1491.

74. **Gournier, H., S. Pascolo, C.-A. Siegrist, J. Jehan, B. Perarnau, Z. Garcia, T. Rose, J. Neefjes, and F.A. Lemonnier.** (1995)*"Restriction of self-antigen presentation to cytolytic T lymphocytes by mouse peptide pumps."* Eur J Immunol 25 : 2019-2026.

75. **Obst, R., E.A. Armandola, M. Nijenhuis, F. Momburg, and G.J. Hämmerling.** (1995)*"TAP polymorphism does not influence the transport of peptide variants in mice and humans."* Eur J Immunol 25 : 2170-2176.

76. **Schumacher, T.N.M., D.V. Kantesaria, D.V. Serreze, D.C. Roopenian, and H.L. Ploegh.** (1994)*"Transporters from H-2^b, H-2^d, H-2^s, H-2^k, and H-2^{g7} (NOD/lt) haplotype translocate similar sets of peptides."* Proc Natl Acad Sci USA 91 : 13004-13008.

77. **Villanueva, M.S., P. Fischer, K. Feen, and E.G. Pamer.** (1995)*"Efficiency of MHC Class I Antigen Processing: A quantitative Analysis."* Immunity 1 : 479-489.

78. **Moss, P.A.H., W.M.C. Rosenberg, and J.I. Bell.** (1992)*"The human T cell receptor in health and disease."* Annu Rev Immunol 10 : 71-96.

79. **Tanaka, Y., C.T. Morita, Y. Tanaka, E. Nieves, M.B. Brenner, and B.B. Bloom.** (1995)*"Natural and synthetic non-peptide antigens recognized by human $\gamma\delta$ T cells."* Nature 375 : 155-158.

80. **Jorgensen, J.L., P.A. Reay, E.W. Ehrich, and M.M. Davis.** (1992)*"Molecular components of T-cell recognition."* Annu Rev Immunol. 10 : 835-873.

81. **Sun, R., S.E. Shepherd, S.S. Geier, C.T. Thomson, J.M. Shell, and S.G. Nathenson.** (1995)*"Evidence that the antigen receptors of cytotoxic T lymphocytes interact with a common recognition pattern on the H-2K^b molecule."* Immunity 3 : 573-582.

82. **Bluestone, J.A., S. Jameson, S. Miller, and R. Dick II.** (1992)*"Peptide-induced conformational changes in class I heavy chains alter major histocompatibility complex recognition."* J Exp Med 176 : 1757-1761.

83. **Catipovic, B., J. Dal Porto, M. Mage, T.E. Johansen, and J.P. Schneck.** (1992)*"Major histocompatibility complex conformational epitopes are peptide specific."* J Exp Med 176 : 1611-1618.

84. **Fremont, D.H., M. Matsumura, E.A. Stura, and P.A. Peterson.** (1992)*"Crystal structures of two viral peptides in complex with murine MHC Class I K^b."* Science 257 : 919-927.

85. **Romero, P., C. Pannetier, J. Herman, C.V. Jongeneel, J.-C. Cerotti, and P.G. Coulie.** (1995)*"Multiple specificities in the repertoire of a melanoma patient's cytolytic T lymphocytes directed against tumor antigen MAGE-1.A1."* J Exp Med 182 : 1019-1028.

86. **Sykulev, Y., A. Brunmark, T.J. Tsomides, S. Kageyama, M. Jackson, P.A. Peterson, and H.A. Eisen.** (1994)*"High-affinity reactions between antigen-specific T-cell receptors and peptides associated with allogeneic and syngeneic major histocompatibility complex proteins."* Proc Natl Acad Aci USA 91 : 11487-11491.

87. **Wu, M.X., T.J. Tsomides, and H.N. Eisen.** (1995)*"Tissue distribution of natural peptides derived from a ubiquitous dehydrogenase, including a novel liver-*

specific peptide that demonstrates the pronounced specificity of low-affinity T-cell reactions." J Immunol 154 : 4495-4502.

88. **Kageyama, S., T.J. Tsomides, Y. Sykulev, and H.N. Eisen.** (1995)*"Variations in the number of peptide-MHC Class I complexes required to activate cytotoxic T cell responses."* J Immunol 154 : 567-576.

89. **Valitutti, S., S. Müller, M. Cella, E. Padovan, and A. Lanzavecchia.** (1995)*"Serial triggering of many T-cell receptors by a few peptide-MHC complexes."* Nature 375 : 148-151.

90. **Evavold, B.D., J. Sloan-Lancaster, K.J. Wilson, J.B. Rothbard, and P.M. Allen.** (1995)*"Specific T cell recognition of minimally homologous peptides: evidence for multiple endogenous ligands."* Immunity 2 : 655-663.

91. **Wucherpfennig, K.W. and J.L. Strominger.** (1995)*"Molecular mimicry in T cell-mediated autoimmunity:viral peptides activate human T cell clones specific for myelin basic protein."* Cell 80 : 695-705.

92. **Quaratino, S., C.J. Thrope, P.J. Travers, and M. Londei.** (1995)*"Similar antigenic surfaces, rather than sequence homology, dictate T-cell epitope mimicry."* Proc Natl Acad Sci USA 92 : 10398-10402.

93. **Croft, M.** (1994)*"Activation of naive, memory and effector T cells."* Curr Opin Immunol 6 : 431-437.

94. **Zamoyska, R.** (1994)*"The CD8 coreceptor revisited: one chain good, two chains better."* Immunity 1 : 243-246.

95. **Sun, J., D.J. Leahy, and P.B. Kavathas.** (1995)*"Interaction between CD8 and major histocompatibility complex (MHC) Class I mediated by multiple contact surfaces that include the α2 and α3 domains of MHC Class I molecules."* J Exp Med 182 : 1275-1280.

96. **Samberg, N.L., E.C. Scarlett, and H.J. Stauss.** (1989)*"The α3 domain of major histocompatibility complex class I molecules plays a critical role in cytotoxic T lymphocyte activation."* Eur J Immunol 19 : 2349-2354.

97. **Rourke, A.M. and M.F. Mescher.** (1993)*"The roles of CD8 in cytotoxic T lymphocyte function."* Immunol Today 14 : 183-188.

98. **Mescher, M.F.** (1995)*"Molecular interactions in the activation of effector and precursor cytotoxic T lymphocytes."* Immunol Rev 146 : 177-210.

99. **Cai, Z. and J. Sprent.** (1994)*"Resting and activated T cells display different requirements for CD8 molecules."* J Exp Med 179 : 2005-2015.

100. **Alexander, M.A., C.A. Damico, K.M. Wieties, T.H. Hansen, and J.M. Connolly.** (1991)*"Correlation between CD8 dependency and determinant densisty using peptide-induced, L^d -restricted cytotoxic T lymphocytes."* J Exp Med 173 : 849-858.

101. **Linsley, P.S. and J.A. Ledbetter.** (1993)*"The role of the CD28 receptor during T cell responses to antigen."* Annu Rev Immunol 11 : 191-212.

102. **Bluestone, J.A.** (1995)*"New perspectives of CD28-B7-mediated T cell costimulation."* Immunity 2 : 555-559.

103. **Asuma, M., M. Cayabyab, J.H. Philips, and L.L. Lanier.** (1993)*"Requirements for the CD8 dependent T cell- mediated cytotoxicity."* J Immunol 150 : 2091-2101.

104. **Harding, F.A. and J.P. Allison.** (1993)*"CD28-B7 interactions allow the induction of CD8$^+$ cytotoxic T lymphocytes in the absence of exogenous help."* J Immunol 177 : 1791-1796.

105. **Chen, L., P. McGowan, P. Ashe, S. Johnston, Y. Li, I. Hellström, and K.E. Hellström.** (1994)*"Tumour immunogenicity determines the effect of B7 co-stimulation on T cell mediated tumor immunity."* J Exp Med 179 : 523-532.

106. **Hellström, K. E., I. Hellström, and L. Chen.** (1995)*"Can co-stimulated tumour immunity be therapeutically efficacious?"* Immunol Rev 145 : 121-143.

107. **Chen, L., S. Ashe, W.A. Brady, I. Hellström, P. McGowan, and P.S. Linsley.** (1992)*"Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4."* Cell 71 : 1093-1102.

108. **Wu, T.-C., A.Y.C. Huang, E.M. Jaffee, H.I. Levitsky, and D.M. Pardoll.** (1995)*"A reasssment of the role of B7-1 expression in tumour rejection."* J Exp Med 182 : 1415-1421.

109. **Guerder, S., J. Meyerhoff, and R. Flavell.** (1994)*"The role of the T-cell costimulator B7-1 in autoimmunity and the induction and maintenance of tolerance to peripheral antigen."* Immunity 1 : 155-166.

110. **Biron, C.A.** (1994)*"Cytokines in the generation of immune responses to, and resolution of, virus infection."* Curr Opin Immunol 6 : 530-538.

111. **Le Gros, G. and F. Erard.** (1994)*"Non-cytotoxic, IL-4, IL-5, IL-10 producing CD8$^+$ T cells: their activation and effector function."* Curr Opin Immunol 6 : 453-457.

112. **Finkelman, F.D.** (1995)*"Relationships among antigen presentation, cytokines, immune deviation, and autoimmune disease."* J Exp Med 182 : 279-282.

113. **Paul, W.E. and R.A. Seder.** (1994)*"Lymphocyte responses and cytokines."* Cell 76 : 241-251.

114. **Trinchieri, G.** (1995)*"Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific immunity."* Annu Rev Immunol 13 : 251-276.

115. **Erard, F., P. Corthesy, M. Nabholz, J.W. Lowenthal, P. Zaech, G. Plaetnick, and H.R. MacDonald.** (1985)*"Interleukin 2 is both necessary and sufficient for the growth and differentation of lectin-stimulated cytolytic T lymphocyte precursors."* J Immunol 134 : 1644-1652.

116. **Stuhler, G. and P. Walden.** (1993)*"Collaboration of helper and cytotoxic T lymphocytes."* Eur J Immunol 23 : 2279-2286.

117. **Heath, W.R., L. Kjer-Nielsen, and M.W. Hoffman.** (1993)*"Avidity for antigen can influence the helper dependence of CD8 T lymphocytes."* J Immunol 151 : 5993-6001.

118. **Young, J.W. and R.M. Steinman.** (1990)*"Dendritic cells stimulate primary human cytolytic lymphocyte responses in the absence of CD4$^+$ helper T cells."* J Exp Med 171 : 1315-1332.

119. **Boog, C.J.P., J. Boes, and C.J.M. Melief.** (1988)*"Stimulation with dendritic cells decreases or obviates the CD4$^+$ helper cell requirement in cytotoxic T cell responses."* Eur J Immunol 18 : 219-223.

120. **Kisielow, P. and H. von Boehmer.** (1995)*"Development and selection of T cells: facts and puzzles."* Adv Immunol 58 : 87-209.

121. **O'Garra, A. and K. Murphy.** (1994)*"Role of cytokines in determining T-lymphocyte function."* Curr Opin Immunol 6 : 458-466.

122. **Openshaw, P., E.E. Murphy, N.A. Hosken, V. Maino, K. Davis, K. Murphy, and A. O'Garra.** (1995)*"Heterogeneity of intracellular cytokine synthesis at the single cell level in polarized T helper 1 and T helper 2 populations."* J Exp Med 182 : 1357-1367.

123. **Okamura, H., H. Tsutsui, T. Komatsu, M. Yutsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, K. Hattori, K. Akita, M. Namba, F. Tanabe, K. Konishi, S. Fukuda, and M. Kurimoto.** (1995)*"Cloning of a new cytokine that induces IFN-γ production by T cells."* Nature 378 : 88-91.

124. **Freeman, G.J., V.A. Boussiotis, A. Anumanthan, G.M. Bernstein, X-Y. Ke, P.D. Rennert, G.S. Gray, J.G. Gribben, and L.M. Nadler.** (1995)*"B7-1 and B7-2 do not deliver identical costimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4."* Immunity 2 : 523-532.

125. **Kumar, V., V. Bhardwaj, L. Soares, J. Alexander, A. Sette, and E. Sercarz.** (1995)*"Major histocompatibility complex binding affinity of an antigenic determinant is crucial for the differential secretion of interleukin 4/5 or interferon γ by T cells."* Proc Natl Acad Sci USA 92 : 9510-9514.

126. **Rudd, C.E., O. Janssen, Y.-C. Cai, A.J. da Silva, M. Raab, and K.V.S. Prasad.** (1994)*"Two-step TCRζ/CD3-CD4 and CD28 signaling in T cells: SH2/SH3 domains, protein tyrosine and lipid kinases."* Immunol Today 15 : 225-234.

127. **Robey, E. and J.P. Allison.** (1995)*"T-cell activation: integration of signals from the antigen receptor and costimulatory molecules."* Immunol Today 16 : 306-310.

128. **Sloan-Lancaster, J., A.S. Shaw, J.B. Rothbard, and P.M. Allen.** (1994)*"Partial T cell signaling: altered phospho-ζ and lack of Zap70 recruitment in APL-induced T cell anergy."* Cell 79 : 913-922.

129. **Berke, G.** (1994)*"The binding and lysis by cytotoxic T lymphocytes: molecular and cellular aspects."* Annu Rev Immunol 12 : 735-773.

130. **Berke, G.** (1995)*"The CTL's kiss of death."* Cell : 9-12.

131. **Müller, C. and J. Tschopp.** (1994)*"Resistance of CTL to perforin-mediated lysis."* J Immunol 153 : 2470-2478.

132. **Griffiths, G.M.** (1995)*"The cell biology of CTL killing."* Curr Opin Immunol 7 : 343-348.

133. **Kägi, D., B. Lederman, K. Bürki, R.M. Zinkernagel, and H. Hengartner.** (1995) *"Lymphocyte-mediated cytotxicity in vitro and in vivo: Mechanisms and significance."* Immunol Rev 146 : 95-115.

134. **Cao, W., S.S. Tykodi, M.T. Esser, V.L. Braciale, and T.J. Braciale.** (1995) *"Partial activation of $CD8^+$ T cells by a self-derived peptide."* Nature 378 : 295-298.

135. **Clark, W.R., C.M. Walsh, A.A. Glass, F. Hayashi, M. Matloubian, and R. Ahmed.** (1995) *"Molecular pathways of CTL-mediated cytotoxicity."* Immunol Rev 146 : 33-44.

136. **Sugarman, B.J., B.B. Aggarwal, P.E. Hass, I.S. Figari, M.A. Palladino, and H.M. Shephard.** (1985) *"Recombinant human tumour necrosis factor-α: effects on proliferation of normal and transformed cells in vitro."* Science 230 : 943-945.

137. **Yamamoto, N., J.-P. Zou, X.-F. Li, H. Takenaka, S. Noda, T. Fujii, S. Ono, Y. Kobayashi, N. Mukaida, K. Matsushima, H. Fujiwara, and T. Hamaoka.** (1995) *"Regulatory mechanisms for production of IFN-γ and TNF by antitumor T cells or macrophages in the tumor-bearing state."* J Immunol 154 : 2281-2290.

138. **Barth, R.J., J.J. Mulé, P.J. Spiess, and S.A. Rosenberg.** (1991) *"Interferon γ and tumour necrosis factor have a role in tumour regressions mediated by murine $CD8^+$ tumour infiltrating lymphocytes."* J Exp Med 173 : 647-658.

139. **Kägi, D., B. Ledermann, K. Bürki, P. Seiler, B. Odermatt, K.J. Olsen, and E.R. Podack.** (1994) *"Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice."* Nature 369 : 31-37.

140. **Mokyr, M.B., A. Prokhovorova, M. Rubin, and J.A. Bluestone.** (1994) *"Insight into the mechanism of TCR-$V\beta8^+$/$CD8^+$ T cell-mediated MOPC-315 tumor eradication."* J Immunol 153 : 3123-3134.

141. **Kawakami, Y., S. Eliyahu, C.H. Delgado, P.F. Robbins, L. Rivoltini, S.L. Topalian, T. Miki, and S.A. Rosenberg.** (1994) *"Cloning of the gene coding for a shared human-melanoma antigen recognized by autologous T-cells infiltrating into tumor."* Proc Natl Acad Sci USA 91 : 3515-3519.

142. **Kawakami, Y., S. Eliyahu, K. Sakaguchi, P.F. Robbins, L. Rivoltini, J.R. Yannelli, E. Appella, and S.A. Rosenberg.** (1994) *"Identification of the immunodominant peptides of the mart-1 human-melanoma antigen recognized by the majority of HLA-A2-restricted tumor-infiltrating lymphocytes."* J Exp Med 180 : 347-352.

143. **Castelli, C., W.J. Storkus, M.J. Mauerer, D.M. Martin, E.C. Huang, B.N. Pramanik, T.L. Nagabhushan, G. Parmiani, and M.T. Lotze.** (1995) *"Mass spectrometric identification of a naturally processed melanoma peptide recognized by cytotoxic T lymphocytes."* J Exp Med 181 : 363-368.

144. **Cox, A.L., J. Skipper, Y. Chen, R.A. Henderson, T.L. Darrow, J. Shabanowitz, V.H. Engelhard, D.F. Hunt, and C.L. Slingluff Jr.** (1994) *"Identification of a peptide recognised by five melanoma-specific human cytotoxic T cell lines."* Science 264 : 716-719.

145. **Bachmann, M.F. and T.M. Kündig.** (1994)*"In vivo versus in vitro assays for assessment of T-and B-cell function."* Curr Opin Immunol 6 : 320-326.

146. **Kane, K.P. and M.F. Mescher.** (1993)*"Activation of CD8-dependent cytotoxic T lymphocyte adhesion and degranulation by peptide class I antigen complexes."* J Immunol 11 : 4788-4797.

147. **Corbella, P., D. Moskophidis, E. Spanapoulou, C. Mamalaki, M. Tolaini, A. Itano, D. Lans, D. Baltimore, E. Robey, and D. Kioussis.** (1994)*"Functional commitment to helper T cell lineage precedes positive selection and is independent of T cell receptor MHC specificity."* Immunity 1 : 269-276.

148. **Jameson, S.C., K.A. Hogquist, and M.J. Bevan.** (1995)*"Positive selection of thymocytes."* Annu Rev Immunol 13 : 93-126.

149. **Jameson, S.C., K.A. Hogquist, and M.J. Bevan.** (1994)*"Specificity and flexibility in thymic selection."* Nature 369 : 750-752.

150. **Ashton-Rickardt, P.G., L. Van Kaer, T.N.M. Schumacher, H.L. Ploegh, and S. Tonegawa.** (1993)*"Peptide contributes to the specificity of positive selection of $CD8^+$ T cells in the thymus."* Cell 73 : 1041-1049.

151. **Sebzda, E., V.A. Wallace, J. Mayer, R.S.M. Yeung, T.W. Mak, and P.S. Ohashi.** (1994)*"Positive and negative thymocyte selection induced by different concentrations of a single peptide."* Science 263 : 1615-1618.

152. **Hogquist, K.A., S.C. Jameson, and M.J. Bevan.** (1994)*"The ligand for positive selection of T lymphocytes in the thymus."* Curr Opin Immunol 6 : 273-278.

153. **Hogquist, K.A., S.C. Jameson, W.R. Heath, J.L. Howard, M.J. Bevan, and F.R. Carbone.** (1994)*"T cell receptor antagonist peptides induce positive selection."* Cell 76 : 17-27.

154. **Hogquist, K.A., S.C. Jameson, and M.J. Bevan.** (1995)*"Strong agonist ligands for the T cell receptor do not mediate positive selection of functional $CD8^+$ T cells."* Immunity 3 : 79-86.

155. **Kuzushima, K., R. Sun, G.M. van Bleek, Z. Vegh, and S.G. Nathenson.** (1995)*"The role of self peptides in the allogeneic cross-reactivity of CTLs."* J Immunol 154 : 594-601.

156. **von Boehmer, H.** (1990)*"Developmental biology of T cells in T cell receptor transgenic mice."* Annu Rev Immunol 8 : 531-556.

157. **Burrows, S.R., S.L. Silinis, D.J. Moss, R. Khanna, I.S. Misko, and V.P. Argaet.** (1995)*"T cell receptor repertoire for a viral epitope in humans is diversified by tolerance to a background major histocompatibility complex antigen."* J Exp Med 182 : 1703-1715.

158. **Cabaniols, J-P., R. Cibotti, P. Kourilsky, K. Kosmatopoulos, and J.M. Kanellopoulos.** (1994)*"Dose-dependent T cell tolerance to an immunodominant self peptide."* Eur J Immunol 24 : 1743-1749.

159. **Cibotti, R., J.M. Kanellopoulos, J.P. Cabaniols, O. Halle-Panenko, K. Kosmatopoulos, E. Sercarz, and P. Kourilsky.** (1992)*"Tolerance to a self-protein involves its immunodominant but does not involve its subdominant determinants."* Proc Natl Acad Sci USA 89 : 416-20.

160. **Heath, W.R., F. Karamalis, J. Donoghue, and J.F.A.P. Miller.** (1995)*"Autoimmunity caused by ignorant CD8$^+$ T cells is transient and depends on avidity."* J Immunol 155 : 2339-2349.

161. **von Herrath, M.G., J. Dockter, M. Nerenberg, J.E. Gairin, and M.B.A. Oldstone.** (1994)*"Thymic selection and adaptability of cytotoxic T lymphocyte responses in transgenic mice expressing a viral protein in the thymus."* J Exp Med 1994 : 1901-1910.

162. **Hsu, B.L., B.D. Evavold, and P.M. Allen.** (1955)*"Modulation of T cell development by an endogenous altered peptide ligand."* J Exp Med 181 : 805-810.

163. **Liu, G.Y., P.J. Fairchild, R.M. Smith, J.R Prowie, D. Kioussis, and D.C. Wraith.** (1995)*"Low avidity recognition of self-antigen by T cells permits escape from central tolerance."* Immunity 3 : 407-415.

164. **Malarkannan, S., M. Afkarian, and N. Shastri.** (1995)*"A rare translation product is presented by K^b major histocompatibility complex class I molecule to alloreactive T cells."* J Exp Med 182 : 1739-1750.

165. **Pirchner, H., U. Hoffman Rohrer, D. Moskophidis, R.M. Zinkernagel, and H. Hengartner.** (1991)*"Lower receptor avidity required for thymic clonal deletion than for effector T-cell function."* Nature 351 : 482-485.

166. **Ferber, I., G. Schönrich, J. Schenkel, A.L. Mellor, G.J. Hämmerling, and B. Arnold.** (1994)*"Levels of peripheral T cell tolerance induced by different doses of tolerogen."* Science 263 : 674-676.

167. **Elliot, E.A. and R.A. Flavell.** (1994)*"Transgenic mice expressing constitutive levels of IL-2 in islet β cells develop diabetes."* Int Immunol 6 : 1629-1637.

168. **Heath, W.R., J. Allison, M.W. Hoffman, G. Schönrich, G. Hämmerling, B. Arnold, and J.F.A.P. Miller.** (1992)*"Autoimmune diabetes as a consequence of locally produced Interleukin-2."* Nature 359 : 547-549.

169. **Andreu-Sanchez, J.L., I.M. de Alboran, M.A.R. Marcos, A. Sanchez-Movilla, C. Martinez-A., and G. Kroemer.** (1991)*"Interleukin 2 abrogates the nonresponsive state of T cells expressing a forbidden T cell receptor repertoire and induces autoimmune disease in neonatally thymectomized mice."* J Exp Med 173 : 1323-1329.

170. **Röcken, M, J.F. Urban, and E.M. Shevach.** (1992)*"Infection breaks T-cell tolerance."* Nature 359 : 79-82.

171. **Röcken, M and E.M. Shevach.** (1993)*"Do parasitic infections break T-cell tolerance and trigger autoimmune disease?"* Parasitol Today 9 : 377-380.

172. **Burns, J. and K. Littlefield.** (1993)*"A role for antigen-presenting cells and bacterial superantigens in reversal of human T lymphocyte anergy."* Eur J Immunol 23 : 3300-3305.

173. **Rocha, B. and H. von Boehmer.** (1991)*"Peripheral selection of the T cell repertoire."* Science 253 : 1225-1228.

174. **Schönrich, G., J. Alferink, A. Klevenz, G. Küblbeck, N. Auphan, A. Schmitt-Verhulst, G.J. Hämmerling, and B. Arnold.** (1994)*"Tolerance induction as a multistep process."* Eur J Immunol 24 : 285-293.

175. **Rocha, B., A. Grandien, and A.A. Freitas.** (1995)*"Anergy and exhaustion are independent mechanisms of peripheral T cell tolerance."* J Exp Med 181 : 992-1003.

176. **Zinkernagel, R.M., D. Moskophidis, T. Kündig, S. Oehen, H. Pircher, and H. Hengartner.** (1993)*"Effector T-cell induction and T-cell memory versus peripheral deletion of T cells."* Immunol Rev 131 : 199-221.

177. **Augustin-Voss, H.G. and B.U. Pauli.** (1994)*"Pathology of neoplasia: morphological and functional attributes."* In Developmental Biology and Cancer, ed. G.M. Hodges and C. Rowlatt. CRC Press, Florida : 3-28.

178. **Donovan, M., S. Demczuk, G. Franklin, and R. Ohlsson.** (1994)*"Physiopathological role of oncogenes in development and neoplasia."* In Developmental biology of cancer, ed. G.M. Hodges and C. Rowlatt. CRC Press, Florida : 313-388.

179. **Hunter, T. and J. Pines.** (1994)*"Cyclins and cancer II: Cyclin D and CDK inhibitors come of age."* Cell 79 : 573-582.

180. **Rodrigues, G.A. and M. Park.** (1994)*"Oncogenic activation of tyrosine kinases."* Curr Opin Genet Devel 4 : 15-24.

181. **Levine, A.J., M.E. Perry, A. Chang, A. Silver, D. Dittmer, M. Wu, and D. Welsh.** (1994)*"The 1993 Walter Hubert Lecture: the role of the p53 tumour-suppressor gene in tumorigenesis."* Br J Cancer 69 : 409-416.

182. **Stauss, H.J. and A.M. Dahl.** (1996)*"Cellular oncogenes for tumour immunity and immunotherapy."* In Tumour Immunology, ed. A.G. Dalgleish. Cambridge University Press, New York : in press.

183. **Cheever, M.A., M.L. Disis, H. Bernhard, J.R. Gralow, S.L. Hand, E.S. Huseby, H.L. Qin, M. Takahashi, and W. Chen.** (1995)*"Immunity to oncogenic proteins."* Immunol Rev 145 : 33-59.

184. **van Pel, A., E. de Plaen, and T. Boon.** (1985)*"Selection of highly transfectable variant from mouse mastocytoma P815."* Somat Cell Mol Genet 11 : 467-475.

185. **Masucci, M.G.** (1993)*"Viral immunopathology of human tumours."* Curr Opin Immunol 1993 : 693-700.

186. **Ressing, M.E., A. Sette, R.M.P. Brandt, J. Ruppert, P.A. Wentworth, M. Hartman, C. Oseroff, H.M. Grey, C.J.M. Melief, and W.M. Kast.** (1995)*"Human CTL epitopes encoded by human papillomavirus type 16 E6 and E7 identified through in vivo and in vitro immunogenicity studies of HLA-A0201 binding peptides."* J Immunol 154 : 5934-5943.

187. **Fossum, B., T. Geddedahl, J. Breivik, J. A. Eriksen, A. Spurkland, E. Thorsby, and G. Gaudernack.** (1994)*"P21-ras-peptide-specific T-cell responses in a patient with colorectal-cancer - CD4(+) and CD8(+) T-cells recognize a peptide corresponding to a common mutation (13 gly-asp)."* Int J Cancer 56 : 40-45.

188. **Wölfel, T., M. Hauer, J. Schneider, M. Serrano, C. Wölfel, E. Klehman-Hieb, E. De Plaen, T. Hankeln, K.-H. Meyer zum Buschenfelde, and D. Beach.** (1995)*"A p16INK^{4a}-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma."* Science 269 : 1281-1285.

189. **Peoples, G.E., P.S. Goedegebuure, R. Smith, D.C. Linehan, I. Yoshino, and T.J. Eberlein.** (1995)*"Breast and ovarian cancer-specific cytotoxic T lymphocytes recognise the same HER2/neu derived peptide."* Proc Natl Acad Sci USA 92 : 432-436.

190. **Disis, M. L., E. Calenoff, G. McLaughlin, A.E. Murphy, W. Chen, B. Groner, M. Jeschke, N. Lydon, E. McGlynn, R.B. Livingston, R. Moe, and M.A. Cheever.** (1994)*"Existent T-cell and antibody immunity to her-2 neu protein in patients with breast-cancer."* Cancer Res 54 : 16-20.

191. **Fisk, B., T.L. Blevins, J.T. Wharton, and C.G. Ioannides.** (1995)*"Identification of an immunodominant peptide of her-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T-lymphocyte lines."* J Exp Med 181 : 2109-2117.

192. **Rivoltini, L., Y. Kawakami, K. Sakaguchi, S. Southwood, A. Sette, P.F. Robbins, F.M. Marincola, M.L. Salgaller, Y.R. Yannelli, and E. Appella.** (1995)*"Induction of tumor-reactive ctl from peripheral-blood and tumor-infiltrating lymphocytes of melanoma patients by in-vitro stimulation with an immunodominant peptide of the human-melanoma antigen mart-1."* J Immunol 154 : 2257-2265.

193. **Bakker, A.B.H., M.W.J. Schreurs, A.J. de Boer, Y. Kawakami, S. A. Rosenberg, G.J. Adema, and C.G. Figdor.** (1994)*"Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes."* J Exp Med 179 : 1005-1009.

194. **van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. de Plaen, B. van den Eynde, A. Knuth, and T. Boon.** (1991)*"A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma."* Science 254 : 1643-1647.

195. **van der Bruggen, P., J-P. Szikora, P. Boël, C. Wildmann, M. Somville, M. Sensi, and T. Boon.** (1994)*"Autologous cytolytic T lymphocytes recognize a MAGE-1 nonapeptide on melanomas expressing HLA-Cw1601."* Eur J Immunol 24 : 2134-2140.

196. **van der Bruggen, P., J. Bastin, T. Gajewski, P.G. Coulie, P. Boël, C. de Smet, C. Traversari, A. Townsend, and T. Boon.** (1994)*"A peptide encoded by the human gene MAGE-3 and presented by HLA-A2 induces cytolytic T lymphocytes that recognise tumor cells expressing MAGE-3."* Eur J Immunol 24 : 3038-3043.

197. **Gaugler, B., B. Van den Eynde, P. van der Bruggen, P. Romero, J.J. Gaforio, E. De Plaen, B. Lethé, F. Brasseur, and T. Boon.** (1994)*"Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes."* J Exp Med 179 : 921-930.

198. **Brichard, V., A. Van Pel, T. Wölfel, C. Wölfel, E. De Plaen, B. Lethe, P. Coulie, and T. Boon.** (1993)*"The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanoma."* J Exp Med 178 : 489-495.

199. **Topalian, S.L., L. Rivoltini, M. Mancini, N.R. Markus, P.F. Robbins, Y. Kawakami, and S.A. Rosenberg.** (1994)*"Human $CD4^+$ T-cells specifically recognize a shared melanoma-associated antigen encoded by the tyrosinase gene."* Proc Natl Acad Sci USA 91 : 9461-9465.

200. **Wölfel, T., A. Van Pel, V. Brichard, J. Schneider, B. Seliger, K. Meyer zum Büschenfelde, and T. Boon.** (1994)*"Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes."* Eur J Immunol 24 : 759-764.

201. **Lane, D.P.** (1994)*"On the expression of the p53 protein in human cancer."* Mol Biol Rep 19 : 23-29.

202. **Lanfrancone, L., G. Pelicci, and P. Pelicci.** (1994)*"Cancer genetics."* Curr Opin Genet Devel 4 : 109-119.

203. **Hu, J., W. Kindsvogel, S. Busby, M.C. Bailey, Y. Shi, and P.D. Greenberg.** (1993)*"An evaluation of the potential to use tumour-associated antigens as targets for antitumour T cell therapy using transgenic mice expressing a retroviral tumor antigen in normal lymphoid tissues."* J Exp Med 177 : 1681-1690.

204. **Koeppen, H., M. Acena, A. Drolet, D.A. Rowley, and H. Schreiber.** (1993)*"Tumors with reduced expression of a cytotoxic T-lymphocyte recognized antigen lack immunogenicity but retain sensitivity to lysis by cytotoxic T-lymphocytes."* Eur J Immunol 23 : 2770-2776.

205. **Mackensen, A., G. Carcelain, S. Viel, S. Raynal, H. Michalaki, F. Triebel, J. Bosq, and T. Hercend.** (1994)*"Direct evidence to support the immunosurveillance concept in human regressive melanoma."* J Clin Invest 93 : 1397-1402.

206. **Hilders, C.G.J.M., L. Ras, J.D.H. Van Eendenburg, Y. Nooyen, and G.J. Fleuren.** (1994)*"Isolation and characterization of tumor-infiltrating lymphocytes from cervical-carcinoma."* Int J Cancer 57 : 805-813.

207. **Lüscher, U., L. Filgueira, A. Juretic, M. Zuber, N.J. Lüscher, M. Heberer, and G.C. Spagnoli.** (1994)*"The pattern of cytokine gene expression in freshly excised human metastatic melanoma suggests a state of reversible anergy of tumor-infiltrating lymphocytes."* Int J Cancer 57 : 612-619.

208. **Vose, B.M., F. Vanky, M. Fopp, and E. Klein.** (1978)*"Restricted autologous lymphocytotoxicity in lung neoplasia."* Br J Cancer 38 : 375-381.

209. **Mazzocchi, A., F. Belli, L. Mascheroni, C. Vegetti, G. Parmiani, and A. Anichini.** (1994)*"Frequency of cytotoxic T-lymphocyte precursors (ctlp) interacting with autologous tumor via the T-cell receptor - limiting dilution analysis of specific ctlp in peripheral-blood and tumor-invaded lymph-nodes of melanoma patients."* Int J Cancer 58 : 330-339.

210. **Itoh, K., M.A. Salmeron, T. Morita, D. Seito, P.F. Mansfield, M.I. Ross, C.M. Balch, and L.B. Augustus.** (1992)*"Distribution of autologous tumor-specific cytotoxic T lymphocytes in human metastatic melanoma."* Int J Cancer 52 : 52-59.

211. **Robbins, P.F., M. El-Gamil, Y. Kawakami, and S.A. Rosenberg.** (1994)*"Recognition of tyrosinase by tumor-infiltrating lymphocytes from a patient responding to immunotherapy."* Cancer Res 54 : 3124-3126.

212. **Lehmann, F., M. Marchand, P. Hainaut, P. Pouillart, X. Sastre, H. Ikeda, T. Boon, and P.G. Coulie.** (1995)*"Differences in the antigens recognized by cytolytic T cells on two successive metastases of a melanoma patient are consistent with immune selection."* Eur J Immunol 25 : 340-347.

213. **Möller, P. and G.J. Hämmerling.** (1992)*"The role of surface HLA-A, B, C molecules in tumor immunity."* Cancer Surveys 13 : 101-127.

214. **Wilson, L., T. Spies, and P.J. van den Elsen.** (1994)*"Cellular immune recognition of HLA-A0201 following gene tranfer into a human embryonal carcinoma cell line."* Hum Immunol 41 : 74-78.

215. **Kaklamanis, L., A. Townsend, I. A. Doussis-Anagnostopoulou, N. Mortensen, A.L. Harris, and K.C. Gatter.** (1994)*"Loss of major histocompatibility complex-encoded transporter associated with antigen presentation (tap) in colorectal-cancer."* Am J Pathol 145 : 505-509.

216. **Garrdio, F., T. Cabrera, A. Concha, S. Glew, F. Ruiz-Cabello, and P.L. Stern.** (1993)*"Natural history of HLA expression during tumour development."* Immunol Today 14 : 491-499.

217. **Baskar, S., L. Glimcher, N. Nabavi, R.T. Jones, and S. Ostrand-Rosenberg.** (1995)*"Major histocompatibility complex class $II^+B7\text{-}1^+$ tumor cells are potent vaccines for stimulating tumor rejection in tumor-bearing mice."* J Exp Med 181 : 619-629.

218. **Cayeux, S., C. Beck, A. Aicher, B. Dorken, and T. Blankenstein.** (1995)*"Tumor cells cotransfected with interleukin-7 and B7.1 genes induce CD25 and CD28 on tumor-infiltrating T lymphocyttes and are strong vaccines."* Eur J Immunol 25 : 2325-2331.

219. **Allison, J.P., A.A. Hurwitz, and D.R. Leach.** (1995)*"Manipulation of costimulatory signals to enhance antitumor T-cell responses."* Curr Opin Immunol 7 : 682-686.

220. **Huang, A. Y.C., P. Golumbek, M. Ahmadzaheh, E. Jaffee, D. Pardoll, and H. Levitsky.** (1994)*"Role of bone marrow-derived cells in presenting MHC Class I-restricted tumor antigens."* Science 264 : 961-965.

221. **Mizoguchi, H., J. O'Shea, D.L. Longo, C.M. Loeffler, D.W. McVicar, and A.C. Ochoa.** (1992)*"Alterations in signal transduction molecules in T lymphocytes from tumour-bearing mice."* Science 258 : 1795-1797.

222. **Bates, S. and G. Peters.** (1995)*"Cyclin D1 as a cellular proto-oncogene."* Semin Cancer Biol 6 : 73-82.

223. **Peters, G.** (1994)*"The D-type cyclins and their role in tumorigenesis."* J Cell Sci 1994 : 89-96.

224. **Baldin, V., J. Lukas, M. J. Marcote, M. Pagano, and G. Draetta.** (1993)*"Cyclin D1 is a nuclear protein required for cell cycle progression in G1."* Genes Dev 7 : 812-821.

225. **Bartkova, J., J. Lukas, M. Strauss, and J. Bartek.** (1994)*"Cell cycle-related variation and tissue-restricted expression of human cyclin D1 protein."* J Pathol 172 : 237-245.

226. **Ajchenbaum, F., K. Ando, J.A. DeCaprio, and J.D. Griffin.** (1993)*"Independent regulation of human D-type cyclin gene expression during G1 phase in primary human T lymphocytes."* J Biol Chem 268 : 4113-4119.

227. **Fantl, V., G. Stamp, A. Andrews, I. Rosewell, and C. Dickson.** (1995)*"Mice lacking cyclin D1 are small and show defects in eye and mammary-gland development."* Genes Dev 9 : 2364-2372.

228. **Sicinski, P., J.L. Donaher, S.B. Parker, T.S. Li, H. Gardner, S.Z. Haslam, R.T. Bronson, S.J. Elledge, and R.A. Weinberg.** (1995)*"Cyclin D1 provides a link between development and oncogenesis in the retina and breast."* Cell 82 : 621-630.

229. **Hinds, P.W., S.F. Dowdy, E.N. Eaton, A. Arnold, and R.A. Weinberg.** (1994)*"Function of a human cyclin gene as an oncogene."* Proc Natl Acad Sci USA 91 : 709-713.

230. **Wang, T.C., R.D. Cardiff, L. Zukerberg, E. Lees, A. Arnold, and E.V. Schmidt.** (1994)*"Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice."* Nature 369 : 669-671.

231. **Filmus, J., A.I. Robles, W. Shi, M.J. Wong, L.L. Colombo, and C.J. Conti.** (1994)*"Induction of cyclin D1 overexpression by activated ras."* Oncogene 9 : 3627-3633.

232. **Han, E. K-H., A. Sgambato, W. Jiang, Y.-J. Zhang, R.M. Santella, Y. Zhang, R.M. Santella, Y. Doki, A.M. Cacace, I. Schiereren, and I.B. Weinstein.** (1995)*"Stable overexpression of cyclin D1 in a human mammary epithelial cell line prolongs the S-phase and inhibits growth."* Oncogene 10 : 953-961.

233. **Quelle, D.E., R.A. Ashmun, S.A. Shurtleff, J. Kato, D. Bar-Sagi, M.F. Roussel, and C.J. Sherr.** (1994)*"Overexpresion of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts."* Genes Dev 7 : 1559-1571.

234. **Lammie, G.A. and G. Peters.** (1991)*"Chromosome 11q13 abnormalities in human cancer."* Cancer Cells 3 : 413-420.

235. **Gillet, C., V. Fantl, R. Smith, C. Fisher, J. Bartek, C. Dickson, D. Barnes, and G. Peters.** (1994)*"Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining."* Cancer Res 54 : 1812-1817.

236. **Bartkova, J., J. Lukas, M. Strauss, and J. Bartek.** (1995)*"Cyclin D1 oncoprotein aberrantly accumulates in malignancies of diverse histogenesis."* Oncogene 10 : 775-778.

237. **Nishida, N., Y. Fukuda, T. Komeda, R. Kita, T. Sando, M. Furukawa, M. Amenomori, I. Shibagaki, K. Nakao, and M. Ikenaga.** (1994)*"Amplification and overexpression of the cyclin D1 gene in aggressive human hepatocellular-carcinoma."* Cancer Res 54 : 3107-3110.

238. **Zhang, Y-J., W. Jiang, C-J. Chen, C.S. Lee, S.M. Kahn, R.M. Santenella, and I.B. Weinstein.** (1993)*"Amplification and overexpression of cyclin D1 in human hepatocellular carcinoma."* Biochem Biophys Res Com 196 : 1010-1016.

239. **Rimokh, R., F. Berger, G. Delsol, C. Charrin, M.-F: Berthéas, M. Ffrench, M. Garoscio, P. Felman, B. Coiffier, P.-A. Bryon, M. Rochet, O. Gentilhomme, D. Germain, and J.-P. Magaud.** (1993)*"Rearrangement and overexpression of the BCL-1/PRAD-1 gene in intermediate lymohocytic lymphomas and in t(11q13)-bearing leukemias."* Blood 81 : 3063-3067.

240. **Levine, A.J. and J. Momand.** (1990)*"Tumor suppressor genes. The p53 and retinoblastoma sensitivity genes and gene products."* Biochim Biophys Acta 1032 : 119-136.

241. **Fakharzadeh, S.F., S.P. Trusko, and D.L. George.** (1991)*"Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumour cell line."* EMBO J 10 : 1565-1569.

242. **Juven, T., Y. Barak, A. Zauberman, D.L. George, and M. Oren.** (1993)*"Wild type p53 can mediate sequence-specific transactivation of an internal promoter within the mdm2 gene."* Oncogene 8 : 3411-3416.

243. **Wu, X., H. Bayle, D. Olson, and A.J. Levine.** (1993)*"The p53-mdm-2 autoregulatory feedback loop."* Genes Dev 7 : 1126-1132.

244. **Martin, K., D. Trouche, C. Hagemeier, T.S. Sorensen, N.B. La Thangue, and T. Kouzardies.** (1995)*"Stimulation of E2F1/DP1 transcriptional activity by mdm2 oncoprotein."* Nature 375 : 691-694.

245. **Oliner, J.D., J.A. Pietonpol, S. Thiagalingam, J. Gyuris, K.W. Kinzler, and B. Vogelstein.** (1993)*"Oncoprotein mdm2 conceals the activation domain of tumour suppressor p53."* Nature 362 : 857-860.

246. **Chen, C.-Y., J.D. Oliner, Q. Zhan, A.J. Fornace, B. Vogelstein, and M.B. Kastan.** (1994)*"Interactions between p53 and mdm2 in a mammalian cell cycle checkpoint pathway."* Proc Natl Acad Sci USA 91 : 2684-2688.

247. **Xiao, Z-X., J. Chen, A.J. Levine, N. Modjtahedi, J. Xing, R. Sellers, and D.M. Livingston.** (1995)*"Interaction between the retinoblastoma protein and the oncoprotein mdm2."* Nature 375 : 694-697.

248. **Jones, S.N., A.E. Roe, L.A. Donehower, and A. Bradley.** (1995)*"Rescue of embryonic lethality in mdm2-deficient mice by absence of p53."* Nature 378 : 206-208.

249. **de Oca Luna, R.M., D.S. Wagner, and G. Lozano.** (1995)*"Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53."* Nature 378 : 203-206.

250. **Cordon-Cardo, C., E. Latres, M. Drobnjak, M.R. Oliva, D. Pollack, J.M. Woodruff, V. Marechal, Chen J., M.F. Brennan, and A.J. Levine.** (1994)*"Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas."* Cancer Res 54 : 794-799.

251. **Floerenes, V.A., G.M. Maelandsmo, A. Forus, A. Andreassen, O. Myklebost, and O. Fodstad.** (1994)*"Mdm2 gene amplification and transcript levels in human sarcomas - relationship to tp53 gene status."* J Natl Cancer Inst 86 : 1297-1302.

252. **Ladanyi, M., S.C. Jhanwar, R. Terek, E. Latres, C. Cordon-Cardo, A. G. Huvos, and J. Healey.** (1993)*"Amplification of the mdm2 gene in human bone sarcomas."* Am J Hum Genet 53 : 317-317.

253. **Leach, F.S., T. Tokino, P. Meltzer, M. Burrell, J.D. Oliner, S. Smith, D.E. Hill, D. Sidransky, K.W. Kinzler, and B. Vogelstein.** (1993)*"p53 mutation and mdm2 amplification in human soft tissue sarcomas."* Cancer Res 53 : 2231-2234.

254. **Oliner, J.D., K.W. Kinzler, P.S. Meltzer, D.L. George, and B. Vogelstein.** (1992)*"Amplification of a gene encoding a p53-associated protein in human sarcomas."* Nature 358 : 80-83.

255. **Bueso-Ramos, C.E., Y. Yang, E. deLeon, P. McCown, S.A. Stass, and M. Albitar.** (1993)*"The human mdm-2 oncogene is overexpressed in leukemias."* Blood 82 : 2617-2623.

256. **Huang, Y.Q., B. Raphael, A. Buchbinder, J.J. Li, W.G. Zhang, and A. E. Friedmankien.** (1994)*"Rearrangement and expression of mdm(2) oncogene in chronic lymphocytic-leukemia."* Am J Hematol 47 : 139-141.

257. **Watanabe, T., T. Hotta, A. Ichikawa, T. Kinoshita, H. Nagai, T. Uchida, T. Murate, and H. Saito.** (1994)*"The mdm2 oncogene overexpression in chronic lymphocytic-leukemia and low-grade lymphoma of B-cell origin."* Blood 84 : 3158-3165.

258. **Marchetti, A., F. Buttitta, S. Girlando, P. Dallapalma, S. Pellegrini, P. Fina, C. Doglioni, G. Bevilacqua, and M. Barbareschi.** (1995)*"Mdm2 gene alterations and mdm2 protein expression in breast carcinomas."* J Pathol 175 : 31-38.

259. **Ebert, M., M. Yokoyama, M. S. Kobrin, H. Friess, M.W. Buchler, and M. Korc.** (1994)*"Increased mdm2 expression and immunoreactivity in human pancreatic ductal adenocarcinoma."* Int J Oncol 5 : 1279-1284.

260. **Donehower, L.A., M. Harvey, B.L. Slagle, M.J. McArthur, C.A. Montgomery, J.S. Butel, and A. Bradley.** (1992)*"Mice deficient for p53 are developmentally normal but susceptible to spontanous tumours."* Nature 356 : 215-221.

261. **Otto, A. and W. Deppert.** (1993)*"Upregulation of mdm-2 expression in Meth A tumor cells tolerating wild-type p53."* Oncogene 8 : 2591-2603.

262. **Levine, A.J., J. Momand, and C.A. Finlay.** (1991)*"The p53 tumour suppressor gene."* Nature 351 : 453- 455.

263. **Crawford, L.W., D.C. Pim, and R.D. Bulbrook.** (1982)*"Detection of antibodies against the cellular protein p53 in sera from patients with breast cancer."* Int J Cancer 30 : 403-408.

264. **Labrecque, S., N. Naor, D. Thomsom, and G. Matlashewski.** (1993)*"Analysis of the anti-p53 antibody response in cancer patients."* Cancer Res 53 : 3468-3471.

265. **Schlichtholz, B., Y. Legros, D. Gillet, C. Gaillard, M. Marty, D. Lane, F: Calvo, and T. Soussi.** (1992)*"The immune response to p53 in breast cancer patients is directed against immunodominant epitopes unrelated to the mutational hot spot."* Cancer Res 52 : 6380-6384.

266. **Lubin, R., B. Schlichtholz, D. Bengoufa, G. Zalcman, J. Trédaniel, A. Hirsch, C.C. De Fromentel, C. Preudhomme, P. Fenaux, G. Fournier, P. Mangin, P. Laurent-Puig, G. Pelletier, M. Schlumberger, F. Desgrandchamps, A. Leduc, J.P. Peyrat, N. Janin, B. Bressac, and T. Soussi.** (1993)*"Analysis of p53 antibodies in patients with various cancers define B-cell epitopes of human p53 - distribution on primary structure and exposure on protein surface."* Cancer Res 53 : 5872-5876.

267. **Mueller, M., M. Meyer, M. Volkmann, U. Rath, B. Kommerell, and P.R. Galle.** (1993)*"Anti-p53 serum antibodies in patients with hepatocellular-carcinoma and gi-tract tumors - clinical relevance and diagnostic usefulness."* Hepatology 18 : A190-A190.

268. **Tilkin, A.-F., R. Lubin, T. Soussi, V. Lazar, N. Janin, M.-C. Mathieu, I. Lefrere, C. Carlu, M. Roy, M. Kayibanda, D. Bellet, J.-G. Guillet, and B. Paillerets.** (1995)*"Primary proliferative T cell responses to wild-type p53 protein in patients with breast cancer."* Eur J Immunol 25 : 1765-1769.

269. **Johnson, D.E. and L.T. Williams.** (1993)*"Structural and functional diversity in the FGF receptor multigene family."* Adv Cancer Res 60 : 1-41.

270. **Reid, H.H., A.F. Wilks, and O. Bernard.** (1990)*"Two forms of the basic fibroblast growth factor receptor-like mRNA are expressed in the developing mouse brain."* Proc Natl Acad Sci USA 87 : 1596-1600.

271. **Mansukhani, A., D. Moscatelli, D. Talarico, V. Levytska, and C. Basilico.** (1990)*"A murine fibroblast growth factor (FGF) receptor expressed in CHO cells is activated by basic FGF and Kaposi FGF."* Proc Natl Acad Sci USA 87 : 4378-4382.

272. **Hughes, S.E. and P.A. Hall.** (1993)*"The fibroblast growth factor and receptor multigene families."* J Pathol 170 : 219-223.

273. **Jacquemier, J., J. Adelaide, P. Parc, F. Penault-Llorca, J. Planche, O. De Lapeyriere, and D. Burnbaum.** (1994)*"Expression of the FGFRI gene in human breast-carcinoma cells."* Int J Cancer 59 : 373-378.

274. **Kobrin, M.S., Y. Yamanaka, H. Friess, M.E. Lopez, and M. Korc.** (1993)*"Aberrant expression of type I fibroblast growth factor receptor in human pancreatic adenocarcinomas."* Cancer Res 53 : 4741-4744.

275. **Morrison, R.S., F. Yamaguchi, J.M. Bruner, M. Tang, W. McKeehan, and M.S. Berger.** (1994)*"Fibroblast growth factor receptor gene expression and immunoreactivity are elevated in human glioblastoma multiforme."* Cancer Res 54 : 2794-2799.

276. **Yamaguchi, F., H. Saya, J.M. Bruner, and R.S. Morrison.** (1994)*"Differential expression of two fibroblast growth factor receptor genes is associated with malignant progression in human astrocytomas."* Proc Natl Acad Sci USA 91 : 484-488.

277. **Di Blasio, A.M., L. Cremonesi, P. Vigano, M. Ferrari, D. Gospodarowicz, M. Vignali, and R.B. Jaffe.** (1993)*"Basic fibroblast growth factor and its receptor messenger ribonucleic acids are expressed in human ovarian epithelial neoplasms."* Am J Obstet Gynecol 169 : 1517-1523.

278. **Fenner, F.** (1992)*"Vaccinia virus as vaccine, and poxvirus pathogenesis."* In Recombinant poxviruses, ed. M.M. Binns and G.L. Smith. CRC Press, Boca Raton, Florida : 1-43.

279. **Moss, B.** (1990)*"Regulation of vaccinia virus transcription."* Annu Rev Biochem 59 : 661-688.

280. **Johnson, G.P., S.J. Goebel, and E. Paoletti.** (1993)*"An update on the vaccinia virus genome."* Virology : 381-401.

281. **VanSlyke, J.K. and D.E. Hruby.** (1990)*"Posttranslational modification of vaccinia virus proteins."* Curr Top Microbiol Immunol 163 : 185-206.

282. **Brutkiewicz, R.R., S.J. Klaus, and R.M. Welsh.** (1992)*"Window of vulnerability of vaccinia virus-infected cells to natural killer (NK) cell-mediated cytolysis*

correlates with enhanced NK cell triggering and is concomitant with a decrease in H-2 Class I antigen expression." Nat Immun 11 : 203-214.

283. **Moss, B. and C. Flexner.** (1987)*"Vaccinia virus expression vectors."* Ann Rev Immunol 5 : 305-324.

284. **Cox, W.I., J. Tartaglia, and E. Paoletti.** (1992)*"Poxvirus recombinants as live vaccines."* In Recombinant poxviruses, ed. M.M. Bins and G.L. Smith. CRC Press, Boca Raton, Florida : 123-162.

285. **Bennink, J.R. and J.W. Yewdell.** (1990)*"Recombinant vaccinia viruses as vectors for studying T lymphocyte specificity and function."* Curr Topics Microbiol Immunol 163 : 153-183.

286. **Sadovnikova, E., X. Zhu, S.M. Collins, J. Zhou, K. Vousden, L. Crawford, P. Beverley, and H.J. Stauss.** (1994)*"Limitations of predictive motifs revealed by cytotoxic T lymphocyte epitope mapping of the human papillomavirus E7 protein."* Int Immunol 6 : 289-296.

287. **Skipper, J. and H.J. Stauss.** (1993)*"Identification of two cytotoxic T lymphocyte-recognised epitopes in the Ras protein."* J Exp Med 177 : 1493-1498.

288. **Tsang, K.Y., S. Zaremba, C. A. Nieroda, M.Z. Zhu, J.M. Hamilton, and J. Schlom.** (1995)*"Generation of human cytotoxic T-cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine."* J Natl Cancer Inst 87 : 982-990.

289. **Spriggs, M.K, B.H. Koller, T. Sato, P.J. Morrissey, W.C. Fanslow, O. Smithies, R.F. Voice, M.B. Widmer, and C.R. Maliszewski.** (1992)*"Beta 2-microglobulin-, CD8$^+$ T-cell-deficient mice survive inoculation with high doses of vaccinia virus and exhibit altered IgG responses."* Proc Natl Acad Sci USA 89 : 6070-6074.

290. **Lechler, R. and M. Pla.** (1995)*"The credentials of a T-cell epitope."* Immunol Today 16 : 561-563.

291. **Carpenter, E.A., J. Ruby, and I.A. Ramshaw.** (1994)*"IFN-γ, TNF and IL-6 production by vaccinia virus immune spleen cells."* J Immunol : 2652-2659.

292. **Chakrabarti, S., K. Brechling, and B. Moss.** (1985)*"Vaccinia virus expression vector coexpression of β-Galactosidase provides visual screening of recombinant virus plaques."* Mol Cell Biol 5 : 3403-3409.

293. **Mackett, M., G.L. Smith, and B. Moss.** (1985)*"The construction and characterisation of vaccinia virus recombinants expressing foreign genes."* In DNA cloning- a practical approach. IRL Press, Oxford, UK : 191-211.

294. **Talavera, A. and J.M Rodriguez.** (1991)*"Vaccinia virus as an expression vector."* In Practical Molecular Virology: viral vectors for gene expression, ed. M. Collins. The Humana Press Inc., Clifton, NJ : 219-233.

295. **Mackett, M., G.L. Smith, and B. Moss.** (1984)*"General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes."* J Virol 49 : 857-864.

296. **Winnacker, E.L.** (1987) *"From genes to clones"*. VCH Verlagsgesellschaft, Weinheim, Germany.

297. **Venkatesan, S., B.M. Baroudy, and B. Moss.** (1981)*"Distinctive nucleotide sequences adjacent to multiple initiation and termination sites of an early vaccinia virus gene."* Cell 125 : 805-813.

298. **Garces, J., K. Masternak, B. Kunz, and R. Wittek.** (1993)*"Reactivation of transcription from a vaccinia virus early promoter late in infection."* J Virol 67 : 5394-5401.

299. **Graham, F.L. and L. Prevec.** (1991)*"Manipulation of adenovirus vectors."* In Gene tranfer and expression protocols, ed. E.J. Murray. The Humana Press Inc., Clifton, NJ : 109-128.

300. **Precious, B. and W.C. Russell.** (1991)*"Growth, purification and titration of adenoviruses."* In Virology, ed. B.W.J. Mahy. IRL press, Oxford, UK : 193-205.

301. **Graham, F.L., D.T. Rowe, R. McKinnon, S. Bacchetti, M Ruben, and P.E. Branton.** (1984)*"Transformation by human adenoviruses."* J Cell Physiol Suppl 3 : 151-163.

302. **Kozarsky, K.F. and J.M. Wilson.** (1993)*"Gene therapy: adenovirus vectors."* Curr Opin Genet Dev 3 : 499-503.

303. **Berencsi, K., R.F. Rando, C. DeTaisne, E. Paoletti, S.A. Plotkin, and E. Gonczol.** (1993)*"Murine cytotoxic T cell response specific for human cytomegalovirus glycoprotein B (gB) induced by adenovirus and vaccinia virus recombinants expressing gB."* J Gen Virol 74 : 2507-2512.

304. **Horwitz, M.S.** (1990)*"Adenoviridae and their replication."* In Virology, ed. B.N. Fields and D.M. Knipe. Raven Press, New York : 1679-1721.

305. **Bett, A.J., L. Prevec, and F.L. Graham.** (1993)*"Packaging capacity and stability of human adenovirus type 5 vectors."* J Virol 67 : 5911-5921.

306. **Graham, F.L. and J. Smiley.** (1977)*"Characterisitics of a human cell line transformed by DNA from human adenovirus type 5."* J Gen Virol 36 : 59-72.

307. **Jacobs, S.C., J.R. Stephenson, and G.W.G. Wilkinson.** (1992)*"High-level expression of the tick-borne encephalitis virus NS1 protein by using adenovirus-based vector: Protection elicited in a murine model."* J Virol 66 : 2086-2095.

308. **Rawle, R.C., B.H. Knowles, R.P. Ricciardi, V. Brahmacheri, P. Duerksen-hughes, W.S.M. Wold, and L.R. Gooding.** (1991)*"Specificity of the mouse cytotoxic T lymphocyte response to adenovirus 5."* J Immunol 146 : 3977-3984.

309. **McFadden, G. and K. Kane.** (1994)*"How DNA viruses perturb functional MHC expression to alter immune recognition."* Adv Cancer Res 63 : 117-209.

310. **Cox, J.H., R.M.L. Buller, J.R. Bennink, J.W. Yewdell, and G. Karupiah.** (1994)*"Expression of adenovirus E3/19K protein does not alter mouse MHC Class I restricted responses to vaccinia virus."* Virology 204 : 558-562.

311. **Wilkinson, G.W.G. and A. Akrigg.** (1992)*"Constitutive and enhanced expression from the CMV major IE promoter in a defective adenovirus vector."* Nucleic Acids Res 20 : 2233-2239.

312. **Spessot, R., K. Inchley, T.M. Hupel, and S. Bacchetti.** (1989)*"Cloning of the Herpes Simplex Virus ICP4 gene in an adenovirus vector: effects on adenovirus gene expression and replication."* Virology 168 : 378-387.

313. **Graham, F.L. and L. Prevec.** (1992)*"Adenovirus-based expression vectors and recombinant vaccines."* In Vaccines, New Approaches to immunological problems, ed. R.W. Ellis. Butterworth-Heinemann, Boston : 363-390.

314. **Jones, N. and T. Shenk.** (1978)*"Isolation of deletion and substitution mutants of adenovirus type 5."* Cell 13 : 181-188.

315. **Graham, F.L.** (1984)*"Covalently closed circles of human adenovirus DNA are infectious."* EMBO J 3 : 2917-2922.

316. **McGrory, W.J., D.S. Bautista, and F.L. Graham.** (1988)*"A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5."* Virology 163 : 614-617.

317. **Wilkinson, G.W.G.** (1991)*"The cytomegalovirus major immediate early promoter and its use in eukaryotic expression systems."* Adv Gene Techn 2 : 287-310.

318. **Sambrook, J., E.J. Fritsch, and T. Maniatis.** (1989) *"Molecular Cloning"*. 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, New York.

319. **Graham, F.L. and A.J. van der Eb.** (1973)*"A new technique for the assay of infectivity of human adenovirus 5 DNA."* Virology 52 : 456-467.

320. **Chomczynski, P. and N. Sacchi.** (1987)*"Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction."* Anal Biochem 162 : 156-159.

321. **Bates, S., L. Bonetta, D. MacAllan, D. Parry, A. Holder, C. Dickson, and G. Peters.** (1994)*"CDK6 (PLSTIRE) and CDK4 (PSK-J3) are a distinct subset of cyclin-dependent kinases that associate with cyclin D1."* Oncogene 9 : 71-79.

322. **Chen, J., V. Marechal, and A.J. Levine.** (1993)*"Mapping of the p53 and mdm-2 interaction domains."* Mol Cell Biol 13 : 4107-4114.

323. **Vogelstein, B.** (1990)*"A deadly inheritance."* Nature 348 : 681-682.

324. **Laemmli, U.K.** (1970)*"Cleavage of structural proteins during the assembly of the head of bacteriophage T4."* Nature 227 : 680-681.

325. **Harlow, E. and D. Lane.** (1988) *"Antibodies, A laboratory Manual"*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, New York.

326. **Ljunggren, H-G. and K. Kärre.** (1985)*"Host resistance directed selectively against H-2 deficient lymphoma variants."* J Exp Med 162 : 1745-1759.

327. **Kärre, K., H-G. Ljunggren, G. Piontek, and R. Kiessling.** (1986)*"Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy."* Nature 319 : 675-678.

328. **Yang, Y., K. Früh, J. Chambers, J.B. Waters, L. Wu, T. Spies, and P.A. Peterson.** (1992)*"Major histocompatibility complex (MHC) encoded HAM2 is necessary for antigenic peptide loading onto class I MHC molecules."* J Immunol 267 : 11669-11672.

329. **Gorer, P.A.** (1950)*"Studies in antibody response of mice to tumour inoculation."* Br J Cancer 4 : 372-379.

330. **Morgenstern, J.P. and H. Land.** (1990)*"Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line."* Nucleic Acids Res 18 : 3587-3596.

331. **Rötzschke, O., K. Falk, S. Stevanovic, G. Jung, P. Walden, and H-G. Rammensee.** (1991)*"Exact prediciton of a natural T cell epitope."* Eur J Immunol 21 : 2891-2894.

332. **Rhim, J.S., H.Y. Cho, and R.J. Huebner.** (1975)*"Non-producer human cells induced by murine sarcoma virus."* Int J Cancer 15 : 23-29.

333. **Medappa, K.C., McLean, C., and R.R. Rueckert** (1971)*"On the structure of rhinovirus 1A"* Virology 44 : 259-270.

334. **Puck, T.T., S.J. Cieciura, and A. Robinson.** (1958)*"Genetics of somatic mammalian cells."* J Exp Med 108 : 945-960.

335. **Stauss, H.J., H. Davies, E. Sadovnikova, B. Chain, N. Horowitz, and C. Sinclair.** (1992)*"Induction of cytotoxic T lymphocytes with peptides in vitro: identification of candidate T-cell epitopes in human papilloma virus."* Proc Natl Acad Sci USA 89 : 7871-7875.

336. **Ozato, K., T.H. Hansen, and D.H. Sachs.** (1980)*"Monoclonal antibodies to mouse MHC antigens.*
II. Antibodies to the H-2L^d antigen, the products of a third polymorphic locus of the mouse major histocompatibility complex." J Immunol 125 : 2473-2477.

337. **Smith, M.H. and B.H. Barber.** (1990)*"The conformational flexibility of class I H-2 molecules as revealed by antipeptide antibodies specific for intracytoplasmic determinants: differential reactivity of beta-2 microglobulin "bound" and "free" heavy chains."* Mol Immunol 27 : 169-180.

338. **Williams, D.B., B.H. Barber, R.A. Flavell, and H. Allen.** (1989)*"Role of beta-2 microglobulin in the intracellular transport and surface expression of murine class I histocompatibility molecules."* J Immunol 142 : 2796-2806.

339. **Hämmerling, G.J., E. Rüsch, N. Tada, S. Kimura, and U. Hämmerling.** (1982)*"Localization of allodeterminants on H-2K^b antigens determined with monoclonal antibodies and H-2 mutant mice."* Proc Natl Acad Sci USA 79 : 4737-4741.

340. **Ozato, K., N.M. Mayer, and D. Sachs.** (1982)*"Monoclonal antibodies to mouse major histocompatibility complex antigens."* Transplantation 34 : 113-120.

341. **Bhattacharya, A., M.E. Dorf, and T.A. Springer.** (1994)*"A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication."* J Immunol 127 : 2488-2495.

342. **Metlay, J.P., M.D. Witmer-Pack, R. Agger, M.T. Crowley, D. Lawless, and R.M. Steinman.** (1990)*"The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies."* J Exp Med 171 : 1753-1771.

343. **Asensi, V., K. Kimeno, I. Kawamura, M. Sakumot, and K. Nomoto.** (1989)*"Treatment of autoimmune MRL/lpr mice with anti-B220 monoclonal antibody reduces the level of anti-DNA antibodies and lymphadenopathies."* Immunology 68 : 204-208.

344. **Coffman, B.** (1982)*"Surface antigen expression and immunoglobulin rearrangement during mouse pre-B cell development."* Immunol Rev 69 : 5-23.

345. **Zuhair, K., K. Ballas, and W. Rasmussen.** (1993)*"Lymphokine-activated killer cells VII. IL-4 induces an NK1.1$^+$ CD8$\alpha^+\beta^+$ TCR$^-\alpha\beta$ B220$^+$ lymphokine-activated killer subset."* J Immunol 150 : 17-30.

346. **Razi-Wolf, Z., G.J. Freeman, F. Galvin, B. Benacerraf, L. Nadler, and H. Reiser.** (1992)*"Expression and function of the murine B7 antigen, the major costimulatory molecule expressed by peritoneal exudate cells."* Proc Natl Acad Sci USA 89 : 4210-4214.

347. **Ho, M-K. and T.A. Springer.** (1982)*"Mac-1 antigen: quantitative expression in macrophage populations and tissues, and immunofluorescent localization in spleen."* J Immunol 128 : 2281-2286.

348. **Springer, T., G. Galfré, D.S. Secher, and C. Milstein.** (1979)*"Mac-1: a macrophage differentiation antigen identified by monoclonal antibody."* Eur J Immunol 9 : 301-306.

349. **Barnstable, C.J., W.F. Bodmer, G. Brown, C. Milstein, A.F. Williams, and A. Ziegler.** (1978)*"Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis."* Cell 14 : 9-20.

350. **Udaka, K., T.J. Tsomides, P. Walden, N. Fokusen, and H.N. Eisen.** (1993)*"A ubiquitious protein is the source of naturally occuring peptides that are recognized by a CD8$^+$ T cell clone."* Proc Natl Acad Sci USA 90 : 11272-11276.

351. **Stauss, H.J. and E. Sadovnikova**, ed. (1996) *"Cytotoxic T lymphocytes"*. In Handbook of Experimental Immunology, ed. D.M. Weir, L.A. Herzenberg, C. Blackwell, and L.A. Herzenberg. Blackwell Science, Cambridge, MA : in press.

352. **Hornung, R.L., D.L. Longo, V.L. Gowda, and L.W. Kwak.** (1996)*"Induction of a CD8$^+$ cytotoxic T lymphocyte response to soluble antigen given together with a novel muramyl dipeptide adjuvant, N-acetyl-D-glucosaminyl-(β1-4)-N-acetylmuramyl-alanyl-D-isoglutamine (GMPD)."* Therapeutic Immunol 1 : in press.

353. **Austyn, J.M.** (1987)*"Lymphoid dendritic cells."* Immunology 62 : 161-170.

354. **Talavera, A. and J.M. Rodriguez.** (1991)*"Isolation and handling of recombinant vaccinia viruses."* In Practical Molecular Virology: Viral vectors for gene expression, ed. M. Collins. The Humana Press Inc., Clifton, NJ : 235-248.

355. **Finlay, C.A.** (1993)*"The mdm-2 oncogene can overcome wild-type p53 suppression of transformed cell growth."* Mol Cell Biol 13 : 301-306.

356. **Jiang, W., S.M. Kahn, P. Zhou, Y. J. Zhang, A. M. Cacace, A.S. Infante, S. Doi, R.M. Santella, and I.B. Weinstein.** (1993)*"Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth-control, cell-cycle progression and gene-expression."* Oncogene 8 : 3447-3457.

357. **Lukas, J., M. Pagano, Z. Staskova, G. Draetta, and J. Bartek.** (1994)*"Cyclin D1 protein oscillliates and is essential for cell cycle progression in human tumour cell lines."* Oncogene 9 : 707-718.

358. **Lammie, G.A., R. Smith, J. Silver, S. Brookes, C. Dickson, and G. Peters.** (1992)*"Proviral insertions near cyclin D1 in mouse lymphomas: a parallel for BCL1 translocations in human B-cell neoplasms."* Oncogene 7 : 2381-2387.

359. **Bernard, O., M. Li, and H.H. Reid.** (1991)*"Expression of two different forms of fibroblast growth factor receptor 1 in different mouse tissues and cell lines."* Proc Natl Acad Sci USA 88 : 7625-7629.

360. **Dittmer, D., S. Pati, G. Zambetti, S. Chu, A.K. Teresky, M. Moore, C. Finlay, and A.J. Levine.** (1993)*"Gain of function mutations in p53."* Nat Genet 4 : 42-45.

361. **Frebourg, T., J. Kassel, K.T. Lam, M.A. Gryka, N. Barbier, T. Ikdahl Andersen, A.-L. Boerresen, and S.H. Friend.** (1992)*"Germ-line mutations of the p53 suppressor gene in patients with high risk for cancer inactivate the p53 protein."* Proc Natl Acad Sci USA 89 : 6413-6417.

362. **Yonish-Rouach, E., D. Grunwald, S. Wilder, A. Kimchi, E. May, J.-J. Lawrence, P. May, and M. Oren.** (1993)*"p53-mediated cell death: relationship to cell cycle control."* Mol Cell Biol 13 : 1415-1423.

363. **Ullrich, S.J., W.E. Mercer, and E. Appella.** (1992)*"Human wild type p53 adopts a unique conformational and phosphorylation state in vivo during growth arrest of glioblastoma cells."* Oncogene 7 : 1635-1643.

364. **Mercer, W.E., M.T. Shileds, D. Lin, E. Appella, and S.J. Ullrich.** (1991)*"Growth suppression induced by wild-type p53 protein is accompagnied by selective down-regulation of proliferating-cell nuclear antigen expression."* Proc Natl Acad Sci USA 88 : 1958-1962.

365. **Mercer, W.E., M.T. Shields, M. Amin, G.J. Sauve, E. Appella, J.W. Romano, and S.J. Ullrich.** (1990)*"Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53."* Proc Natl Acad Sci USA 87 : 6166-6170.

366. **Shaulsky, G., N. Goldfinger, and V. Rotter.** (1991)*"Alterations in tumor development in vivo mediated by expression of wil type or mutant p53 proteins."* Cancer Res 51 : 5232-5237.

367. **Barak, Y. and M. Oren.** (1992)*"Enhanced binding of a 95 kDa protein to p53 in cells undergoing p53-mediated growth arrest."* EMBO J 11 : 2115-2121.

368. **Barak, Y., T. Juven, R. Haffner, and M. Oren.** (1993)*"mdm2 expression is induced by wild type p53 activity."* EMBO J 12 : 461-468.

369. **Haines, D.S., J.E. Landers, L.J. Engle, and D.L. George.** (1994)*"Physical and functional interaction between wild-type p53 and mdm2 proteins."* Mol Cell Biol 14 : 1171-1178.

370. **Smith, R., G. Peters, and C. Dickson.** (1995)*"Genomic organization of the mouse cyclin D1 gene (cyl-1)."* Genomics 25 : 85-92.

371. **Betticher, D.C., N. Thatcher, H.J. Altermatt, P. Hoban, W.D.J. Ryder, and J. Heighway.** (1995)*"Alternate splicing produces a novel cyclin D1 transcript."* Oncogene 11 : 1005-1011.

372. **Lebwohl, D.E., R. Muise-Helmericks, L. Sepp-Lorenzino, S. Serve, M. Timaul, R. Bol, P. Borgen, and N. Rosen.** (1994)*"A truncated cyclin D1*

gene encodes a stable messenger-RNA in a human breast-cancer cell-line." Oncogene 9 : 1925-1929.

373. **Farabaugh, P.J.** (1993)*"Alternative readings of the genetic code."* Cell 74 : 591-596.

374. **Krug, R.M.** (1993)*"The regulation of export of mRNA from nucleus to cytoplasm."* Curr Opin Cell Biol 5 : 944-949.

375. **Rosbash, M. and R.H. Singer.** (1993)*"RNA travel: tracks from DNA to cytoplasm."* Cell 75 : 399-401.

376. **Kozak, M.** (1992)*"Regulation of translation in eukaryotic systems."* Annu Rev Cell Biol 8 : 197-225.

377. **Townsend, A., J. Bastin, K. Gould, G. Brownlee, M. Andrew, B. Coupar, D. Boyle, C. Chan, and G. Smith.** (1988)*"Defective presentation to class I-restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen."* J Exp Med 168 : 1211-1224.

378. **Fetten, J.V., N. Roy, and E. Gilboa.** (1991)*"A frameshift mutation at the NH_2- terminus of the nucleoprotein gene does not affect generation of cytotoxic T lymphocytes epitopes."* J Immunol 147 : 2697-2705.

379. **Shastri, N. and F. Gonzalez.** (1993)*"Endogenous generation and presentation of the ovalbumin peptide/K^b complex to T cells."* J Immunol. 150 : 2724-2736.

380. **Shastri, N., V. Nguyen, and F. Gonzalez.** (1995)*"Major histocompatibility class I molecules present cryptic translation products to T-cells."* J Biol Chem 270 : 1088-1091.

381. **Jiang, W., W.J. Swiggard, C. Heufler, M. Peng, A. Mirza, R.M. Steinman, and M.C. Nussenzweig.** (1995)*"The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing."* Nature 375 : 151-155.

382. **Ohashi, P.S., S. Oehen, K. Buerki, H. Pircher, C.T. Ohashi, B. Odermatt, B. Malissen, R.M. Zinkernagel, and H. Hengartner.** (1991)*"Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice."* Cell 65 : 305-317.

383. **Tarpey, I., S. Stacey, J. Hickling, H.D.L. Birley, A. Renton, A. McIndoe, and D.H. Davies.** (1994)*"Human cytotoxic T lymphocytes stimulated by endogenously processed human papillomavirus type 11 E7 recognize a peptide containing a HLA-A2 (A*0201) motif."* Immunology 81 : 222-227.

384. **Ashton-Rickardt, P.G. and S. Tonegawa.** (1994)*"A differential-avidity model for T-cell selection."* Immunol Today 15 : 362-366.

385. **Kuhröber, A., R. Schirmbek, and J. Reimann.** (1994)*"A self-reactive class I-restricted T-cell response of H-2^b mice to determinants of the Vβ8.2 domain of the T-cell receptor for antigen."* Immunology 83 : 532-539.

386. **Panina-Bordignon, P., R. Lang, P.M. van Endert, E. Benazzi, A.M. Felix, R.M. Pastore, G.A. Spinas, and F. Sinigaglia.** (1995)*"Cytotoxic T Cells specific for glutamic acid decarboxylase in autoimmune diabetes."* J Exp Med 181 : 1923-1927.

387. **Tsuchida, T., K.C. Parker, R.V. Turner, H.F. McFarland, J.E. Coligan, and W.E. Biddison.** (1994)*"Autoreactive CD8+ responses to human myelin protein-derived peptides."* Proc Natl Acad Sci USA 91 : 10859-10863.

388. **Boon, T., T.F. Gajewski, and P.G. Coulie.** (1995)*"From defined human tumour antigens to effective immunization."* Immunol Today 16 : 334-335.

389. **van Pel, A., P. van der Bruggen, P.G. Coulie, V.G. Brichard, B. Lethe, B. van den Eynde, C. Uyttenhove, J.-C. Renauld, and T. Boon.** (1995)*"Genes coding for tumour antigens recognized by cytolytic T lymphocytes."* Immunol Rev 145 : 229-250.

390. **Moskophidis, D., E. Laine, and R.M. Zinkernagel.** (1993)*"Peripheral clonal deletion of antiviral memory CD8+ T cells."* Eur J Immunol 23 : 3306-3311.

391. **Hämmerling, G.J., G. Schönrich, I. Ferber, and B. Arnold.** (1993)*"Peripheral tolerance as a multistep mechanism."* Immunol Rev 133 : 93-104.

392. **Pichler, W.J. and T. Wyss-Coray.** (1995)*"T cells as antigen-presenting cells."* Immunol Today 15 : 312-315.

393. **Barnaba, V., C. Watts, M. de Boer, P. Lane, and A. Lanzavecchia.** (1994)*"Professional presentation of antigen by activated human T cells."* Eur J Immunol 24 : 71-75.

394. **Sarvetnick, N., D. Liggit, S.L. Pitts, S.E. Hansen, and T.A. Stewart.** (1988)*"Insulin-dependent diabetes mellitus induced in transgenic mice by ectopic expression of class II MHC and interferon-gamma."* Cell 52 : 773-782.

395. **Unutmaz, D., P. Pileri, and S. Abrignani.** (1994)*"Antigen-independent activation of naive and memory resting T cells by a cytokine combination."* J Exp Med 180 : 1159-1164.

396. **Doherty, P.C., S. Hou, and R.A. Tripp.** (1994)*"CD8+ T-cell memory to viruses."* Curr Opin Immunol 6 : 545-552.

397. **Isobe, H., T. Morna, S. Li, A. Young, S. Nathenson, P. Palese, and C. Bona.** (1995)*"Presentation by a major histocompatibility complex class I molecule of nucleoproteins peptide expressed in two different genes of an influenza virus transfectant."* J Exp Med 181 : 203-213.

398. **Tanchot, C. and B. Rocha.** (1995)*"The peripheral T cell repertoire: independent homeostatic regulation of virgin and activated CD8+ T cell pools."* Eur J Immunol 25 : 2127-2136.

399. **Dick, L.R., C. Aldrich, S.C. Jameson, Moomaw. C.R., B.C. Pramanik, C.K. Doyle, G.N. DeMartino, M.J. Bevan, J.M. Forman, and C.A. Slaughter.** (1994)*"Proteolytic processing of ovalbumin and β–galactosidase by the proteasome to yield antigenic peptides."* J Immunol 152 : 3884-3894.

400. **Koup, R.A.** (1994)*"Virus escape from CTL recognition."* J Exp Med 180 : 779-782.

401. **Jameson, S.C. and M.J. Bevan.** (1995)*"T cell receptor antagonists and partial agonists."* Immunity 2 : 1-11.

402. **Inaba, K., M. Witmer-Pack, M. Inaba, K.S. Hathcock, H. Sakuta, M. Azuma, H. Yagita, K. Okumura, P.S. Linsley, S. Ikehara, S.Hodes**

Muramatsu R.J., and R.M. Steinman. (1994)*"The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro."* J Exp Med 180 : 1849-1860.

403. **Cocks, B.G., C-C. J. Chang, J.M. Carballido, H. Yssel, and J.E. de Vries.** (1995)*"A novel receptor involved in T-cell activation."* Nature : 260-262.

404. **Manjunath, N., M. Correa, M. Ardman, and B. Ardman.** (1995)*"Negative regulation of T cell adhesion and activation by CD43."* Nature 377 : 535-538.

405. **Macatonia, S.E., N.A. Hosken, M. Litton, P. Vieira, C-S. Hsieh, J.A. Culpepper, M. Wysocka, G. Trinchieri, K.M. Murphy, and A. O'Garra.** (1995)*"Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4$^+$ T cells."* J Immunol 154 : 5071-5079.

406. **Noble, A., P.A. Macary, and D.M. Kemeny.** (1995)*"IFN-γ and IL-4 regulate the growth and differentiation of CD8$^+$ T cells into subpopulations with distinct cytokine profiles."* J Immunol 155 : 2928-2937.

407. **McDonagh, M. and E.B. Bell.** (1995)*"The survival and turnover of mature and immature CD8 T cells."* Immunology 84 : 514-520.

408. **Brichard, V.G., G. Warnier, A. Van Pel, G. Morlighem, S. Lucas, and T. Boon.** (1995)*"Individual differences in the orientation of the cytolytic T cell response against mouse tumor P815."* Eur J Immunol 25 : 664-671.

409. **Falk, K., O. Rötzschke, K. Deres, J. Metzger, G. Jung, and H-G. Rammensee.** (1991)*"Identification of naturally processed viral nonapeptides allows their quantification in infected cells and suggests an allele-specific T cell epitope forecast."* J Exp Med 174 : 425-434.

410. **Wallny, H-J., K. Deres, S. Faath, G. Jung, A. van Pel, T. Boon, and H-G. Rammensee.** (1992)*"Identification and quantitation of a naturally presented peptide as recognized by cytotoxic T lymphocytes specific for an immunogenic tumor variant."* Int Immunol 4 : 1085-1090.

411. **Lie, W.-R., N.B. Myers, J. Gorka, R.J. Rubocki, J.M. Connolly, and T.H. Hansen.** (1990)*"Peptide ligand-induced conformation and surface expression of the L^d class I molecule."* Nature 344 : 439-441.

412. **Gabathuler, R., G. Reid, G. Kolaitis, J. Driscoll, and W.A. Jefferies.** (1994)*"Comparison of cell lines deficient in antigen presentation reveals a functional role for TAP-1 alone in antigen processing."* J Exp Med 180 : 1415-1425.

413. **Carbone, F.R. and M.J. Bevan.** (1989)*"Induction of ovalbumin specific cytotoxic T cells by in vivo peptide immunization."* J Exp Med 169 : 603-612.

414. **van Kaer, L., P.G. Ashton-Rickardt, H.L. Plough, and S. Tonegawa.** (1992)*"TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4$^-$CD8$^+$ T cells."* Cell 71 : 1205-1214.

415. **Kast, W.M., L. Roux, J. Curren, H.J.J. Blom, A.C. Voordouw, R.H. Meloen, D. Kolakofsky, and C.J.M. Melief.** (1991)*"Protection against lethal Sendai virus infection by in vivo priming of virus -specific cytotoxic T lymphocytes with a free synthetic peptide."* Proc Natl Acad Sci USA 88 : 2283-2287.

416. **Schumacher, T.N.M., M.L.H. de Bruijn, L.N. Vernie, M.W. Kast, C.J.M. Melief, J.J. Neefjes, and H.L. Ploegh.** (1991)*"Peptide selection by MHC class I molecules."* Nature 350 : 703-706.

417. **Cole, G.A., T.L. Hogg, and D.L. Woodland.** (1995)*"T-cell recognition of the immunodominant sendai-virus NP324-332/K-b epitope is focused on the center of the peptide."* J Immunol 155 : 2841-2848.

418. **Widmann, C., J.L. Maryanski, P. Romero, and G. Corradin.** (1991)*"Differential stability of antigenic MHC Class I-restricted synthetic peptides."* J Immunol 147 : 3745-3751.

419. **Kozlowski, S., M. Corr, T. Takeshita, L. F. Boyd, C.D. Pendleton, R.N. Germain, J.A. Berzofsky, and D.H. Margulies.** (1992)*"Serum angiotensin-1 converting enzyme activity processes a human immunodeficiency virus 1 gp160 peptide for presentation by major histocompatibility complex class I molecules."* J Exp Med 175 : 1417-22.

420. **Sherman, L.A., T.A. Burke, and J.A. Bigge.** (1992)*"Extracellular processing of antigens that bind class I major histocompatibility molecules."* J Exp Med 175 : 1221-1226.

421. **Zhang, W., A.C.M. Young, M. Imarai, S.G. Nathenson, and J.C. Sacchettini.** (1992)*"Crystal structure of the major histocompatibility complex class I H-2K^b molecule containing a single viral peptide: implications for peptide binding and T cell receptor recognition."* Proc Natl Acad Sci USA 89 : 8403-8407.

422. **Deres, K., T.N.M. Schumacher, K-H. Wiesmüller, S. Stevanovic, G. Greiner, G. Jung, and H.L. Plough.** (1992)*"Preferred size of peptides that bind to H-2K^b is sequence dependent."* Eur J Immunol 22 : 1603-1608.

423. **Fremont, D.H., E. Stura, M. Matsumara, P.A. Peterson, and I. A. Wilson.** (1995)*"Crystal structure of an H-2K^b -ovalbumin peptide complex reveals the interplay of primary and secondary anchor positions in the major histocompatibility complex binding groove."* Proc Natl Acad Sci USA 92 : 2479-2483.

424. **Jameson, S.C. and M.J. Bevan.** (1992)*"Dissection of major histocompatibility complex (MHC) and T cell receptor contact residues in a K^b restricted ovalbumin peptide and an assessment of the predictive power of MHC binding motifs."* Eur J Immunol 22 : 2663-2667.

425. **Elliott, T., J. Elvin, V. Cerundolo, H. Allen, and A. Townsend.** (1992)*"Structural requirements for the peptide-induced conformational change of free major histocompatibility complex class I heavy chains."* Eur J Immunol 22 : 2085-2091.

426. **Chen, W., S. Khilko, J. Fecondo, D.H. Margulies, and J. McCluskey.** (1994)*"Determinant selection of major histocompatibility complex class I-restricted antigenic peptides is explained by class I-peptide affinity and is strongly influenced by nondominant anchor residues."* J Exp Med 180 : 1471-1483.

427. **Elvin, J.G., V. Cerundolo, T.J. Elliott, and A.R.M. Townsend.** (1991)*"A quantitative assay of peptide dependent class I assembly."* Eur J Immunol 21 : 2025-2031.

428. **De Bruijn, M.L.H., J.D. Nieland, T.N.M. Schumacher, H.L. Plough, W.M. Kast, and C.J.M. Melief.** (1992)*"Mechanisms of induction of primary virus-specific cytotoxic T lymphocyte responses."* Eur J Immunol 22 : 3013-3020.

429. **Macatonia, S.E., P.M. Taylor, S.E. Knight, and B.A. Askonas.** (1989)*"Primary stimulation by dendritic cells induces antiviral proliferative and cytotoxic T cell responses in vitro."* J Exp Med 169 : 1255-1264.

430. **Macatonia, S.E., S. Patterson, and S.C. Knight.** (1991)*"Primary proliferative and cytotoxic T cell-responses to HIV induced in vitro by human dendritic cells."* Immunology 74 : 399-406.

431. **Mehta-Damani, A., S. Markowicz, and E.G. Engleman.** (1994)*"Generation of antigen-specific CD8+ ctls from naive precursors."* J Immunol 153 : 996-1003.

432. **Nonacs, R., R. Humborg, J.P. Tam, and R.M. Steinman.** (1992)*"Mechanisms of mouse spleen dendritic cell function in the generation of influenza-specific cytolytic T lymphocytes."* J Exp Med 176 : 519-529.

433. **Klaus, G.G.B.**, ed. (1987) *"Lymphocytes"*. A practical approach. IRL Press, Oxford, UK.

434. **Day, P.M., F. Esquivel, J. Lukszo, J.R. Bennink, and J.W. Yewdell.** (1995)*"Effect of tap on the generation and intracellular trafficking of peptide-receptive major histocompatibility complex class-i molecules."* Immunity 2 : 137-147.

435. **Al-Ramadi, B.K., M.T. Jelonek, L.F. Boyd, D.H. Margulies, and A.L.M. Bothwell.** (1995)*"Lack of strict correlation of functional sensitization with the apparent affinity of MHC/peptide complexes for the TCR."* J Immunol 155 : 662-673.

436. **Ribaudo, R.K. and D.H. Margulies.** (1995)*"Polymorphism at position nine of the MHC Class I heavy chain affects the stability of association with β2-microglobulin and presentation of viral peptide."* J Immunol 155 : 3481-3493.

437. **Bix, M. and D. Raulet.** (1992)*"Functionally conformed free class I heavy chains exist on the surface of β2 microglobulin negative cells."* J Exp Med 176 : 829-834.

438. **Neefjes, J.J., J. Dierx, and H.L. Ploegh.** (1993)*"The effect of anchor residue modifications on the stability of major histocompatibility complex class I- peptide interactions."* Eur J Immunol 23 : 840-845.

439. **Sijts, A.J.A.M., M.L.H. De Bruijn, J.D. Nieland, W.M. Kast, and C.J.M. Melief.** (1992)*"Cytotoxic T lymphocytes against the antigen-processing-defective RMA-S tumor cell line."* Eur J Immunol 22 : 1639-1642.

440. **Motal, U.M.A., X. Zhou, A. Joki, A.R. Siddiqi, B.R. Srinivasa, K. Stenvall, J. Dahmen, and M. Jondal.** (1993)*"Major histocompatibility complex class I-binding peptides are recycled to the cell surface after internalisation."* Eur J Immunol 23 : 3224-3229.

441. **Gagliardi, M.C., G. de Petrillo, S. Salemi, L. Boffa, M. G. Longobardi, P. Dellabona, G. Casorati, N. Tanigaki, R. Harris, A. Lanzavecchia, and V. Barnaba.** (1995)*"Presentation of peptides by cultured monocytes or activated T cells allows specific priming of human cytotoxic T lymphocytes in vitro."* Int Immunol 7 : 1741-1752.

442. **Ossevoort, M.A., M.C.W. Feltkamp, K.J.H. van Veen, C.J.M. Melief, and W.M. Kast.** (1995)*"Dendritic cells as carriers for a cytotoxic T-lymphocyte epitope-based peptide vaccine in protection against a human papillomavirus type 16-induced tumor."* J Immunother 18 : 86-94.

443. **Cassell, D.J and R.H. Schwartz.** (1994)*"A quantitative analysis of antigen-presenting cell function: activated B cells stimulate naive CD4 T cells but are inferior to dendritic cells in providing costimulation."* J Exp Med 180 : 1829-1840.

444. **Fuchs, E.J. and P. Matzinger.** (1992)*"B cells turn off virgin but not memory T cells."* Science 258 : 1156-1159.

445. **Feltkamp, M.C.W., M.P.M. Vierboom, W.M. Kast, and C.J.M. Melief.** (1994)*"Efficient MHC Class I-peptide binding is required but does not ensure MHC Class I restricted immunogenicity."* Mol Immunol 31 : 1391-1401.

446. **Lipford, G.B., S. Bauer, H. Wagner, and K. Heeg.** (1995)*"In vivo CTL induction with point substituted ovalbumin peptides: immunogenicity correlates with peptide-induced MHC class I stability."* Vaccine 13 : 313-320.

447. **Rötzschke, O., K. Falk, K. Deres, H. Schild, M. Norda, J. Metzger, G. Jung, and H.-G. Rammensee.** (1990)*"Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells."* Nature 348 : 252-254.

448. **Falk, K., O. Rötzschke, and H.-G. Rammensee.** (1990)*"Cellular peptide composition governed by major histocompatibility complex class I molecules."* Nature 348 : 248-251.

449. **Malarkannan, S., S. Goth, D.R. Buchholz, and N. Shastri.** (1995)*"The role of MHC class I molecules in the generation of endogenous peptide/MHC complexes."* J Immunol 154 : 585-598.

450. **Wallny, H.-J., O. Rötzschke, K. Falk, G. Hämmerling, and H.-G. Rammensee.** (1992)*"Gene transfer experiments imply instructive role of major histocompatibility complex class I molecules in cellular peptide processing."* Eur J Immunol 22 : 655-659.

451. **Eberl, G., M. A. Roggero, and G. Corradin.** (1995)*"A simple mathematical-model for the functional peptide/MHC/TCR interactions."* J Immunol 154 : 219-225.

452. **Janeway, C.A.** (1994)*"Thymic selection: two pathways to life and two to death."* Immunity 1 : 3-6.

453. **Bhardwaj, V., V. Kumar, H.M. Geysen, and E.E. Sercarz.** (1993)*"Degenerate recognition of a dissimilar antigenic peptide by myelin basic protein-reactive T cells."* J Immunol 151 : 5000-5010.

454. **Hagerty, D.T. and P.M. Allen.** (1995)*"Intramolecular mimicry."* J Immunol 155 : 2993-3001.

455. **Burrows, S.R., R. Khanna, J.M. Burrows, and D.J. Moss.** (1994)*"An alloresponse in humans is dominated by cytotoxic T lymphocytes (CTL) crossreactive with a single Epstein-Barr virus CTL epitope: implications for Graft-versus-Host Disease."* J Exp Med 179 : 1155-1161.

456. **Schild, H., O. Rötzschke, H. Kalbacher, and H-G. Rammensee.** (1990)*"Limit of T cell tolerance to self proteins by peptide presentation."* Science 247 : 1587-1589.

457. **Calin-Laurens, V., M.C. Tréscol-Biémont, D. Gerlier, and C. Rabourdin-Combe.** (1993)*"Can one predict antigenic peptides for MHC class I-restricted cytotoxic T-lymphocytes useful for vaccination."* Vaccine 11 : 974-978.

458. **Feltkamp, M.C.W., G.R. Vreugdenhil, M.P.M. Vierboom, E. Ras, S. van der Burg, J. ter Schegget, C.J.M. Melief, and W.M. Kast.** (1995)*"Cytotoxic T lymphocytes raised a subdominant epitope offered as a synthetic peptide eradicate human papillomavirus type16-induced tumours."* Eur J Immunol 25 : 2638-2642.

459. **Irvine, K.R., B.J. McCabe, S.A. Rosenberg, and N.P. Restifo.** (1995)*"Synthetic oligonucleotide expressed by a recombinant vaccinia virus elicits therapeutic ctl."* J Immunol 154 : 4651-4657.

460. **Restifo, N.P., I. Bacik, K.R. Irvine, J.W. Yewdell, B.M. McCabe, R. W. Anderson, L.C. Eisenlohr, S.A. Rosenberg, and J.R. Bennink.** (1995)*"Antigen processing in vivo and the elicitation of primary CTL responses."* J Immunol 154 : 4414-4422.

461. **McCabe, B.J., K.R. Irvine, M.I. Nishimura, J.C. Yang, P.J. Spiess, E.P. Shulman, S.A. Rosenberg, and N.P. Restifo.** (1995)*"Minimal determinant expressed by a recombinant vaccinia virus elicits therapeutic antitumor cytolytic T-lymphocyte responses."* Cancer Res 55 : 1741-1747.

462. **Salgaller, M.L., A. Afshar, F.M. Marincola, L. Rivoltini, Y. Kawakami, and S.A. Rosenberg.** (1995)*"Recognition of multiple epitopes in the human melanoma antigen gp100 by peripheral blood lymphocytes stimulated in vitro with synthetic peptides."* Cancer Res 55 : 4972-4979.

463. **Wu, Y. and Y. Liu.** (1994)*"Viral induction of co-stimulatory activity on antigen-presenting cells bypasses the need for $CD4^+$ T-cell help in $CD8^+$ T cell responses."* Curr Biol 4 : 499-505.

464. **Wada, H., T. Ono, A. Uenaka, M. Monden, and E. Nakayama.** (1995)*"Requirement of $CD4^+$ T cells and antigen presenting cells for primary in vitro generation of $CD8^+$ cytotoxic T cells against L^d binding self peptide p2Ca."* Immunology 84 : 633-637.

465. **Grohman, U., R. Bianchi, M.C. Fioretti, L. Binaglia, C. Uyttenhove, A. Van Pel, T. Boon, and P. Puccetti.** (1995)*"$CD8^+$ cell activation to a major mastocytoma rejection antigen, P815AB: requirement for tum^- or helper peptides in priming for skin test reactivity to a P815AB-related peptide."* Eur J Immunol 25 : 2797-2802.

466. **Sutter, G. and B. Moss.** (1992)*"Nonreplicating vaccinia vector efficiently expresses recombinant genes."* Proc Natl Acad Sci USA 89 : 10847-10851.

467. **Erickson, A.L. and C.M. Walker.** (1993)*"Class I major histocompatibility complex-restricted cytotoxic T cell responses to vaccinia virus in humans."* J Gen Virol 74 : 751-754.

468. **Pan, Z.K., G. Ikonomidis, A. Lazenby, D. Pardoll, and Y. Paterson.** (1995)*"A recombinant Listeria monocytogenes vaccine expressing a model tumor antigen protects mice against lethal tumor cell challenge and causes regression of established tumors."* Nat Med 1 : 471-477.

469. **Falo, L.D., M. Kovacsovics-Bankowski, K. Thompson, and K.L. Rock.** (1995)*"Targeting antigen into the phagocytic pathway in vivo induces protective tumor immunity."* Nat Med 1 : 649-653.

470. **Pardoll, D.M. and A.M. Beckerleg.** (1995)*"Exposing the immunology of naked DNA vaccines."* Immunity 3 : 165-169.

471. **Culver, K.W. and R.M. Blaese.** (1994)*"Gene therapy for cancer."* Trends Genet 10 : 174-178.

472. **Crystal, R.G.** (1995)*"Transfer of genes to humans: early lessons and obstacles to success."* Science 270 : 404-410.

473. **Srivastava, P.K. and H. Udono.** (1994)*"Heat shock protein-peptide complexes in cancer immunotherapy."* Curr Opin Immunol 6 : 728-732.

474. **Udono, H., D.L. Levey, and P.K. Srivastava.** (1994)*"Cellular requirements for tumor-specific immunity elicited by heat shock proteins: tumor rejection antigen gp96 primes CD8+ T cells in vivo."* Proc Natl Acad Sci USA 91 : 3077-81.

475. **Edgington, S.M.** (1995)*"Therapeutic applications of heat shock proteins."* Biotechnology 13 : 1442-1444.

476. **Pardoll, D.M.** (1993)*"Cancer vaccines."* Immunol Today 14 : 310-316.

477. **Papadopoulos, E.B., M. Ladanyi, D. Emanuel, S. Mackinson, F. Boulad, M.H. Carabasi, H. Castro-Malaspina, B.H. Childs, A.P. Gillio, T.N. Small, J.W. Young, N.A. Kernan, and R.J. O'Reilly.** (1994)*"Infusions of donor leukocytes to treat Epstein -Barr virus associated lymphoproliferative disorders after allogeneic bone marrow transplantation."* N Engl J Med 330 : 1185-91.