

THE STABILITY OF IMMUNOGLOBULIN MESSENGER RNA IN
B CELL LINES

A thesis presented by

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ABSTRACT

An important determinant of the level of protein produced from any particular gene is the cellular content of its messenger RNA, which in turn may be regulated at the levels of both synthesis and degradation. For example, as B cells differentiate, there is a large increase in the abundance of messenger RNA coding for immunoglobulin, which is not fully accounted for by an increase in the rate of gene transcription. The aim of this work has been to investigate the role of changes in messenger RNA stability in the regulation of immunoglobulin gene expression.

Initial experiments used a model system, consisting of mouse fibroblast cells containing a high copy number recombinant human growth hormone gene, to develop a method for measuring RNA half-lives in mammalian cells. Pulse chase analysis was carried out using a ribonuclease protection assay to detect specific transcripts. This method was then used to investigate the rates of decay of immunoglobulin heavy chain RNA in two cell lines representing the resting B cell and the plasma cell stages of differentiation. It was found that heavy chain RNA had a significantly longer half-life in the cell line representing the plasma cell stage. This increase in half-life makes a significant contribution to the increase in heavy chain mRNA levels in these cells.

To investigate factors which might control the stability of heavy chain RNA in plasma cells, a transfection system was employed to facilitate half-life measurements on a variety of mutated heavy chain RNA molecules. Heavy chains altered at their 5' and 3' ends were placed under the control of a heat shock promoter, and transfected into cells representing the plasma cell stage. This allowed a pulse of heavy chain mRNA to be generated upon treatment of the cells with elevated temperature, the decay of which could be monitored by Northern blot analysis. It was found that sequences at the 5' end of the molecule are important for its stability in cell lines representing the plasma cell stage.

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ABBREVIATIONS

A	amperes
A ₂₆₀	absorbance at wavelength 260nm
A ₆₀₀	absorbance at wavelength 600nm
A ₄₅₀	absorbance at wavelength 450nm
ATP	adenosine 5'-triphosphate
bis-acrylamide	N,N'-methylene-bis-acrylamide
bp	base pair(s) of DNA
BSA	bovine serum albumin
°C	degrees Celsius
CDR	complementarity-determining region
Ci	Curies
C μ	μ heavy chain constant region
c.p.m.	counts per minute
CTP	cytidine 5'triphosphate
D	diversity region
dATP	2-deoxyadenosine 5'-triphosphate
dCTP	2-deoxycytidine 5'-triphosphate
dGTP	2-deoxyguanosine 5'-triphosphate
dTTP	2-deoxythymidine 5'-triphosphate
DEPC	diethyl pyrocarbonate
DH	heavy chain diversity region
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
d.p.m.	disintegrations per minute
DRB	5,6-di-chloro1- β -D-ribofuranosyl benzimidazole
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylenediaminetetra acetic acid, disodium salt
F	Farad
FCS	foetal calf serum
Fig.	figure
g	gram
GTP	guanosine 5'-triphosphate
hr(s)	hour(s)
hGH	human growth hormone
h.p.l.c	high pressure liquid chromatography

IPTG	isopropyl β -D-thiogalactoside
IRE	iron response element
J	joining region
JH	heavy chain joining region
JL	light chain joining region
k-	kilo-
kDa	kilodalton
kb	kilobase pairs of DNA
K_m	Michaelis constant
l	litre
log	logarithm (base 10)
\log_e	logarithm (base e)
m-	milli-
M	molar
MHC	major histocompatibility complex
mMTI	mouse metallothionein-I gene
MOPS	3-[N-morpholino]propanesulphonic acid
mRNA	messenger RNA
μ -	micro-
μ_m	membrane form of μ chain
μ_s	secreted form of μ chain
n-	nano-
NIP	[4-hydroxy-5-iodo-3-nitrophenyl]acetyl
NP	[4-hydroxy-3-nitrophenyl]acetyl
NP40	nonidet P40
p-	pico-
PABP	poly(A) binding protein
PAGE	polyacrylamide-gel electrophoresis
PBS	phosphate-buffered saline
PEG	polyethylene glycol
pH	hydrogen ion concentration, minus log of
poly(A)	homopolymer of ATP
REP	repetitive extragenic palindromic
RF	replicative form
r.p.m.	revolutions per minute
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
S	Svedberg unit
SSC	standard saline citrate
$t_{1/2}$	half-life
TAE	Tris-acetate/EDTA

TBE	Tris-borate/EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
tk	thymidine kinase
TMB	3,3',5,5'-tetramethylbenzidine
TNES	Tris.Cl/sodium chloride/EDTA/SDS
TE	Tris.Cl/EDTA
T _d	dissociation temperature
Tris.Cl	Tris[hydroxymethyl]aminomethane chloride
tRNA	transfer RNA
UTP	uridine 5'-triphosphate
uv	ultra-violet
V	variable region
V/cm	volts per centimetre
VH	heavy chain variable region
VL	light chain variable region
X-gal	5-bromo-4-chloro-3-indolyl β -D-galactoside

CONTENTS

	PAGE
TITLE	1
ABSTRACT	2
ACKNOWLEDGEMENTS	3
PUBLICATION	4
ABBREVIATIONS	5
CONTENTS	8
LIST OF FIGURES	12
LIST OF TABLES	16
CHAPTER 1: INTRODUCTION	17
1.1: Introduction	17
1.2: Antibody structure and gene assembly	17
1.3: B cell development	22
1.4: Immunoglobulin gene expression in B cells	26
1.4.1: Transcription of immunoglobulin genes and the control of rearrangement	26
1.4.2: Transcription of rearranged immunoglobulin genes	27
1.4.3: Post-transcriptional regulation of immunoglobulin gene expression	29
1.5: The role of mRNA stability in gene expression	35
1.5.1: Introduction	35
1.5.2: The importance of mRNA stability in hormone induced changes in gene expression	37
1.6: The role of terminal structures in controlling the rate of decay	39
1.6.1: The 7-methylguanosine cap	39
1.6.2: Stem loops	40
1.6.3: The poly(A) tail	45
1.6.4: The 3' untranslated region	49
1.7: The role of ribosomes	52
1.7.1: Bacteria	52
1.7.2: Yeast	54
1.7.3: Translation and the control of β -tubulin expression	55
1.8: Summary and aims	57

CHAPTER 2: MATERIALS AND METHODS	60
2.1: General materials	60
2.1.1: Reagents and equipment	60
2.1.2: Bacterial strains	60
2.1.3: Plasmids	61
2.1.4: Buffers and solutions	61
2.1.5: Bacterial growth media	62
2.1.6: Nucleic acid and protein size markers	63
2.2: DNA methods	64
2.2.1: Purification of DNA	64
2.2.2: Preparation of plasmid DNA	64
2.2.3: Enzyme reactions of DNA manipulation	66
2.2.4: Agarose gel electrophoresis	68
2.2.5: Recovery of DNA from agarose	69
2.2.6: Preparation of competent cells	70
(<i>Escherichia coli</i> LM1035)	
2.2.7: Transformation of bacteria	70
2.2.8: Site-directed mutagenesis	71
2.3: RNA methods	76
2.3.1: Removal of ribonuclease activity from	76
glassware and solutions	
2.3.2: Isolation of RNA	77
2.3.3: Isolation of polyadenylated RNA	79
2.3.4: Agarose gel electrophoresis of RNA	81
2.3.5: Northern blotting	81
2.3.6: Slot blotting	83
2.3.7: <i>In vitro</i> transcription using SP6	84
polymerase	
2.3.8: Ribonuclease protection analysis	87
2.4: Protein methods	90
2.4.1: Western blotting	90
2.4.2: Antibody assays	92
2.5: Cell culture methods	93
2.5.1: Materials	93
2.5.2: General procedures	93
2.5.3: Cloning by limiting dilution	94
2.5.4: Cell labelling and pulse-chase	94
experiments	
2.5.5: Generation of stable cell lines	95
2.5.6: Analysis of transfectants containing	97
the μ gene under the control of the heat	
shock promoter	

2.5.7: Measurement of mRNA decay using the heat shock promoter	97
2.5.8: Growth of cells in the presence of tunicamycin after heat shock induction	97
2.5.9: Specific activity of the UTP pool	98
CHAPTER 3: DEVELOPMENT OF PULSE-CHASE METHOD AND RIBONUCLEASE PROTECTION ASSAY	99
3.1: Introduction	99
3.2: Results	103
3.2.1: Ribonuclease protection assay	103
3.2.2: Pulse-chase experiments with CBMG cells	112
3.3: Discussion	119
CHAPTER 4: THE HALF-LIFE OF μ mRNA IN TWO B CELL LINES	129
4.1: Introduction	129
4.2: Results	131
4.2.1: Construction of probes for Northern blot analysis and ribonuclease protection	131
4.2.2: Northern blots of RNA from WEHI231 and MXW231 cells	133
4.2.3: Optimisation of ribonuclease protection assay	142
4.2.4: Pulse-chase analysis on WEHI231 and MXW231 cells	149
4.2.5: Decay of UTP pool	155
4.2.6: Decay of total polyadenylated RNA	162
4.3: Discussion	165
CHAPTER 5: TRANSFECTED CELL LINES CONTAINING THE μ GENE	174
5.1: Introduction	174
5.2: Results	178
5.2.1: Construction of control plasmids	178
5.2.2: Mutagenesis of the μ leader sequence	185
5.2.3: Transfection experiments	194
5.3: Discussion	207
CHAPTER 6: MEASUREMENT OF μ mRNA HALF-LIFE USING HEAT SHOCK INDUCTION	211
6.1: Introduction	211

6.2: Results	211
6.2.1: Probes used for Northern blot analysis	211
6.2.2: Quantitative Northern blot analysis	212
6.2.3: Induction of μ mRNAs containing leader sequence from the tk gene, and SV40 late region polyadenylation signal	215
6.2.4: Induction of μ mRNAs containing the mutated leader sequence	225
6.2.5: Nuclear RNA	230
6.2.6: Protein produced from the heat shock- induced μ mRNA	237
6.3: Discussion	245
CHAPTER 7: CONCLUSIONS AND FUTURE WORK	250
REFERENCES	256
APPENDIX	291

LIST OF FIGURES

	PAGE
Fig. 1.1: Structure of a murine IgG molecule	18
Fig. 1.2: Organisation of the murine heavy chain locus	21
Fig. 1.3: A schematic representation of the major stages in B-cell development	23
Fig. 3.1: Plasmids containing the hGH "minigene"	101
Fig. 3.2: hGH probes used for ribonuclease protection experiments	104
Fig. 3.3: Ribonuclease protection experiment with different amounts of ribonuclease	106
Fig. 3.4: Tritiated poly(A)+ RNA hybridised to an unlabelled probe	108
Fig. 3.5: Ribonuclease protection with a longer probe	109
Fig. 3.6: Ribonuclease protection with increasing amounts of tritiated total RNA	111
Fig. 3.7: Peak area vs. RNA input for gel shown in Fig. 3.6	113
Fig. 3.8: Protected c.p.m. vs. RNA input for increasing amounts of tritiated total RNA	114
Fig. 3.9: Pulse-chase analysis of hGH mRNA in CBMG cells	116
Fig. 3.10: Ribonuclease protection of pulse-chase samples using a labelled probe	117
Fig. 3.11: Decay of hGH mRNA in CBMG cells	118
Fig. 3.12: Decay of total mRNA in CBMG cells, in the presence and absence of glucosamine	120
Fig. 3.13: Pulse-chase experiment to test the effect of glucosamine on the chase conditions	121
Fig. 3.14: Decay of hGH mRNA in CBMG cells, in the presence and absence of glucosamine	122
Fig. 3.15: Summary graph	123
Fig. 4.1: Probes used in Northern blot analysis and ribonuclease protection	132
Fig. 4.2: Northern blot of RNA from WEHI231 and MXW231 cells	135
Fig. 4.3: Northern blot of a range of amounts of RNA from WEHI231 and MXW231 cells	136
Fig. 4.4: Quantitation of Northern blots probed with pSP64 μ NP	137
Fig. 4.5: Quantitation of Northern blot probed with	139

pSP64 μ NP

Fig. 4.6: Northern blot probed with the pSP65rRNA probe	140
Fig. 4.7: Quantitation of ribosomal RNA Northern blots	141
Fig. 4.8: Ribonuclease protection using the rRNA probe	143
Fig. 4.9: Ribonuclease protection using the μ NP probe	145
Fig. 4.10: Ribonuclease protection experiment to titrate the ribonuclease digestion conditions	147
Fig. 4.11: Relationship between hybridisation signal and amount of RNA hybridised	148
Fig. 4.12: Pulse-chase analysis on WEHI231 and MXW231 cells	150
Fig. 4.13: Quantitation of signal from two pulse-chase experiments	153
Fig. 4.14: Decay of a putative rRNA precursor	154
Fig. 4.15: h.p.l.c. analysis of UTP	157
Fig. 4.16: h.p.l.c. analysis of MXW231 cell lysate	159
Fig. 4.17: Calibration of UTP measurements	161
Fig. 4.18: Decay of UTP pool	163
Fig. 4.19: Northern blot analysis of polyadenylated RNA from a pulse-chase experiment	164
Fig. 4.20: Decay of poly(A)+ RNA	166
Fig. 5.1: Starting plasmids for transfection constructs	179
Fig. 5.2: 3-way ligation	182
Fig. 5.3: Results of 3-way ligations	183
Fig. 5.4: Deletion of immunoglobulin heavy chain enhancer	184
Fig. 5.5: Alternative selectable marker for control construct	186
Fig. 5.6: Summary of μ genes contained in various plasmids	187
Fig. 5.7: Site-directed mutagenesis	188
Fig. 5.8: Mutagenesis oligonucleotides	189
Fig. 5.9: Oligonucleotides used for sequencing	193
Fig. 5.10: Plasmids containing the mutated μ gene	195
Fig. 5.11: Heat shock induction from p372 μ_s gpt in J558L cells	197
Fig. 5.12: Heat shock induction from p516neo Δ E and p516 μ_s neo Δ E	199
Fig. 5.13: Heat shock induction from p516neo Δ E and p516 μ_s neo Δ E; Northern blot	200
Fig. 5.14: Transfectants derived from plasmids linearised at the <i>Hind</i> III site	202
Fig. 5.15: Construction of plasmids containing the SV40	204

late region polyadenylation site

Fig. 5.16: Experiment to identify mRNA species containing SV40 sequences	206
Fig. 5.17: Heat shock induction from mutant and control plasmids linearised at the <i>Eco</i> RI site	208
Fig. 6.1: Actin probe	213
Fig. 6.2: Quantitation of Northern blots	214
Fig. 6.3: Analysis of culture supernatants by ELISA assay	216
Fig. 6.4: Northern blot analysis of total RNA isolated after heat shock induction; μ NP probe	218
Fig. 6.5: Northern blot analysis of total RNA isolated after heat shock induction; actin probe	219
Fig. 6.6: Typical scanning results from heat shock inductions	221
Fig. 6.7: Decay of μ mRNA in B8 cells	222
Fig. 6.8: Decay of μ mRNA in B3 cells	223
Fig. 6.9: Decay of μ mRNA in D3 cells	224
Fig. 6.10: Northern blot analysis of total RNA after heat shock induction of Z5 and D6 cells; μ NP probe	227
Fig. 6.11: Northern blot analysis of total RNA after heat shock induction of Z5 and D6 cells; actin probe	228
Fig. 6.12: Decay of μ mRNA in Z5 cells	229
Fig. 6.13: Northern blot analysis of RNA isolated after heat shock induction of D1 and D6 cells; μ NP probe	231
Fig. 6.14: Northern blot analysis of RNA isolated after heat shock induction of D1 and D6 cells; actin probe	232
Fig. 6.15: Decay of μ mRNA in D1 and D6 cells	233
Fig. 6.16: Northern blot of nuclear RNA from Z5 cells	234
Fig. 6.17: Northern blot of nuclear RNA from D1 and D6 cells: μ NP probe	236
Fig. 6.18: Northern blot of nuclear RNA from D1 and D6 cells: actin probe	238
Fig. 6.19: Western blot of cell lysates and culture supernatant from B3 cells	240
Fig. 6.20: Western blot of cell lysates and culture supernatant from Z5 cells	242
Fig. 6.21: Western blot of cell lysates and culture	243

supernatant from D6 cells

Fig. 6.22: The effect of tunicamycin on μ protein from 244
Z5 and D6 cells

LIST OF TABLES

	PAGE
Table 3.1: The half-life of hGH mRNA in CBMG cells	124
Table 4.1: Ratio of μ mRNA levels between WEHI231 and MXW231 cells	142
Table 4.2: The half-life of μ mRNA in MXW231 and WEHI231 cells	155
Table 5.1: Transfection efficiencies	196
Table 6.1: The half-life of altered μ mRNA in B8 cells	225
Table 6.2: The half-life of wild-type μ mRNA in Z5 cells	226
Table 6.3: Summary of heat shock induction data	247

CHAPTER 1 INTRODUCTION

1.1: Introduction.

The mammalian immune system consists of a number of cell types, including B and T lymphocytes, macrophages, mast cells and granulocytes, which differentiate from stem cells found in the bone marrow or foetal liver. Each cell type performs a particular function, and the overall immune response is regulated by a complex network of interactions between the cells, mediated both by soluble molecules and cell-to-cell contact. The major role of B lymphocytes is to produce antibodies which bind to specific antigens and target them for destruction by other components of the immune system. To this end, during B cell ontogeny, a highly ordered programme of development and differentiation culminates in the production of terminally differentiated plasma cells which secrete large amounts of specific antibodies. The work described in this thesis was aimed at increasing our understanding of the molecular biology of antibody production.

1.2: Antibody structure and gene assembly.

The simplest antibody molecules (illustrated schematically in Fig. 1.1) consist of two identical heavy chains and two identical light chains, linked by disulphide bonds. Both heavy and light chains are divided into structural domains that each form a conserved structure known as the "antibody fold", which is stabilised by an internal disulphide linkage. The sequences of the amino-terminal domains of the heavy and light chains are highly variable, whereas the carboxy-terminal regions are confined to one of several types within a given species (Hilschmann and Craig, 1965; Putnam and Easley, 1965). The variable region contains the antigen binding site, which consists of three hypervariable subregions whose amino acid residues are thought to contact the antigen; these are termed complementarity-determining regions or CDRs (Wu and Kabat,

FIGURE 1.1

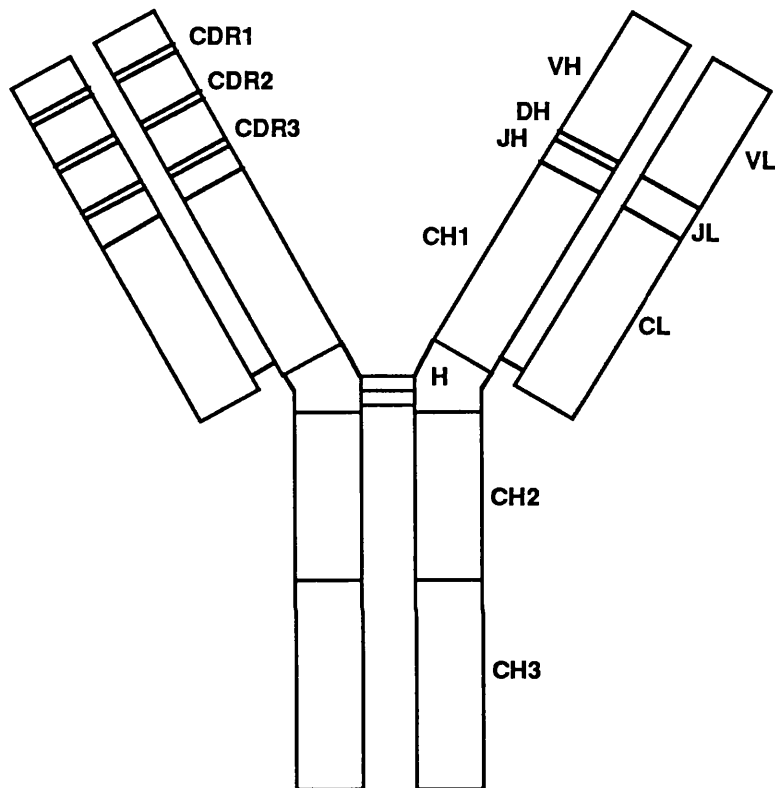


Figure 1.1: Structure of a murine IgG molecule.

A generalised IgG molecule is shown in schematic form (not to scale). The heavy chain variable (VH), diversity (DH) and joining (JH) region domains are indicated on the right hand side, as are the light chain variable (VL) and joining (JL) region domains. On the left hand side the positions of the 3 complementarity determining regions, CDRs 1,2 and 3, are indicated. CL indicates the light chain constant region, and CH1, CH2 and CH3 indicate the heavy chain constant region domains. H indicates the hinge region. Not to scale.

1970). In the three-dimensional structure, the heavy and light chain CDRs form loops which together form the antigen binding site. The CDRs are bounded in the linear peptide sequence by four framework regions, which are less variable.

The carboxy termini of heavy chains of the mouse fall into 5 isotype classes, IgM, IgD, IgG, IgE, and IgA, which provide different biological effector functions, including complement fixation and Fc receptor binding. There are two classes of light chain constant region, κ and λ , but no functional differences have been identified for these. Heavy chain constant regions contain between two and four domains, and some isotypes also contain a hinge region between the first two constant region domains. In addition, each isotype is found in two alternative forms, one of which is secreted from the cell, and the other which has a hydrophobic carboxy-terminus which anchors the antibody molecule in the cell membrane (Vassalli *et al*, 1979). It is the membrane form which serves as the B cell receptor for antigen.

Each newly-generated B cell produces antibody molecules of a single specificity and all the progeny of this cell produce antibodies with the same specificity. The enormous range of specificities necessary to combat the wide variety of antigens that may be encountered is generated by a complex process of somatic gene rearrangement producing unique immunoglobulin genes, and hence unique antibodies, in each B cell clone. According to the clonal selection theory, the immune response to a particular antigen is initiated when that antigen is recognised by a B lymphocyte receptor of pre-existing specificity (Burnet, 1957). This stimulates the cell to proliferate, producing a clone of cells which recognise the antigen. During the proliferation, somatic mutation occurs, and the resulting clones producing high-affinity antibodies are selected to proliferate further by their improved ability to bind antigen. Some of these cells become long-lived memory cells, which remain in the bloodstream and are able to provide a rapid, high-affinity response when the antigen is encountered again.

The genetic information encoding immunoglobulin polypeptides exists in the germ-line in multiple gene segments which are assembled in B lymphocytes to form a

complete gene (for reviews, Tonegawa 1983; Alt *et al*, 1986; Alt *et al*, 1987). The genes for the heavy chain, and the two types of light chain, κ and λ , are at distinct chromosomal locations. A schematic representation of the mouse heavy chain locus is illustrated in Fig. 1.2. The variable region of the heavy chain is coded for by three gene segments, the variable, diversity, and joining segments, which are found in separate clusters on chromosome 12 in the mouse (D'Eustachio *et al*, 1980). The four joining segments are approximately 7 kb upstream of the first constant region gene, $C\mu$, and the 12 known diversity segments, which are divided into three families, are found within an 80kb region beginning 1kb upstream of the joining segments (Kurosawa *et al*, 1981; Kurosawa and Tonegawa, 1982; Wood and Tonegawa, 1983). The number of variable gene segments may be as high as 1000 or more (Livant *et al*, 1986), and these are divided into nine families based on sequence homology of their coding regions (Brodeur and Riblet, 1984; Winter *et al*, 1985). However, the functional repertoire is likely to be smaller than this figure, as there are a high percentage of pseudogenes. There are five constant region genes, $\mu, \delta, \gamma, \epsilon$, and α , which give rise to the isotypes IgM, IgD, IgG, IgE and IgA, respectively. The μ gene is closest to the variable region segments and is the first isotype to be expressed during development. The light chain gene segments are organised in a similar manner, but contain only variable, joining, and constant region segments (Tonegawa, 1983).

Each heavy chain gene is assembled by a recombination process that brings together one variable, diversity, and joining segment 5' to the constant region genes. The V and J regions of light chain genes are assembled in a similar manner. The combinatorial assortment of the various segments makes an important contribution to the generation of diversity necessary for the immune system to be able to respond to an almost unlimited array of antigens. Further diversity is generated by imprecise joining of the segments and the insertion and removal of additional bases at the junctions (Alt and Baltimore, 1982; Desiderio *et al*, 1984), and the random association of different light and heavy chains.

FIGURE 1.2

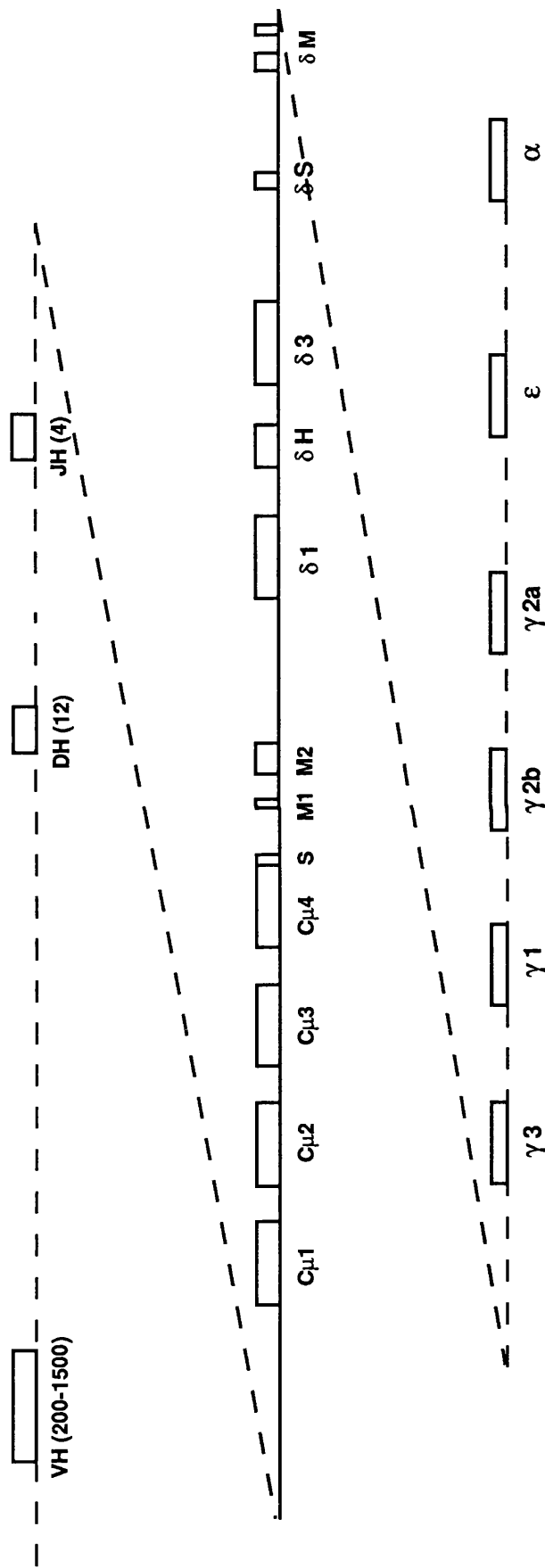


Figure 1.2: Organisation of the murine heavy chain locus.

The VH, DH and JH boxes represent one member of each family, and the number of family members is indicated in brackets. The 4 C μ constant region exons are shown, followed by the exons encoding the membrane-bound form of the μ polypeptide, and further downstream, the δ exons. The other constant region isotype genes are shown underneath, but their detailed exon structure is not shown. Not to scale.

The V, D, and J regions are flanked by recombination recognition sequences which are conserved between immunoglobulin and T cell receptor genes (reviewed in Tonegawa, 1983; Alt *et al*, 1987; Alt *et al*, 1986), and function in both T and B cell lines, indicating that they are probably the target of a common "recombinase" (Yancopoulos *et al*, 1986; Lieber *et al*, 1987; Zúñiga *et al*, 1982; Kemp *et al*; 1980; Forster *et al*, 1980; Kurosawa *et al*, 1981). Two genes, RAG-1 and RAG-2, which encode the recombinase itself, or an activator of it, have recently been cloned (Schatz *et al*, 1989; Oettinger *et al*, 1990). Several lines of evidence indicate that the products of the RAG-1 and RAG-2 are part of the recombination machinery. The carboxy terminal portion of RAG-1 shows homology to a topoisomerase (Wang *et al*, 1990), and RAG-2 expression in the chicken bursa correlates with the gene conversion process which generates chicken immunoglobulin diversity (Carlson *et al*, 1991). The availability of these genes will contribute greatly to our understanding of the mechanism of somatic immunoglobulin gene rearrangement.

1.3: B cell development

The major stages of B cell development are illustrated schematically in Fig. 1.3. The first phase of development is antigen independent, and involves the differentiation of stem cells to mature B lymphocytes. In mice, this process occurs in the liver of the foetus until just after birth, but is maintained in the bone marrow for the rest of adult life (Owen *et al*, 1974; Raff *et al*, 1976; Kincade 1981; Verladi and Cooper 1984). The mature B lymphocytes express antibody molecules on their cell surface which serve as receptors for antigen (Vitetta *et al*, 1971). The second phase of development is antigen dependent, and begins after the mature B cells have migrated from the bone marrow to peripheral lymphoid organs like the spleen and lymph nodes. Here they come into contact with circulating antigens and are stimulated to divide and differentiate further by the binding of antigen to their surface antibody molecules, and the appropriate signals from T cells. This results in the

FIGURE 1.3

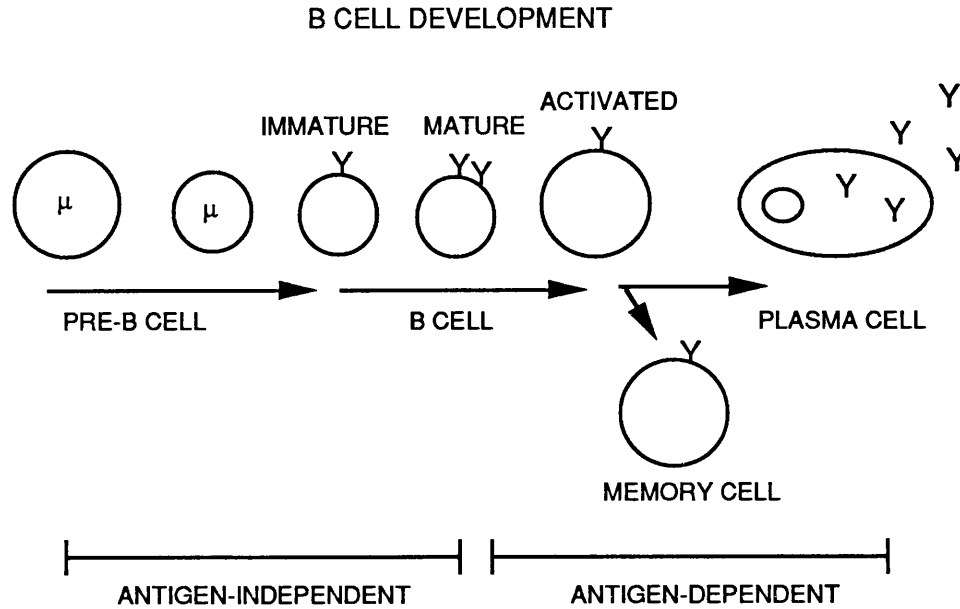


Figure 1.3: A schematic representation of the major stages in B-cell development.

The B cell differentiation pathway in mammals is shown. The arrows indicate the direction of maturation. μ indicates cytoplasmic μ heavy chains, and Y indicates complete immunoglobulin molecules. For detailed explanation, see text.

production of plasma cells which secrete antibody, and memory B cells, which form the basis of the secondary response if the antigen is encountered again later in life. Some of the major characteristics of each stage of B cell development will now be considered in more detail.

One of the earliest events in B cell development is rearrangement of the immunoglobulin gene loci to produce functional genes; this is a highly ordered process. First, D to J joining occurs on both heavy chain alleles, and V to DJ joining then follows (Alt *et al*, 1984). When a rearrangement has occurred such that the initiating V region methionine codon is in frame with the D and J segments, production of μ protein occurs; these cells are referred to as pre-B cells (Raff *et al*, 1976; Burrows *et al*, 1979; Levitt and Cooper, 1980). The production of the membrane form of μ protein has been shown to be responsible for the phenomenon of allelic exclusion, whereby further rearrangement on the other heavy chain allele is prevented (Pernis *et al*, 1965; Cebra *et al*, 1966; Nussenzweig *et al*, 1987). This is an important mechanism for ensuring that each B cell clone produces only a single antibody specificity, a feature central to the process of clonal selection (Burnet, 1957). The production of the membrane form of μ chain is also the trigger for the initiation of kappa chain rearrangement (Reth *et al*, 1987). The pre-B cells are large, proliferating cells which give rise to smaller, non-dividing, μ^+ progeny which rearrange their light chain genes (Coffman and Weissman, 1983). Thus a particular heavy chain rearrangement can be passed to several progeny and thereby combine with different light chains (Sauter and Paige 1987; Caton, 1990).

After successful light chain rearrangement, the heavy and light chains combine and are expressed on the cell surface; this is the immature B cell stage (Spear *et al*, 1973, Nossal and Pike, 1973). Upon further maturation, the cells express IgD isotype as well as IgM, of the same antigenic specificity, and both these membrane-bound immunoglobulins act as B cell receptors for antigen (Kearney *et al*, 1977; Layton *et al*, 1978; Scher *et al*, 1980; Blattner and Tucker 1984).

The mature, resting B cells remain in the G_0 phase of

the cell cycle until they encounter antigen which binds specifically to their receptors, and the subsequent responses depend on the nature of the antigen. The process of B cell activation is complex and as yet poorly understood, but the essential features are as follows (reviewed in Noelle and Snow 1990). B cell activation by so-called T-independent antigens occurs via the cross-linking of several immunoglobulin molecules on each cell, by virtue of the fact that the antigens are polymeric, with repetitive antigenic determinants on each molecule. This cross-linking results in the B cell being in an "excited" state, able to respond to lymphokines produced by macrophages and other accessory cells which have taken up and degraded the antigen. T-dependent antigens, on the other hand, contain only a single antigenic determinant, and B cell activation by these antigens occurs in two steps. When the antigen binds to the B cell receptor, it is taken up and degraded, and the resulting peptides are presented on the surface of the B cell in association with major histocompatibility complex (MHC) class II molecules. This complex is then recognised by the T cell receptor molecule on helper T cells, and the binding of the T cell receptor initiates a second activation signal via the MHC molecule. In addition, the T cell begins secreting lymphokines, to which the activated B cell can now respond, by proceeding through the cell cycle and into mitosis. Further proliferation depends on the continued presence of antigen, and differentiation into plasma cells is controlled by various lymphokine signals. Interleukins 2 and 5 in particular are required for the secretion of pentameric IgM (Lernhardt *et al*, 1987; Matsui *et al*, 1989). A percentage of the activated B cells revert to the resting state and become long-lived memory B cells, some of which undergo class switching to produce surface antibodies of other isotypes (Gathings *et al*, 1977; Calvert *et al*, 1983).

Two important questions regarding immunoglobulin gene expression concern the control of its tissue specificity, and the regulation of gene expression during development and differentiation. Both of these topics are under extensive study, and the following section outlines some of the current knowledge on these questions.

1.4: Immunoglobulin gene expression in B cells.

1.4.1: Transcription of immunoglobulin genes and the control of rearrangement.

Immunoglobulin genes are transcribed at the early stages of B cell development, before they are rearranged, and this early transcription is thought to be important for the control of the ordered rearrangement process (Yancopoulos and Alt 1985; Blackwell et al, 1986; Alt et al, 1987). Transcription of heavy chain variable region genes occurs before their rearrangement, is independent of the heavy chain intronic enhancer element, and is switched off in mature B cells (Yancopoulos and Alt, 1985). Unrearranged C μ genes and partly rearranged D-J loci are also transcribed (Alt et al, 1982; Nelson et al, 1983; Reth and Alt, 1984; Lennon and Perry, 1985), as are light chain constant region genes (Van Ness et al, 1981; Martin and Van Ness, 1989). The heavy chain enhancer is able to function as a cryptic promoter for C μ transcripts (Su and Kadesh, 1990). Furthermore, activation of κ gene rearrangement correlates with the induction of germline transcription of the gene (Schlissel and Baltimore, 1989; Blackwell et al, 1989), and heavy chain gene recombination has been shown to be enhanced by transcription (Blackwell et al, 1986).

These observations are consistent with the model that immunoglobulin loci are targetted for rearrangement by transcriptional activation at the appropriate stage of development (Yancopoulos and Alt, 1985; Blackwell et al, 1986; Alt et al, 1987). The model also predicts that V κ genes should be transcribed in cells undergoing κ chain rearrangement, but not in cells undergoing V to DJ joining, or in mature B cells. As yet, these transcripts have not been detected (Schlissel and Baltimore, 1989; Mather and Perry, 1981), although V λ genes are transcribed after κ gene rearrangement (Picard and Schaffner, 1984a), at the time when they would be rearranging if κ rearrangement was non-productive. Either specific transcription factors, or the chromatin configuration associated with active transcription, might be required for the recombinase to gain access to the recognition sequences. The availability of cloned

recombinase will assist greatly in attempts to answer these questions (Oettinger *et al*, 1990; Schatz *et al*, 1989). Transcription of the appropriate constant region genes has also been shown to precede isotype class switching, which occurs via a separate mechanism, highlighting the close relationship between transcription and recombination (Stavnezer-Nordgren and Sirlin, 1986; Lutzker *et al*, 1988; Berton *et al*, 1989; Esser and Radbruch, 1989).

1.4.2: Transcription of rearranged immunoglobulin genes.

From the data described above it is clear that immunoglobulin gene transcription in pre-B cells has an important role to play in the control of rearrangement and the early development of the B cell. High level transcription of immunoglobulin genes occurs when the variable region promoters come under the influence of transcriptional enhancer sequences located in the J-C intron of the heavy chain and κ genes (Gillies *et al*, 1983; Banerji *et al*, 1983; Neuberger, 1983; Queen and Baltimore, 1983; Picard and Schaffner, 1984b; Queen and Stafford, 1984). Both the immunoglobulin promoters (found upstream of each variable region gene) and enhancer sequences have preferential activity in B lymphocytes, but the promoter alone can confer tissue specific expression in conjunction with a viral enhancer (Mason *et al*, 1985; Grosschedl and Baltimore, 1985). In addition, the heavy chain promoter/enhancer combination have greater activity together than when either is paired with heterologous promoters or enhancers (Garcia *et al*, 1986). Intragenic regions of the μ gene also contribute to the tissue specificity of expression, presumably acting via post-transcriptional mechanisms (Grosschedl and Baltimore, 1985). Several DNA binding sites have been identified in the promoter and enhancer elements, and the protein factors which bind to these are being identified and cloned, giving some insight into the mechanism of enhancer action (for reviews see Sen and Baltimore, 1989; Calame, 1989).

Recently, novel 3' enhancer elements have been identified some distance downstream of the rodent heavy chain, κ and λ constant region loci (Meyer and Neuberger,

1989; Petterson *et al*, 1990; Hagman *et al*, 1990; Eccles *et al*, 1990). The first of these to be discovered was the downstream κ enhancer, which is a B cell specific enhancer 9kb 3' to $C\kappa$, and is stronger than the κ intron enhancer (Meyer and Neuberger, 1989). The heavy chain locus 3' enhancer was first identified in the rat, and is 25 kb downstream of the most 3' constant region gene, $C\alpha$. A homologous enhancer has also been identified in a similar position in the mouse (Lieberson *et al*, 1991). The heavy chain 3' enhancer is retained after all types of heavy chain switch recombination events, and therefore its presence explains the activation of *c-myc* translocations to this region in rat immunocytomas, mouse plasmacytomas and Burkitt lymphomas (Kakkis *et al*, 1986; Croce, 1987; Kakkis *et al*, 1988; Pear *et al*, 1988). It also accounts for the continued expression of heavy chain genes in cell lines which have deleted the heavy chain intron enhancer (Klein *et al*, 1984; Zaller and Eckhardt, 1985). The λ locus in the mouse was found to contain two elements, one 3' to λ_1 , and one 3' to λ_2 (Hagman *et al*, 1990; Eccles *et al*, 1990). The λ locus contains no intron enhancer, unlike the other immunoglobulin loci, but the λ gene requires an enhancer to be expressed in transfected cell lines and transgenic mice (Picard and Schaffner, 1983, 1984b; Neuberger *et al*, 1989) so the discovery of the 3' enhancers sheds some light on the question of what controls λ gene expression *in vivo*.

The downstream enhancers could simply represent redundancy in the control system. However, transgenic mouse studies indicate that the heavy chain promoter and intron enhancer are insufficient to confer full level expression of the transgenes (Storb, 1987; Petterson *et al*, 1989), indicating that the 3' enhancer elements may serve an important function. The presence of multiple control elements is reminiscent of the findings of Grosveld *et al*, (1987), who have identified sequences flanking the globin locus which confer cell type specific, position independent, high level expression on β -globin transgenes and transfected β -globin genes. These sequences, termed dominant control regions, are located some distance from the genes, and are

associated with DNaseI super-hypersensitive regions. They are thought to control the chromatin organisation of the globin locus in a tissue specific manner (Grosveld *et al*, 1987; Forrester *et al*, 1987; Blom Van Assendelft *et al*, 1989). Similar control elements have been isolated from the chicken lysozyme gene (Bonifer *et al*, 1990), indicating that this may be a general mechanism of control. Like globin genes, immunoglobulin genes are highly expressed in a tissue specific manner, and it therefore seems likely that they will be subject to a similar mode of regulation. The 3' enhancers are the most likely candidates for components of dominant control regions. It seems likely that the dominant control regions may confer the overall competence for transcription to a particular locus in the correct cell type, and that more local control elements, and post-transcriptional processes, regulate gene expression within that locus during development.

1.4.3: Post-transcriptional regulation of immunoglobulin gene expression

Regulation of the synthesis, assembly, and secretion of antibodies in B cells occurs at a variety of levels. Some aspects of these controls will now be considered in more detail, concentrating on the characteristic status of immunoglobulin gene rearrangement and expression associated with each developmental stage.

(i): Pre-B cells.

Transcription of rearranged heavy chain alleles, of which μ is the first to be expressed, can give rise to two mRNA species with alternative 3' termini, one of which codes for secreted μ protein, and the other encodes a hydrophobic 3' terminus which anchors the protein in the membrane (Vassalli *et al*, 1979; Alt *et al*, 1980; Rogers *et al*, 1980; Early *et al*, 1980). These mRNAs are produced by alternative processing at the 3' end of the μ constant region (see Guise *et al* 1989, for review). At the pre-B cell stage, μ mRNA encoding both the membrane and secreted forms of μ chain are produced, with μ_m being the predominant species (Perry *et al*, 1981; Lamson and Koshland 1984; Mather *et al*, 1984). The

distance between the μ_m and μ_s cleavage/polyadenylation sites and their affinities for a limiting cellular factor seems to govern their relative usage at this early stage (Galli et al, 1987, 1988). The μ_m cleavage/polyadenylation site is the stronger, and in transfection experiments it has been shown that the levels of μ_s mRNA can be increased if the μ_s polyadenylation site is available for longer on the nascent transcript, i.e. if the distance between the sites is increased (Peterson and Perry, 1986; Galli et al, 1988). Deletion of μ_m splice sites does not affect the levels of μ_s mRNA, indicating that the splicing reaction does not compete with the polyadenylation at the μ_s site (Galli et al, 1988).

At this stage of development, although approximately one third of the transcription of the μ locus proceeds into the δ region, the primary transcripts are processed to the two forms of μ mRNA, the δ portion being degraded (Mather et al, 1984; Yuan and Tucker, 1984a; Yuan, 1986; Mather 1987), so only μ protein is produced (Raff et al, 1976; Burrows et al, 1979; Levitt and Cooper 1980). This protein undergoes preliminary glycosylation in the endoplasmic reticulum, but as no light chain is available for assembly, it is degraded (Dulis 1983). The μ chain is thought to bind a protein known as heavy chain binding protein (BiP) in the endoplasmic reticulum, which targets it for degradation until BiP is displaced by light chain (Haas and Wabl, 1983; Bole et al, 1986; Munro and Pelham, 1986; Hendershot et al, 1987). BiP is a glucose-regulated protein which apparently prevents improperly glycosylated and improperly folded proteins entering the Golgi. However, evidence is accumulating that not all the μ protein binds with BiP; a small proportion of it associates with a λ -like molecule which may be important in the regulation of B cell development. Some of this evidence is outlined below.

A gene termed λ_5 , because of its homology to C_λ and J_λ sequences, has been shown to be expressed in neoplastic pre-B cells (Sakaguchi and Melchers, 1986; Kudo et al, 1987), as has the V_{pre-B1} gene, to which it is closely linked (Kudo and Melchers, 1987). A candidate for the λ_5 gene product has been shown to be covalently associated with μ heavy chains in murine and human pre-B lymphoma cell lines (Pillai and

Baltimore, 1987; Hendershot *et al*, 1988). Recently, the V_{pre-B} , λ_5 , and μ chain have been demonstrated to be associated in a complex which is transported to the cell surface and can be immunoprecipitated with μ specific antibodies; μ and λ_5 being disulphide bonded, and V_{pre-B} being non-covalently associated (Karasuyama *et al*, 1990; Tsubata and Reth, 1990; Misener *et al*, 1990). This complex may form part of the signalling pathway which mediates allelic exclusion and triggers light chain rearrangement, and may also provide other regulatory functions via its interaction with the stromal cell layer (which provides important B cell growth factors) (Glozak *et al*, 1990). Therefore this μ polypeptide, although it is not assembled into functional antibody with light chain, does seem to be important in the control of B cell development.

(ii): Immature B cells.

After productive rearrangement of a light chain gene, light chain mRNA and protein are produced. Light chain polypeptide combines with the heavy chain, allowing terminal glycosylation to occur followed by transport to the cell membrane (Dulis *et al*, 1982; Dulis 1983). At this stage, both μ_s and μ_m mRNA are produced and translated. However, only μ_m protein is assembled with light chain and expressed on the cell surface, and μ_s protein is degraded in the endoplasmic reticulum (Dulis *et al*, 1982; Sitia *et al*, 1988). The mechanism of the distinction between μ_s and μ_m polypeptides is beginning to be understood. The μ_s carboxy terminus has been shown to be responsible for the retention of the secretory form of μ chain in B cells, in particular the cysteine-575 residue, and it is possible that this retention occurs via the binding of BiP to the μ_s tailpiece (Sitia *et al*, 1990). The μ_m transmembrane segment, on the other hand, (which does not bind BiP), is required for the surface expression of IgM in B cells (Sitia *et al*, 1990; Williams *et al*, 1990). μ_m protein is not expressed on the cell surface in non-B cell types, or at the plasma cell stage, although μ_m mRNA is translated, indicating that factors specific to the B cell stage of differentiation are required to facilitate cell surface expression of μ_m (Sitia *et al*, 1987; Williams *et al*, 1990). The B cell specific

factors have not been identified, but they could mediate insertion of the transmembrane segment in the membrane, or displace a protein which retains the μ_m molecules in the endoplasmic reticulum (Williams *et al*, 1990). Clearly post-translational regulation is a very important mechanism for controlling the surface expression of IgM.

(iii): Mature B cells.

Mature B cells are characterised by the presence of IgD on their cell surface in addition to IgM (Kearney *et al*, 1977; Layton *et al*, 1978), and also by their ability to respond to antigen by entering the cell cycle; development of immature B cells on the other hand is easily aborted by antigen (Raff *et al*, 1975; Nossal and Pike, 1975). As mentioned earlier, the μ and δ genes are part of the same transcription unit, and at an early stage of B cell development, the level of transcription of δ is set at one third that of μ . In other words, two thirds of the polymerase molecules terminate before reaching the δ exons (Yuan, 1986). δ mRNA is not expressed at significant levels until the mature B cell stage, indicating that prior to this stage either the δ mRNA is unstable, or the primary transcript is not processed efficiently to δ mRNA (Mather, 1987). In addition, inefficient δ peptide processing also contributes to the low levels of IgD at early developmental stages (Yuan, 1986). Even at the mature B cell stage, the steady state level of IgD mRNA is still 10 fold less than that of IgM (Yuan and Tucker, 1984b), but the level of IgD protein on the cell surface is higher than that of IgM, as a result of a higher turnover rate of IgM protein (Yuan, 1984). So IgD expression is regulated by a complex combination of post-transcriptional controls.

(iv): Plasma cells.

When B cells are stimulated to differentiate into plasma cells, a series of qualitative and quantitative changes take place in their immunoglobulin gene expression, which results in the cessation of cell surface immunoglobulin production, and the secretion of pentameric IgM (Melchers and Andersson, 1974). At the mRNA level, there is a switch from producing

both μ_s and μ_m mRNA to the production of μ_s mRNA only, with little or no μ_m mRNA (Lamson and Koshland, 1984). Also, *de novo* synthesis of mRNA encoding J chain (the polypeptide joining chain required for the assembly of pentameric IgM) begins (Mestecky *et al*, 1971; Koshland, 1975; Mather *et al*, 1981; Lamson and Koshland, 1984). The lymphokines interleukin 5 and 2 have a role in the control of the switch to μ_s mRNA and J chain transcription respectively (Matsui *et al*, 1989; Yuan *et al*, 1990). These events are followed by a very large increase in the cytoplasmic levels of μ_s and J chain mRNA, and a parallel increase in the levels of protein production (Tsuda *et al*, 1978; Perry *et al*, 1981; Yuan and Tucker, 1982; Lamson and Koshland, 1984; Yuan and Tucker, 1984b). The mechanism of high level μ gene expression is considered later in this Section.

The control of the switch from μ_m to μ_s mRNA at this stage of development, unlike at earlier stages, is transcriptional, and seems to involve a gradual increase in transcriptional termination 5' to the μ_m exons (Guise *et al*, 1988; Weiss *et al*, 1989; Yuan and Dang, 1989). This also has the effect of preventing IgD mRNA expression, (as the IgD exons are part of the same transcription unit), leading to the disappearance of IgD from the cell surface (Mather *et al*, 1984; Yuan and Tucker, 1984b).

Further regulation of μ_s and μ_m gene expression occurs at the protein level, as low levels of μ_m mRNA may be expressed in differentiated cell types (Yuan and Tucker, 1982; Sitia *et al*, 1988). As mentioned in Section (ii), surface IgM expression is prevented in plasma cells by the retention and degradation of μ_m polypeptide in the endoplasmic reticulum (Sitia *et al*, 1987). Also IgM cannot be secreted until it is assembled in polymeric form; as mentioned above, the cysteine 575 residue in the carboxy terminus of μ_s causes retention of monomeric IgM by a mechanism involving BiP (Sitia *et al*, 1990). Assembly of IgM into polymers was initially thought to require the presence of J chain, but this has been shown not to be the case, as both pentameric and hexameric IgM can be produced in glioma cells in the absence of J chain (Cattaneo and Neuberger 1987). Therefore the inability of undifferentiated B cells

to produce secreted IgM is probably only partly due to lack of J chain. This is supported by evidence that levels of immunoglobulin protein and mRNA can be high prior to IgM secretion, indicating that other factors are required (King and Corley, 1989). These are likely to be enzymes necessary for IgM assembly, for example sulfhydryl oxidase (Roth and Koshland, 1981).

It is clear from the preceding paragraphs that regulation of immunoglobulin gene expression occurs at all levels; transcriptional, post-transcriptional, and post-translational, and the relative contributions of each style of regulation varies during development. The end product of this development and differentiation process is the production of plasma cells which have a short lifespan of one or two days, during which time they secrete thousands of antibody molecules per second (Fazekas de St. Groth and Scheidegger, 1980). It is therefore of interest to investigate how this high level gene expression is achieved. Various lines of evidence indicate that the increase in μ mRNA levels between the B cell and plasma cell is mediated largely by post-transcriptional mechanisms. This evidence will now be considered in more detail.

Using two immortalised cell lines which represent different stages of differentiation, a difference of around 150 fold in the steady state cytoplasmic levels of heavy chain mRNA has been observed between the plasma cell stage and the pre-B cell stage (Perry and Kelley, 1979). However, the difference in nuclear μ RNA levels was only 6 fold, suggesting that mRNA abundance is being regulated at the post-transcriptional level. Transcriptional run-off assays on various cell lines have shown that there is only a 2-5 fold increase in transcription between plasmacytoma cell lines (representing the plasma cell stage) and B cell lymphoma lines representing B cell stages (Mather et al, 1984; Kelley and Perry, 1986). The change in steady state levels in these cell lines varies between 20-100 fold. In addition, the heavy chain enhancer is as active in pre-B cell lines as it is at later stages, consistent with the transcription data (Mason et al, 1985; Grosschedl and Baltimore, 1985; Gerster et al, 1986). Data obtained from

polyclonal activation of B cell populations qualitatively agrees with the data from immortalised cell lines, although there may be a greater transcriptional contribution to the increase in μ mRNA levels in this case (Lamson and Koshland, 1984; Yuan and Tucker, 1984a; Yuan and Tucker, 1984b; Chen-Bettecken *et al*, 1985; Weiss *et al*, 1989).

Taken together, these data suggest that post-transcriptional processes are the major factors which control the level of immunoglobulin mRNA production in the differentiated B cell lines, and may also be involved to a lesser extent in normal B cells. Possible control points could involve nuclear processing, nuclear to cytoplasmic transport, or cytoplasmic stability (Darnell 1982). The work described in this thesis was aimed at determining the role of mRNA stability in this regulation. At present, control of mRNA stability, particularly in mammalian cells, is poorly understood compared with our level of understanding of the control of gene transcription. However, the processes involved are beginning to be unravelled in a number of systems in which regulation of mRNA stability has been shown to be the dominant control mechanism. Some examples of these systems will now be discussed.

1.5: The role of mRNA stability in gene expression

1.5.1: Introduction.

Clearly the steady state level of an mRNA represents a balance between its rate of synthesis and its rate of degradation, and it is clear from studies comparing the rate of synthesis of a variety of mRNAs with their abundance that post-transcriptional processes are involved in the control of steady-state mRNA levels (Harpold *et al*, 1979; Carneiro and Schibler, 1984). Early studies of average mRNA stability in mammalian cells, before the availability of cloned sequences as probes, revealed a variety of half-lives, and some conflicting results. Murphy and Attardi (1973) found total mRNA decayed with a half-life of 2-3 days, whereas a much shorter half-life of 10 hours has been observed (Greenberg, 1972). Further studies revealed the existence of two or more populations of mRNA with different average rates of decay.

Singer and Penman, using a pulse-chase method, determined that 33% of mRNA decayed with a half-life of 7 hours, and 67% had a half-life of 24 hours in HeLa cells, but these values were found to be 4 hours and 12 hours respectively in the presence of actinomycin D (Singer and Penman 1972; 1973). Other workers detected the existence of a third class of mRNA with an even shorter half-life, of 1-2 hours (Puckett et al, 1975; Puckett and Darnell, 1976), or even less (Berger and Cooper, 1975). In addition, mRNA decay has been shown to depend on the rate of cell division in L cells but not in 3T6 cells (Perry and Kelley, 1973; Abelson et al, 1974).

These disparities reflect the difficulties associated both with the analysis of mixed populations of molecules and the various problems associated with the different methods used for measuring half-life. The first problem has been resolved by the use of cloned sequences as probes for specific mRNAs, which has revealed that mRNAs in mammalian cells can have half-lives ranging from as little as a few minutes to several hundred hours (Wilson and Treisman, 1988; Brock and Shapiro, 1983a). The second problem of methodology still exists, however. Various methods for measuring mRNA stability introduce different errors into the result, so that some methods are useful for mRNAs with short half-lives, and some are more appropriate for long-lived mRNAs.

Synthesis of new mRNA can be prevented with transcriptional inhibitors, to allow the measurement of the disappearance of pre-existing molecules with time. The disadvantage of this system is that transcriptional inhibitors like actinomycin D, α -amanitin or 5,6-di-chloro-1- β -D-ribofuranosyl benzimidazole (DRB) may affect the measured half-lives by interfering with cellular processes other than transcription. Actinomycin D, for example, which inhibits transcription by intercalating DNA (Sobell, 1985), also interferes with translation (Singer and Penman, 1972; Kostura and Craig, 1986). Therefore it is toxic after extended exposure, and not suitable for measuring long half-lives.

An alternative method is to pulse-label mRNA with radioactive precursors and observe the loss of radiolabel from the mRNA species under study, under chase conditions in which no further radiolabel is incorporated. However, the

use of such pulse-chase protocols in mammalian cells is complicated by the large size of the ribonucleotide precursor pool, and the fact that the labelled nucleotide cannot be chased out of the cells effectively. This makes the method unsuitable for determining very short half-lives, as radiolabel is still incorporated into mRNA during the early part of the chase (Warner et al, 1966; Schimke, 1970; Scholtissek, 1971; Levis and Penman, 1977). This method is discussed more fully in Chapters 3 and 4.

Continuous labelling to steady state can also be used. The time taken to reach equilibrium (when no further radiolabel is incorporated into the mRNA of interest) can be used to calculate the half-life of the message (Greenberg, 1972). This method suffers from the disadvantage that the specific activity of the precursor pool must remain constant throughout the labelling period; this is difficult to achieve, and difficult to approximate to for a short-lived mRNA.

These problems can be overcome by using a method appropriate for the half-life being determined, and where possible by carrying out the determination by more than one method. It is also worth noting that all of the above methods measure chemical and not functional stability.

1.5.2: The importance of mRNA stability in hormone induced changes in gene expression.

The regulation of mRNA levels by hormones occurs at both the transcriptional and post-transcriptional levels, and provides a prime example of the importance of mRNA degradation as a control point in expression. For example, oestrogen induces ovalbumin mRNA in the chick oviduct, and the mRNAs for yolk precursor proteins, apolipoprotein II and vitellogenin II, in avian liver. As well as increasing the rate of gene transcription, oestrogen selectively stabilises the induced mRNAs against degradation (Palmiter and Carey, 1974; Wiskocil et al, 1980; Brock and Shapiro, 1983a, 1983b; Shapiro and Brock, 1985). This conclusion is based on the observation that the mRNAs were degraded rapidly when the hormone was withdrawn. In *Xenopus laevis* liver, for example, vitellogenin mRNA is very stable after several days

oestrogen treatment (half-life = 500 hours), and decays with a half-life of 16 hours when oestrogen is removed (Shapiro and Brock, 1985; Brock and Shapiro, 1983a). The stabilisation in the presence of the hormone requires ribosome loading but not ongoing protein synthesis (Blume and Shapiro, 1989). The rate of degradation of apolipoprotein II and vitellogenin II mRNAs on hormone withdrawal in avian liver depends on the duration of the prior hormone treatment (Gordon *et al*, 1988), and there is evidence that this is also the case for ovalbumin induction in the oviduct (Palmiter and Carey, 1974).

In avian liver, the half-life of apolipoprotein II mRNA is 13 hours if oestrogen is withdrawn during the first two days of hormone induction, and drops to 1.5 hours when the hormone is withdrawn after 3 days or more. Gordon *et al* argue that oestrogen therefore does not selectively stabilise the mRNA, but rather that it induces a nuclease degradative capacity which only becomes active upon withdrawal (Gordon *et al*, 1988). This seems to contradict the results with vitellogenin mRNA in *Xenopus*, as it is hard to believe that such a long half-life of 500 hours is not due to some selective stabilisation effect. The apparent discrepancy may reflect species differences, but the data do not rule out the possibility that a degradative capacity is also induced in *Xenopus* liver which becomes active on withdrawal.

The mechanism of decay of these hormone induced mRNAs is beginning to be elucidated. Apolipoprotein II mRNA is initially cleaved in the 3' non-coding region at AAU and UAA elements whether it is in fast or slow decay mode, and these elements are thought to lie in single stranded loop regions (Bakker *et al*, 1988; Binder *et al*, 1989; Hwang *et al*, 1989; Cochrane and Deeley, 1989). This suggests the possibility that the mRNA is targeted for degradation by the initial endonuclease cleavage which removes the poly(A) tail, leaving the mRNA susceptible to exonucleases. The control mechanism therefore would involve an increase in the rate of the initial cleavage. Williams' group (Binder *et al*, 1989) have not observed any changes in the length of the poly(A) tail after withdrawal, but Cochrane and Deeley observed interesting transient effects of the hormone. The poly(A)

tail is 100 nucleotides longer during the first 24 hours of induction than after chronic stimulation. They also propose that the half-life decreases during the approach to steady state, using a computer algorithm to model this situation, correlating with the change in the poly(A) tail. This transient effect of the hormone during induction is also consistent with the half-life data (Cochrane and Deeley, 1988), and provides another model to be tested.

Hormonal control of mRNA half-life is therefore a complex system which will be fertile ground for clarifying the mechanisms involved in the regulation of mRNA stability. A variety of aspects of mRNA structure and translation have been implicated in the control of the decay of different mRNAs, such as the role of the 3' non-coding region in the example described above. At present, it seems that different expression systems vary in their modes of regulation of RNA stability, each one being appropriate for the function of the particular gene product. Some examples of the different modes of regulation will now be described, grouped under headings according to the principle determinant of stability in each system.

1.6: The role of terminal structures in controlling the rate of decay

1.6.1: The 7-methylguanosine cap

It has long been thought that the terminal structures of mRNA, in eukaryotic cells the 5' cap and the poly (A) tail, might be important for cytoplasmic stability of the mRNA. The "cap" structure composed of a 7-methylguanosine at the 5' end of eukaryotic mRNAs (Shatkin, 1976) is required for translation initiation and stability of the mRNA in wheat germ extracts and *Xenopus laevis* oocytes, and may be providing protection against a 5' exonuclease which is able to cleave unblocked mRNA (Furuichi et al, 1977). Such an exonuclease has been detected in yeast (Stevens, 1980). Therefore although the 5' cap is required for the production of functional mRNA, it is unlikely to be involved in the control of differential mRNA stability, as it is present on most eukaryotic mRNAs, with the exception of some viral mRNAs

(Shatkin, 1976) which presumably have alternative protective structures at their 5' ends. Recent evidence indicates that the 5' cap facilitates mRNA export from the nucleus (Hamm and Mattaj, 1990).

1.6.2: Stem loops

(i): Bacteria.

There are several cases documented where stem loop structures, or sequences with the potential to form stable hairpin loops in RNA, have been shown to play a role in controlling mRNA stability. The bacteriophage λ *int* gene expression from p_L is retroregulated by cleavage of a stem loop structure in the *sib* region by RNase III (Guaneros et al, 1982; Schmeissner et al, 1984). This cleavage renders the mRNA susceptible to degradation by 3' to 5' exonucleases, (for example RNase II and polynucleotide phosphorylase (Kaplan and Apirion, 1974; Deutscher, 1985; Donovan and Kushner, 1986;)), and results in low expression of *int*. The *int* gene is also transcribed from the p_I promoter, and these transcripts terminate at the t_I terminator stem loop which is not a substrate for RNase III. This therefore results in high level expression of the *int* gene product. RNase III is also involved in the regulation of expression of the bacteriophage T7 gene 1.2 (Saito and Richardson, 1981), and may be an important regulatory enzyme, as several proteins change their rate of synthesis in RNAase III deficient strains (Gitelman and Apirion, 1980).

A highly conserved inverted repeat which has the potential to form stable stem-loop structures in mRNA is the repetitive extragenic palindromic (REP) sequence of *E. coli* and *Salmonella typhimurium*. It is present at 500 to 1000 copies per cell and found in about 25% of all transcription units (Stern et al, 1984). The REP sequence has been shown to be capable of stabilising upstream mRNA by protecting it from 3' to 5' exonucleolytic attack in the histidine transport operon of *Salmonella typhimurium*, the *malEGF* operon of *E. coli*, and when cloned into other operons (Newbury et al, 1987a,b). Although this stabilisation seems to be important for controlling gene expression, the level of conservation of the REP sequences suggests that they may have

an additional function; recently DNA gyrase has been shown to bind REP sequences, indicating a possible role in the maintenance of supercoiling (Yang and Ames, 1988).

(ii): Control of iron metabolism in mammalian cells.

The importance of mRNA stem loops and their binding proteins in the regulation of gene expression is exemplified by the expression of proteins involved in iron metabolism in mammalian cells. The iron responsive element (IRE) is a proposed stem loop structure present as a single copy in the 5' untranslated region of ferritin mRNA, and as 5 variants in the 3' untranslated region of the transferrin receptor mRNA (Casey et al, 1988). When iron is in excess, ferritin synthesis and iron storage increase and uptake of iron decreases, via increased translation of ferritin mRNA and increased degradation of the transferrin receptor mRNA, respectively (Zahringer et al, 1976; Müllner and Kühn, 1988). There is also some evidence that prolonged exposure to iron causes the mRNA for ferritin to become more stable, perhaps as a result of its translocation to polysomes, or via the induction of a stabilising factor (Mattia et al, 1989, 1990). In recent years a great deal of information about the mechanism of concerted regulation of ferritin and transferrin receptor expression has been obtained.

Two of the IRE's from the 3' untranslated region of the transferrin receptor mRNA can independently confer iron-dependent translation of an indicator gene transcript when inserted into its 5' untranslated region, indicating that a common *trans*-acting factor may mediate the regulation of the stability of transferrin receptor mRNA and the translation of ferritin mRNA (Casey et al, 1988). In addition, the ferritin IRE has been shown to interact specifically, in an iron dependent manner, with a cytosolic protein in an *in vitro* gel shift assay, and the 3' untranslated region of the transferrin receptor mRNA competes for this binding (Roualt et al, 1988; Leibold and Munro, 1988; Koeller et al, 1989; Leibold et al, 1990). The transferrin receptor IREs vary in their affinities for this factor (Koeller et al, 1989), which has been purified by affinity purification from human liver and shown to be a 90kDa protein (Roualt et al, 1989).

The IRE binding protein can exist in two states, of high and low affinity, and is switched from one to the other by a redox mechanism (Hentze et al, 1989; Haile et al, 1989). The reduced form shows high affinity binding (10-30pM) and the oxidised form exhibits low affinity binding (2-5nM); increased binding activity correlates with decreased translation of ferritin mRNA, and increased stability of transferrin receptor mRNA (Haile et al, 1989; Casey et al, 1989; Barton et al, 1990). For the iron dependent regulation of the stability of transferrin receptor mRNA, three of the five IREs are required, and a cytosine residue at the base of the IRE loop is essential for high affinity binding of the regulatory protein. Loss of this residue leads to loss of iron regulation and low levels of mRNA, but deletions which do not prevent the high affinity interaction lead to loss of iron regulation with high levels of mRNA (Casey et al, 1989).

These data are consistent with the interpretation that the high affinity IRE/binding protein interaction protects the mRNA from a rapid degradation process which occurs via a determinant in the 3' untranslated region. An intriguing possibility raised by this hypothesis is that the instability determinant is in fact an alternative secondary structure; an alternative structure with very similar predicted energy has been proposed by others (Müllner and Kühn, 1988). The IRE binding protein may affect the equilibrium between these two structures as it switches between its high and low affinity forms. The recent cloning of the gene for the IRE binding protein will provide the experimental means of answering some of these questions (Roualt et al, 1990), and will no doubt produce valuable results, as this system is one of the few examples in eukaryotes where so much is known about the mRNA-protein interaction involved in the control of mRNA translation and stability.

(iii): Histone gene expression.

The expression of replication variant histone genes is restricted to the late G₁ and S phases of the cell cycle, and is tightly coupled to DNA synthesis. Drugs which inhibit DNA synthesis cause the rapid decay of histone mRNA with a half-life of approximately fifteen minutes, and histone mRNAs

disappear with a similar half-life at the end of S phase (Sittman et al, 1983; DeLisle et al, 1983; Heintz et al, 1983; Baumbach et al, 1984; Graves et al, 1985; Bandyopadhyay et al, 1987).

It has been shown with a temperature-sensitive cell cycle mutant that the 3' terminus of histone H4 mRNA is important for this regulation, and that similar sequences are important for both 3' end processing and correct regulation (Lüscher et al, 1985; Stauber et al, 1986; Capasso et al, 1987; Levine et al, 1987). The histone 3' hairpin loop has been highly conserved in evolution (Hentschel and Birnstiel, 1981), and polyadenylated histones and a replacement variant H3.3, are not cell cycle regulated (Sittman et al, 1983; Lüscher et al, 1985; Bird et al, 1985; Alterman et al, 1985; Cheng et al, 1989). Recent work by Morris et al (1991) has shown that the half-life of histone H3 mRNA is actively regulated during the cell cycle. These workers used a heat shock promoter so that synthesis of histone H3 mRNA could be induced at different points in the cell cycle. During late S phase a half-life of 10-15 minutes was observed, compared to 110 minutes early in S phase (Morris et al, 1991).

In vivo but not *in vitro*, protein synthesis is required for the destabilisation of histone mRNA when DNA synthesis stops, leading some workers to suggest that free histones autogenously regulate the stability of their mRNAs (DeLisle et al, 1983; Baumbach et al, 1984; Sive et al, 1984; Graves and Marzluff, 1984; Wu and Bonner, 1985; Sariban et al, 1985; Ross and Kobs, 1986). The lack of an effect of inhibition of protein synthesis *in vitro* is explained by the fact that the rate of protein synthesis *in vitro* is anyway very slow, so production of histone protein will be insufficient to yield an effect (Ross and Kobs, 1986). Direct evidence has been obtained that the concentration of free histone protein does indeed affect the rate of decay of histone mRNA *in vivo* and *in vitro* (Osley and Hereford, 1981; Peltz and Ross, 1987). In addition, the interactions of ribosomes with histone mRNA itself also seem to be important, as premature termination leads to histone mRNA stability in the absence of DNA synthesis (Graves et al, 1987). In addition, the hairpin loop must be at the 3' end,

and the distance from the termination codon to the 3' end must be less than 300 nucleotides. The degrading activity may be associated with the ribosome, and recognise the 3' end of the mRNA, or translation may alter the secondary structure, rendering the mRNA a target for the nuclease. The nuclease activity and the signal for its activation remain to be identified, but the same signal may also control deoxynucleotide metabolism (Graves and Marzluff, 1984).

An *in vitro* system for studying mRNA decay has been developed, based on polysomes and many of the components required for *in vitro* translation (Ross and Kobs, 1986). In this system, the relative rates of decay of several mRNAs are maintained, indicating that differential decay is obtainable *in vitro*. These workers have studied the decay of histone mRNA using this system, and shown that decay initiates with one or more cleavages at the 3' end; this result has since been confirmed *in vivo* (Ross and Kobs 1986; Ross *et al*, 1986). The two prominent cleavage products, 5 and 12 bases away from the 3' terminus, map to the stem loop, and are only detected in cells which are rapidly degrading their histone mRNA. The nuclease which degrades histone mRNA at its 3' terminus has been further characterised, and requires the presence of divalent cation, but not ATP or GTP, and can be released from polysomes in an active form by a high salt wash (Ross *et al*, 1987). In addition, an activity in the extract degrades a variety of non-polyadenylated mRNAs, whereas polyadenylated histone mRNA was at least 10 fold more stable than unmodified histone mRNA (Peltz *et al*, 1987). The nuclease activity itself is stable, and its activity *in vitro* is not affected by alterations in protein or DNA synthesis in cells from which the extract is made (Peltz *et al*, 1989). This indicates that either other *trans*-acting factors are involved in the changes in stability, or that the nuclease activity *in vitro* differs in some way from the *in vivo* activity.

These factors may relate to the location of the histone polysomes in the cell. Histone mRNA-containing polysomes are located predominantly on non-membrane bound polysomes which are associated with the cytoskeletal framework (Zambetti *et al*, 1985). This location is crucial for the correct

degradation of the mRNA when DNA synthesis is inhibited, as a histone mRNA bearing a signal sequence is targetted to membrane bound polysomes and is stable after DNA synthesis inhibition (Zambetti et al, 1987).

In summary, then, a working hypothesis can be drawn up for the mechanism of regulation of histone mRNA levels. The high concentration of free histones at the end of DNA synthesis probably provides the signal to degrade histone mRNA. This signal acts via polysomes associated with the cytoskeleton, and may activate a nuclease associated with the ribosomes. The nuclease then cleaves the mRNA close to the 3' end, destroying the stem loop and thus exposing the mRNA to attack by 3' to 5' exonucleases.

1.6.3: The poly(A) tail

(i): Introduction.

The function of the poly(A) tail at the 3' end of most eukaryotic messages has been implicated in the control of mRNA processing/transport, stability and translation (Brawerman, 1981). Nuclear processing of most eukaryotic mRNAs involves cleavage in the 3' untranslated region downstream of a AAUAAA sequence and the subsequent addition of on average 260 polyadenylate residues by poly(A) polymerase (reviewed in Brawerman and Diez, 1975; Humphrey and Proudfoot 1988). The poly(A) tail is shortened when the mRNA reaches the cytoplasm, rapidly at first, and then more slowly, to give a tail length distribution characteristic of each mRNA species (Mendecki et al, 1972; Sheiness and Darnell, 1973). Adenylate residues can also be added to mRNA in the cytoplasm (Brawerman, 1975), and presumably it is the equilibrium between these two processes which determines the steady state distribution of poly(A) length (Brawerman, 1975; Greenberg and Perry, 1972; Sheiness and Darnell, 1973; Jeffery and Brawerman, 1974).

Zeevi et al have concluded that newly synthesised adenovirus type 2 mRNA which lacks poly(A) can be spliced and transported to the cytoplasm, but has a shorter half-life than wild-type mRNA (Zeevi et al, 1982). This argues against a direct role for poly(A) in mRNA transport, although the presence of a short poly(A) tract could not be ruled out. A

bacterial mRNA was transported in *Xenopus* oocytes only when it was polyadenylated, but it is possible that poly(A)- mRNA was transported and then rapidly degraded (Wickens and Stephenson, 1984).

Experiments on ovalbumin and globin mRNAs have shown that the presence of a poly(A) tail can affect the efficiency of translation (Doel and Carey, 1976; Soler et al, 1987). In addition, more recent data clearly indicates that translational recruitment of mRNAs during *Xenopus laevis* oocyte maturation depends on changes in poly(A) tail length (McGrew et al, 1989; Fox and Wickens, 1990; McGrew and Richter, 1990; Paris and Richter, 1990). These effects may be mediated by the poly(A) binding protein, PABP, which will now be considered in slightly more detail.

(ii): Poly(A) binding protein.

The existence of poly(A) binding protein (PABP) has been known for some time (Kwan and Brawerman, 1972; Blobel, 1973). It exists in cytoplasmic and nuclear forms, both encoded by the same gene in yeast, and contains four tandemly repeated 90 amino acid domains (Sachs et al, 1986; Adam et al, 1986). These 90 amino acid domains have been identified in other RNA binding proteins (reviewed by Bandziulis et al, 1989). A single domain is sufficient for poly(A) binding, and the possession of several domains allows the molecule to move between poly(A) tracts via an interstrand exchange mechanism (Sachs et al, 1987). This is important for the regulation of expression of PABP, which occurs at the level of translation. The protein binds to a poly(A) rich region in the 5' untranslated region of its own mRNA and represses its translation. When new poly(A)+ mRNAs appear at the G₀ to G₁ transition, PABP binds to these and thus relieves the repression of its own synthesis (Sachs et al, 1986; Grange et al, 1987; Ullrich et al, 1988). Consistent with this, the increase in levels of PABP during *Xenopus laevis* development correlates with the observed increase in total poly(A)+ mRNA.

In reticulate lysates free poly(A) can inhibit translation, an effect which is relieved by the addition of PABP (Jacobsen and Favreu, 1983; Grossi de Sa et al, 1988). This suggests that poly(A)+ mRNA requires PABP for its

translation, and further work by Sachs and Davis (1989) provides *in vivo* evidence that this is the case. They showed that in yeast containing mutated PABP, mRNA with long poly(A) tails accumulated, and in addition, there was a decrease in the amount of large polysomes, and an increase in monosomes. Revertants were isolated and these were found to contain altered 60S ribosomal subunits which allowed normal translation initiation, but did not suppress the accumulation of long poly(A) tails. The suppressor mutation was located in the ribosomal protein gene L46. Another revertant had an altered gene encoding a putative rRNA helicase involved in the maturation of 25S rRNA (Sachs and Davis, 1990). Therefore it seems that translation in the absence of PABP requires some alteration in the ribosomal subunits. These results are consistent with the hypothesis that the requirement for PABP for translation of poly(A)+ mRNA prevents the translation of partly degraded mRNA, but it is also possible that poly(A) and PABP act as a translational enhancer (Munroe and Jacobsen, 1990).

(iii): Relationship between poly(A) tail length and mRNA stability.

Data on several specific mRNAs shows that the rate of shortening of their poly(A) tails determines their stability (Wilson *et al*, 1978; Brewer and Ross, 1988; Wilson and Treisman, 1988; Swartout and Kinniburgh, 1989). This effect seems to be specific for certain mRNAs, and may be independent of the shortening of the poly(A) tail of newly transported mRNA. The poly(A) tail of actin mRNA, for example, is rapidly shortened when it enters the cytoplasm, but the mRNA is stable (Krowczynska *et al*, 1985). The poly(A) tail of metallothionein mRNA is also shortened in two steps, initially rapidly, but more slowly when the tails are below a certain length (Mercer and Wake, 1985). An association between rate of poly(A) tail shortening and mRNA stability was first observed by Wilson *et al* (1978). The rate of poly(A) tail shortening of two Adenovirus type 2 mRNAs with different stabilities was studied in a pulse-chase experiment by measuring the rate of elution of the mRNA from poly(U) sepharose. The mRNA with the fastest turnover rate

accumulated molecules with short poly(A) faster than the longer-lived mRNA. In this case, it is not clear whether the authors were observing the initial loss of poly(A) when the molecules entered the cytoplasm, or a separate event. Their labelling time was 2.5 hours, and it is conceivable that the cytoplasmic molecules were not labelled to equilibrium during this time.

Human growth hormone (hGH) mRNA provides an example of an mRNA whose poly(A) tail increases in length at the same time as the mRNA is stabilised. hGH expression in the pituitary can be induced by the hormone dexamethasone, and this induction, like that of many hormone inducible genes (see Section 1.5.2), is controlled at both the transcriptional and post-transcriptional levels (Evans *et al*, 1982; Yaffe and Samuels, 1984; Diamond and Goodman, 1985). The mechanisms controlling hGH mRNA expression have been dissected using a model system in which thymidine kinase/hGH fusion genes have been transfected into mouse L cells (Paek and Axel, 1987). This system allows delineation of sequences important for induction, and shows that the coding sequence of hGH, coupled with a tk promoter and 3' region, retains inducibility. In the presence of the hormone, a subpopulation of 25% of the hGH mRNA was stabilised completely, compared with its half-life of 9 hours in the absence of the hormone. The mRNA was observed to increase in size during induction, due to a reversible increase in length of the poly(A) tail. It is not clear whether this is due to initial addition of a longer poly(A) tail in the nucleus, a decrease in the rate of its degradation, or addition of adenylate residues in the cytoplasm, but it is tempting to speculate that the increased poly(A) tail length is responsible for the enhanced stability. It is also possible that a pre-existing subpopulation of mRNA with a long poly(A) tail is selectively stabilised upon hormone induction; two distinct sizes of rat growth hormone mRNA have been observed in cultured pituitary cells, and the difference has been attributed to the poly(A) tail length (Eberhardt *et al*, 1982). Sequences in the coding region must be affecting the stability of the mRNA, but the mechanism of this is unknown. The regulation of hGH mRNA expression is discussed further in

Chapter 3, and the rate of poly(A) tail shortening and its relationship to mRNA stability is discussed further in the following section, in specific examples where the 3' untranslated region seems to affect poly(A) tail metabolism.

1.6.4: The 3' untranslated region.

Conservation of sequences in the 3' untranslated regions of homologous proteins in distantly related species indicates some functional role for this region of the mRNA (Yaffe et al, 1985). In particular, the mRNAs for transiently expressed gene products contain particular instability determinants in this region. These are described below, with particular reference to the *c-myc* and *c-fos* genes. These sequences seem to act by controlling the rate of shortening of the poly(A) tail, and therefore provide a hint that control of the rate of shortening of the poly(A) tail may be a more general control mechanism which is affected by different parameters in different systems.

A well-studied example of a gene which is regulated in part by control of the stability of its mRNA is that of *c-fos*. The *c-fos* protein is induced in response to many signals; mitogens, hormones and ionophores, and the induction is rapid and transient (Greenberg and Ziff, 1984; Kruijer et al, 1984). The transient nature of its expression is vital for normal control of cell growth and division, as it has been shown that over-expression leads to cell transformation (Miller et al, 1984; Meijlink et al, 1985). Removal of the 3' untranslated region has been shown to cause over-expression of the protein by stabilising the mRNA (Treisman, 1985; Fort et al, 1987; Rahmsdorf et al, 1987; Lee et al, 1988). In addition, mRNAs for other transiently expressed proteins including various lymphokines, cytokines and proto-oncogene mRNAs, contain a run of A and U bases in their 3' untranslated region, and in particular, the motif AUUUA is reiterated several times. This A-U rich region has been shown to impart instability to an otherwise stable β -globin mRNA (Caput et al, 1986; Shaw and Kamen, 1986). Many workers have noted that cycloheximide and other protein synthesis inhibitors prolong the half-life of *c-fos* mRNA, as well as increasing its rate of synthesis, a phenomenon known as

superinduction (Fort *et al*, 1987; Rahmsdorf *et al*, 1987). This indicates that either a labile protein is required for destabilisation, or that the *c-fos* mRNA is stable when it is not being actively translated. Work to distinguish between these possibilities has been carried out by Wilson and Treisman (1988), who demonstrated that cycloheximide acts instantly to block mRNA turnover, suggesting that ongoing translation of the *c-fos* mRNA itself is required for it to be unstable. It has also been observed that the *c-fos* mRNA decreases in length before turnover (Treisman 1985; Mitchell *et al*, 1986), and Wilson and Treisman have shown that this is due to a decrease in the length of the poly(A) tail, using a non-denaturing gel system to analyse poly(A) tail lengths. When the A-U rich sequence is removed from the 3' untranslated region, the mRNA is more stable and the poly(A) tail shows a markedly decreased rate of shortening, but degradation of the mutant mRNA is still dependent on ongoing translation (Wilson and Treisman, 1988). These data indicate, then, that the A-U rich sequences act to direct rapid shortening of the poly(A) tail in a translation dependent manner, and that the resulting de-adenylated RNA is labile.

Although the A-U rich region in the 3' untranslated region is clearly important for determining mRNA stability, *trans*-acting cellular factors are able to distinguish between different mRNAs of the rapid-turnover class. This is illustrated by the constitutive expression of GM-CSF mRNA in a monocyte tumour, due to selective stabilisation of its mRNA, in the absence of stabilisation of the *c-myc* and *c-fos* genes (Schuler and Cole, 1988). In spite of the similarity in overall base composition, and the presence of AUUUA motifs, there are no extended regions of homology between these sequences, and clearly there is scope for more detailed mapping of the region with respect to the control of mRNA stability.

In addition, sequences outside the 3' untranslated region of the *c-fos* mRNA influence its stability, as shown by Kabnick and Housman (1988) and Shyu *et al* (1989). Chimeric mRNAs between *c-fos* and β -globin showed that the 3' untranslated region of *c-fos* had a dramatic destabilising

effect on β -globin mRNA, but that *c-fos* was only partly stabilised by the 3' end of β -globin. The 5' untranslated region of *c-fos* was also able to destabilise β -globin mRNA, whereas the 5' region of β -globin had little effect on the rate of decay of *c-fos*. These results indicate that several individual sequence elements may be important for the instability of *c-fos* mRNA, but the higher order structure may also play a role.

Although the kinetics of induction of the *c-myc* gene are slower than those of *c-fos*, some aspects of their regulation are similar. *c-myc* is expressed at high levels in malignant cells, but transiently in normal cells when they enter the cell cycle (Campisi et al, 1984; Keath et al, 1984; Rabbitts et al, 1985a; Dean et al, 1986a). When HL-60 cells are induced to differentiate, transcriptional elongation is blocked in the first exon of the *c-myc* gene, and later, transcriptional initiation is inhibited (Bentley and Groudine, 1986; Eick and Bornkamm, 1986; Nepveu and Marcu, 1986; Bentley and Groudine, 1988). These data support the notion that *c-myc* expression drives proliferation, and that its absence allows differentiation. Like *c-fos*, *c-myc* is regulated at both the transcriptional and post-transcriptional levels, and cytoplasmic *c-myc* mRNA is very unstable (Dani et al, 1984; Dony et al, 1985; Blanchard et al, 1985; Dean et al, 1986b). Its stability can be further decreased by interferon treatment of some cells (Dani et al, 1985; Knight et al, 1985). As with *c-fos*, the 3' untranslated region primarily determines the short half-life, (Jones and Cole, 1987), but alterations at the 5' end of the mRNA can override the 3' signal (Piechaczyk et al, 1985; Rabbitts et al, 1985b; Eick et al, 1985; Jones and Cole, 1987).

The mechanism of *c-myc* mRNA degradation is also strikingly similar to that observed for *c-fos*. *In vitro* studies have shown that the poly(A) tail of *c-myc* is degraded first, followed by nuclease attack in the A-U rich region; γ -globin, on the other hand, shows no such poly(A) tail shortening, correlating with its longer stability (Brewer and Ross, 1988). These results are supported by *in vivo* data from Swartwout and Kinniburgh (1989), which show

that in growing cells, *c-myc* mRNA is deadenylated before it is degraded, but that in differentiating cells, poly(A)+ *c-myc* mRNA is directly degraded. The latter mode of decay may act as a failsafe mechanism to shut off *c-myc* expression in differentiating cells. Laird-Offringa et al (1989) also observed loss of the poly(A) tail prior to degradation *in vivo*. Like *c-fos*, *c-myc* is stabilised by treatment with cycloheximide (Linial et al, 1985), and it has recently been shown that loss of polyadenylate is the translation-dependent step (Laird-Offringa et al, 1990). *In vitro* data indicate that a soluble cytoplasmic component is responsible for *c-myc* mRNA decay which is inactivated by cycloheximide (Brewer and Ross, 1989). The role of this component is at present unclear, as the rapidity of the effect of cycloheximide on *c-myc* mRNA decay makes it likely that translation of the *c-myc* mRNA itself is required for its instability, as is the case with *c-fos*. The effect of translation on mRNA stability will now be discussed in more detail, again with reference to examples in which translation has been shown to be important.

1.7: The role of ribosomes

1.7.1: Bacteria.

As ribosomes are in intimate contact with the mRNA, it would not be surprising if they were involved in the biochemistry of mRNA stability. There is some evidence in bacteria that ribosomes play a passive role of protection of the mRNA from ribonucleases (Schneider et al, 1978). Using protein synthesis inhibitors which blocked ribosome initiation (kasugamycin) or elongation (chloramphenicol), these workers showed that the endonucleolytic cleavage of *lac* mRNA (Blundell and Kennell, 1974) was blocked when the ribosomes were stalled on the mRNA, but increased when initiation was prevented. This particular cleavage is thought to be by RNase III (Shen et al, 1981). The phenomenon of polarity in bacterial operons containing a nonsense mutation, in which distal genes are not expressed due to cleavage and degradation of that region of the mRNA, also supports the view that ribosomes protect mRNA from endonucleases (Morse and Yanofsky, 1969).

However, other evidence from bacteria and eukaryotes demonstrates that the involvement of ribosomes in mRNA decay is more complex than is suggested by this simple model. For example, the 5' untranslated region of the outer membrane protein A (*omp A*) mRNA was found to be as least as stable as the translated portion in rapidly growing cells (von Gabain et al, 1983). Also, mRNAs with different ribosome loadings were found to have no differences in their stability (Stanssens et al, 1986). The site of a premature stop codon on the mRNA can markedly alter the effect it has on the stability of the mRNA, as shown for nonsense mutations in β -lactamase (*bla*) mRNA (Nilsson et al, 1987). When the mutation is 5' to codon 26, the mRNA is destabilised, but if it is downstream (codon 56 or beyond) the mRNA has the same half-life as the wild-type. No difference was observed in the decay rates of the translated and non-translated regions of the message, demonstrating that a large unprotected region of mRNA can be stable.

Therefore the role of ribosomes in the decay of different mRNAs may depend on the mRNA in question. Alternatively, assembly of ribosomes on mRNA and their transit past a particular mRNA site might be required to maintain stability, or a minimum number of ribosomes on the message may be required for the whole mRNA to be stable. More work is required to distinguish between these possibilities, but the 5' untranslated region of bacterial mRNAs seems to play a role in controlling mRNA stability, possibly exerting its effect by affecting ribosome binding.

Studies by Belasco et al, (1986), on stabilities of *bla* and *omp A* mRNAs neatly illustrate that different mRNAs have different modes of decay, and that stability determinants can be localised to specific mRNA segments. The *omp A* message has a half-life of 6 minutes in slow growing cells and 15 minutes in rapidly growing cells, whereas *bla* mRNA (which is similar in length) decays with a half-life of 3 minutes regardless of growth rate (Nilsson et al, 1984; von Gabain et al, 1983). The 5' portions of each of these mRNAs are able to confer their particular mode of decay on the other when it is fused downstream, indicating the presence of distinct stability determinants at the 5' end of each message (Belasco

et al, 1986). More recently, specific endonuclease cleavage sites have been found in the 5' non-coding regions of both these mRNAs, and those in *omp A* are thought to regulate its growth rate dependent stability, as the rate of production of the cleavage products correlates with the rate of degradation (Melefors and von Gabain, 1988; Nilsson et al, 1988). The data are consistent with a model in which the initial endonucleolytic cleavages disrupt the binding of ribosomes to the mRNA, thereby rendering the remainder of the message unstable. Recently, a novel ribonuclease has been identified, ribonuclease K, which carries out these cleavages (Lundberg et al, 1990).

This model could also apply to the regulation of the bacteriophage T4 gene 32 product, another example in which sequences close to the initiation codon control expression. The protein product of gene 32 binds to the mRNA in this region and represses its translation (Krisch and Allet, 1982), and this 5' sequence also acts independently in a cis-dominant manner to stabilise the mRNA, but only in the presence of a phage *trans*-acting factor. Late in infection, these transcripts become unstable, perhaps due to cleavage at or near the ribosome binding site (Gorski et al, 1985). More recently, another RNA processing enzyme, RNase E, has been implicated in the decay of bacteriophage T4 mRNAs (Mudd et al, 1990).

1.7.2: Yeast.

Ribosomes or other *trans*-acting factors can therefore protect specific sequences at the 5' end of a bacterial mRNA which are the target sequences for rate-limiting endonuclease cleavage. A series of studies on the relationship between translation and mRNA stability has been carried out in *Saccharomyces cerevisiae* which seem to rule out a passive role for ribosomes in protecting mRNA from decay. Santiago et al (1986) studied the half-lives of a series of random mRNAs using the transcriptional inhibitor 1,10-phenanthroline. They found that the mRNAs fell into two classes, of long and short half-life, with an inverse correlation between mRNA length and half-life within each class. They could not account for the existence of the two populations by

differences in poly(A) tail lengths, and there was no correlation either between the level of ribosome loading and stability (Santiago *et al*, 1987). Looking at pyruvate kinase mRNA, the introduction of a deletion or stable stem loop into the 3' untranslated region drastically reduced the ribosome loading but did not affect transcript stability. Likewise, introduction of a premature termination codon close to the initiation codon aborted translation but did not alter the chemical half-life (Purvis *et al*, 1987). These observations are in contrast to those of others with the URA1 and URA3 genes (Losson and Lacroute, 1979; Pelsey and Lacroute, 1984), but a possible reason for this may be that the URA3 transcript belongs to the unstable class of mRNA; the effects of translation may differ between the two classes. In any case, a simple case of ribosome protection of mRNA does not seem to be operating in yeast.

1.7.3: Translation and the control of β -tubulin expression.

A striking example of where the translation of the mRNA plays a crucial role in the control of its stability is provided by the autoregulation of β -tubulin expression. α and β -tubulin are the principle constituents of microtubules, and the appropriate level of their production is achieved in response to the level of unpolymerised subunits in the cell (Ben Ze'ev *et al*, 1979; Cleveland *et al*, 1981). The response occurs at the level of the stability of the mRNA rather than at the level of transcription (Cleveland and Havercroft, 1983; Pachter *et al*, 1987; Caron *et al*, 1985; Pittenger and Cleveland, 1985). Furthermore, it has been shown by the elegant work of Cleveland and colleagues that sequences in the first exon of β -tubulin, 5' to codon 18, are essential for this regulation (Gay *et al*, 1987), and that only tubulin mRNAs associated with polysomes are regulated in this way (Pachter *et al*, 1987). They found that protein synthesis inhibitors which dissociate polysomes, (puromycin and pactomycin), prevented subunit-dependent regulation of β -tubulin mRNA, whereas cycloheximide, which interferes with ribosome translocation, did not interfere with the regulation. Further experiments have revealed that the instability of tubulin mRNA depends on recognition of the

first four amino acids of β -tubulin, met-arg-glu-ile, on the nascent polypeptide, rather than on the mRNA itself (Yen et al, 1988). Mutations which alter this polypeptide sequence abolish regulation, but mutations which conserve these four amino acids do not effect regulation. Frameshift mutations, or those which position the four amino acids internally in the peptide, also abolish regulation. Finally, the sequence must be followed by sufficient peptide for it to emerge from the ribosome.

The model proposed for tubulin autoregulation is that the unpolymerised subunits (or factors activated by them), bind the met-arg-glu-ile peptide as it emerges from the ribosome, thus activating a ribonuclease activity which is probably associated with the ribosome itself. Alternatively, the binding could cause transient ribosome stalling which leaves the mRNA exposed to non-specific ribonuclease. By slowing elongation to various extents it has now been demonstrated that continuous elongation (even at a very slow rate) is required for regulation; this is still compatible with either model (Gong and Brandhorst, 1988; Gay et al, 1989).

In *Chlamydomonas reinhardi*, tubulin is transiently induced after deflagellation, and the mRNA decays rapidly thereafter; once again, translation is required for this rapid degradation, and the data is consistent with the hypothesis that flagellar precursors regulate the stability of their mRNAs (Baker et al, 1986). Interestingly, a correlation has been observed between the loss of the poly(A) tail of tubulin mRNA and the increased rate of degradation of the mRNA during the cell cycle of *Physarum polycephalum*, (Green and Dove, 1988), and the poly(A) tail of tubulin mRNA is also shortened in *Chlamydomonas* during the process of accelerated degradation of tubulin mRNA after deflagellation. However, somewhat unexpectedly, the rate of shortening of the tubulin mRNA poly(A) tail is increased rather than decreased after cycloheximide treatment in *Chlamydomonas*, unlike the situation described for *c-fos* and *c-myc* (Baker et al, 1989; Section 1.6.4). More work is required in this area to assess the significance of this observation.

1.8: Summary and Aims.

From the data described above it is clear that control of mRNA stability is an important means of regulating gene expression in a variety of systems. These different expression systems have been discussed under headings according to the main factors that have been identified as important for the regulation mechanism. However, it is clear that some recurring themes are evident in several of these systems, and of particular interest are the sites in the mRNA molecules which control the degradation of the rest of the mRNA.

The stability of some bacterial mRNAs can be differentially regulated in polycistronic operons by stem loops at the 3' ends of the coding sequences. Other mRNA species can be regulated by endonucleolytic cleavages at their 5' ends, and the enzymes involved in this process are currently being identified. Differential susceptibility to the endonucleases seems to result at least in part from mRNA secondary structure in the region (Lundberg et al, 1990). The endonucleolytic cleavages could control mRNA stability by preventing ribosome loading, or by initiating degradation in a 5' to 3' direction. However, no 5' to 3' endonuclease has yet been identified in *E. coli* (Deutscher, 1988).

In mammalian cells, the 3' untranslated region of the mRNA has an important regulatory function in many cases. In apolipoprotein II mRNA, secondary structure in this region is the site of initial cleavages, which may target the mRNA for degradation by removal of the poly(A) tail. The transferrin receptor mRNA stability is regulated by stem loops in the 3' untranslated region, for which a specific binding protein has been identified, but it is not yet known how high affinity binding of this protein protects the mRNA from degradation. The A-U rich region in the 3' untranslated region has been shown to direct rapid shortening of the poly(A) tail of *c-fos*, leading to the decay of the rest of the mRNA, and *c-myc* mRNA decays in a similar manner. What is at present not clear is the role of translation of the mRNAs in this process. The work with yeast mutants indicates that PABP may have a role to play in coupling of translation and poly(A)

tail shortening.

Translation has been shown to be crucial for mRNA instability not only in the *c-myc* and *c-fos* systems, but also in β -tubulin and histone expression. One hypothesis is that a ribonuclease is associated with the ribosomes, and that it reacts to the appropriate signal by cleaving at a specific site in the mRNA. In histone mRNA, this site would be the 3' stem-loop, thus leaving the mRNA susceptible to exonucleases. Location of histone polysomes on the cytoskeleton is important for correct regulation, so it is possible that the signal which activates the ribonuclease acts via the cytoskeleton. The location of polysomes may be a more general feature in regulation of mRNA stability, and this question is raised in Chapters 5 and 6. Other workers have proposed that a nuclease activity is tightly associated with the mRNA itself, in the form of mRNP, and not bound to the ribosomes (Bandyopadhyay *et al*, 1990). The activity seems to be specific for each mRNA, for example cleaving at A-U sites in the 3' untranslated region of β -globin mRNA, and cleaving internally in actin mRNA. The nuclease seems to be inhibited by a cytoplasmic factor, so activation of the nuclease would involve disruption of the interaction of this factor with the nuclease. This could be affected by the secondary structure of the mRNA, which in turn could be altered by translating ribosomes. All of these possibilities remain to be investigated.

Immunoglobulin gene expression provides an ideal system for studying mRNA stability, as the high levels of mRNA produced in differentiated cells have been shown to be dependent on post-transcriptional processes, and the appropriate stable cell lines are available to study. Cloned genes are also available, and the cell lines are readily transfectable, allowing the effects of *in vitro* mutagenesis to be analysed. The work described in this thesis was aimed at arriving at a better understanding of the role, if any, of mRNA stability in the control of immunoglobulin gene expression in B cell lines representing different stages of differentiation. The work is divided into three parts:

(i) Development of an assay system useful for measuring mRNA half-lives in mammalian cells, using a model transfected

cell line.

(ii) Use of this assay system to compare the half-lives of μ heavy chain mRNA in two cell lines representing early and late stages of B cell differentiation.

(iii) Examination of the effect of mutations in the μ mRNA on its half-life in the differentiated cell type, in order to determine which factors are important for controlling its stability, with a particular interest in the effect of the localisation of the polysomes in the cell.

CHAPTER 2

MATERIALS AND METHODS

2.1: General materials

2.1.1: Reagents and equipment

General chemicals were obtained from B.D.H. Ltd., Poole, Dorset, or Sigma Chemical Co. Ltd., Poole, Dorset, unless otherwise stated. Ethanol was obtained from James Burrough (F.A.D.) Ltd.

Radioisotopes were from Amersham International, Little Chalfont, Bucks, or New England Nuclear (supplied by DuPont (U.K.) Ltd., Stevenage, Herts. "Protosol" tissue and gel solubiliser was also obtained from New England Nuclear and "Amplify" autoradiographic enhancer was obtained from Amersham International. X-ray film was supplied by Fuji Photo Film Co., Japan.

Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase and calf intestinal phosphatase were purchased from N.B.L., Cramlington, Northumberland, Boehringer Mannheim Ltd., Lewes, East Sussex, or New England Biolabs, Beverly, Mass., USA. SP6 polymerase, and ribonuclease A and T1, were purchased from Boehringer Mannheim Ltd., and "RNasin" ribonuclease inhibitor from Pharmacia LKB Biotechnology, Uppsala, Sweden.

Nitrocellulose filters and slot-blot apparatus were obtained from Schleicher and Schüll GmbH, Einbeck, West Germany and "Hybond-N" from Amersham International. Filter papers and DE81 paper circles were from Whatmann Ltd., Maidstone, Kent.

Horizontal and vertical gel electrophoresis equipment was made by Bethesda Research Laboratories Inc., Maryland USA.

"GeneClean" kits were supplied by BIO101 Inc., La Jolla, California, USA, and "Sequenase" kits from United States Biochemical Corporation, Cleveland, Ohio, USA.

Oligodeoxyribonucleotides were synthesised using an Applied Biosystems DNA synthesizer, model number 380B.

Bacto-tryptone, bacto-agar and yeast extract were obtained from Difco Ltd., Detroit, Michigan, USA. Foetal calf serum was obtained from Imperial Laboratories, Andover, Hampshire, and all other tissue culture medium components

were obtained from Gibco-BRL., Uxbridge, Middx.

2.1.2: Bacterial strains

Escherichia coli LM1035: a highly transforming derivative of *E. coli* HB101 (A. Docherty, personal communication).

E. coli HB101: F^- , *hsdS20* ($r_B^-m_B^-$), *leu*, *recA13*, *ara14*, *proA2*, *lacY1*, *galK2*, *rpsL20* (Str^r), *xyl-5*, *mtl-1*, *mcrB*, *supE44*. (Boyer and Roulland-Dussoix, 1969)

E. coli JM101: F' , *thi*, $\Delta(lac-proAB)$, *traD36*, *proAB*, *supE*, *lacI^q* $\Delta(lacZ)M15$ (Messing 1979, Gronenborn and Messing 1978)

E. coli HB2151: K12, *ara*, $\Delta(lac-pro)$, *thi*, F' , *proAB⁺*, *lacI^q* $\Delta(lacZ)M15$. (Carter et al 1985).

E. coli HB2154: as HB 2151 but *mutL::Tn10*. (Carter et al 1985)

2.1.3: Plasmids

pSP64 and pSP65 were obtained from Promega Biotech Ltd., Madison, USA.

pSVMTGH8 (Pavlakis and Hamer, 1983b) was a kind gift from G. Pavlakis, National Institute for Health, Bethesda, Maryland, USA.

pNP1 (Wood et al, 1984), was constructed at Celltech Ltd. p516, p372 (M. Neuberger, unpublished) and pMEB3 (Michot et al, 1982) were a kind gift from M. Neuberger, M.R.C. Laboratory of Molecular Biology, Hills Rd., Cambridge.

pAM1 (Minty et al, 1981) was a kind gift from The Patterson Laboratory, Manchester.

2.1.4: Buffers and solutions

In general, buffers and solutions for DNA work were made up in sterile deionised water, and where possible, were autoclaved or filtered through 0.2 μ m filters.

TE: 10mM Tris.Cl, pH 7.5, 1mM EDTA (1.21g Tris base, 0.372g EDTA per litre, adjusted to pH 7.5 with hydrochloric acid).

TNES: 10mM Tris.Cl, pH 7.3, 1mM EDTA, 100mM sodium chloride, 0.1% (w/v) SDS (1.21g Tris base, 0.372g EDTA, 5.84g sodium chloride, 1g SDS per litre, adjusted to pH 7.3 with

hydrochloric acid).

20X SSC: 3M sodium chloride, 0.3M trisodium citrate pH 7.0 (175.2g sodium chloride, 88.2g trisodium citrate per litre).

100X Denhardt's Solution: 20g Ficoll 400000, 20g polyvinylpyrrolidone, and 20g bovine serum albumin per litre.

Deionised Formamide: Formamide, obtained from B.D.H. Ltd., was deionised by stirring for approximately 30 minutes with 2g/60ml "Amberlite" MB-1 ion exchange resin (B.D.H.), until the pH was neutral. The resin was then removed by filtration and the formamide stored in tightly capped tubes at -20°C.

Phenol: Phenol, (B.R.L. nucleic acid grade) was equilibrated with Tris.Cl pH 7.0 as follows. 100g of phenol was thawed at 60°C and 100ml of water and 100µg of hydroxyquinoline were added. The mixture was shaken, then allowed to separate into two layers. The upper aqueous layer was removed by aspiration, and 100ml of 1M Tris.Cl pH 7.0 was added and mixed to form an emulsion as before. When the layers had separated once more, the aqueous layer was removed and more 1M Tris.Cl was added. This process was repeated until the pH of the aqueous layer was 7.0 or above. Finally the phenol was equilibrated in 0.1M Tris.Cl pH 7.0, and stored at 4°C for up to one month, or at -20°C indefinitely.

10mg/ml Salmon Sperm DNA: Salmon sperm DNA was dissolved by stirring overnight on a magnetic stirrer, then sheared by passing it through a 19 guage needle 20 times. It was then denatured by boiling for 20 minutes, cooled rapidly and stored in aliquots at -20°C.

10mg/ml Yeast RNA: Yeast RNA from Boehringer Mannheim Ltd. was dissolved at 10mg/ml in 0.06M sodium hydroxide, and stored at -20°C.

2.1.5: Bacterial growth media

L Broth: 10g bacto-tryptone, 5g yeast extract, 5g sodium chloride, 1ml 1M sodium hydroxide per litre.

LB Agar: As for L Broth but with 20g bacto-agar added per litre.

2X TY Broth: 16g bacto-tryptone, 10g yeast extract, and 5g sodium chloride per litre.

2X TY Agar: As for 2X TY broth but with 15g bacto-agar

added per litre.

H-top medium: 10g bacto-tryptone, 8g sodium chloride, 6g bacto-agar per litre.

Minimal Medium: 5X M9 salts were made up by dissolving 64g disodium hydrogen orthophosphate (heptahydrate), 15g potassium dihydrogen orthophosphate, 2.5g sodium chloride and 5g ammonium chloride in deionised water to a final volume of 1l. This solution was sterilised by autoclaving as described below. 200ml was then made up to 1l with sterile deionised water and the addition of 20ml 20% (w/v) glucose.

Bacterial culture media were sterilised by autoclaving at 120°C, 1.05kg/cm (15psi). Antibiotics were added after the media had cooled to below 55°C. Carbenicillin was added where appropriate to a final concentration of 100µg/ml. All manipulations involving bacterial growth media and cultures were carried out using standard microbiological aseptic technique.

2.1.6: Nucleic acid and protein size markers

λ/*Cla* I DNA markers were prepared by *Cla* I digestion of unmethylated bacteriophage λ DNA (obtained from Boehringer-Mannheim Ltd.). Similarly, pAT153/*Hpa* II markers were prepared by *Hpa* II digestion of pAT153 DNA.

The 0.24-9.5kb RNA ladder was obtained from Bethesda Research Labs., Maryland, USA.

Prestained high molecular weight protein markers were also obtained from B.R.L.

The sizes of these markers were as follows.

λ/*Cla* I:- 11381, 10493, 4398, 4203*, 3674, 2614, 2057*, 1916, 1806, 1703, 1112, 972, 657, 621, 537 and 354 bp.

* indicates the fragments containing λ cohesive ends; these are present in submolar amounts unless the markers are denatured by heating before use. Together they form a band of 6260 bp.

pAT153/*Hpa* II:- 622, 492, 403, 242, 238, 217, 201, 190, 147, 122, 110, 90, 76 and 67 bp.

RNA ladder:- 9.5, 7.5, 4.4, 2.4, 1.4 and 0.24 nucleotides.

Protein markers:- 200, 97.4, 68, 43, 25.7, 18.4, and 14.3 kDa.

2.2: DNA methods

2.2.1: Purification of DNA

DNA was routinely purified after various procedures by phenol and chloroform extraction followed by ethanol precipitation. This was carried out as follows. The solution containing DNA was added to an equal volume of phenol that had been pre-equilibrated with 0.1M Tris.Cl pH 7.0 (see Section 2.1.4). This was then mixed vigourously to form an emulsion, and the two phases were separated by centrifugation for 3 minutes in the Eppendorf centrifuge. The aqueous phase was then removed to a clean tube; if there was a lot of denatured protein at the interface, it could be further extracted with phenol or a 1:1 mixture of phenol and chloroform. To remove any remaining phenol, the aqueous phase was finally extracted with an equal volume of chloroform, mixing and centrifuging as before. The DNA was then precipitated from the aqueous phase by addition of sodium acetate to a final concentration of 0.3M, followed by 2 volumes of ethanol. It was allowed to precipitate at -20°C for 16-20 hours, or on dry ice for 10 minutes, then recovered by centrifugation for 15 minutes in an Eppendorf centrifuge. The supernatant was carefully removed and the pellet washed in 70% ethanol and recentrifuged. The pellet was then dried briefly under vacuum and resuspended in sterile water or the appropriate buffer.

2.2.2: Preparation of plasmid DNA

Plasmid DNA was prepared from bacterial cultures by the alkaline-lysis method (Maniatis et al, 1982). The following stock solutions were used:-

10X Solution 1: 500mM glucose, 100mM EDTA, 200mM Tris.Cl pH8.0. This was stored at 4°C and before each use, the solution was diluted down to 1X and lysozyme was added to a final concentration of 5mg/ml .

Solution II: 0.2M sodium hydroxide, 1% (w/v) SDS. This was made up fresh for each use.

Solution III: 3M potassium/5M acetate made up as follows. 294.42g potassium acetate was dissolved in approximately 600ml water, then 115ml glacial acetic acid was added, and the volume made up to 1l with water (final

pH=4.8). The solution was stored at 4°C until use.

Small scale preparations were carried out as follows. 5ml of L broth containing the appropriate antibiotic was inoculated with a single bacterial colony, and grown to saturation overnight by shaking vigorously at 37°C. The bacteria were harvested from 1.5ml of culture by centrifugation for 5 minutes in the Eppendorf centrifuge, and the remainder of the culture was stored at 4°C. The medium was removed from the bacterial pellet by aspiration, and the pellet resuspended by vortexing in 100µl of ice cold solution I to remove the bacterial cell walls. After 5 minutes at room temperature, 200µl of solution II was added and the contents of the tubes mixed by gentle inversion to lyse the cells. The chromosomal DNA and most of the protein was then precipitated by immediate addition of 150µl of solution III with mixing, and the tubes put on ice for 5 minutes. The lysates were then centrifuged in the Eppendorf centrifuge for 5 minutes, and the supernatant containing the plasmid DNA carefully transferred to a fresh tube. Protein was removed by extraction with an equal volume of phenol/chloroform (1:1), and the supernatant transferred to a fresh tube once more. Two volumes of ethanol were added, and, after 5 minutes at room temperature, the DNA was recovered by centrifugation for 5 minutes in the Eppendorf centrifuge. The pellets were washed in 70% (v/v) ethanol and dried briefly under vacuum, then resuspended in 50µl of sterile water. 5µl was then digested with the appropriate restriction enzyme. As the preparations also contain RNA, 50µg/ml ribonuclease A was routinely added to the restriction enzyme digestion mix.

For large scale preparations, 200ml of L broth containing antibiotic was inoculated at 1/100 with an overnight culture, and incubated overnight with vigorous shaking. Essentially the same procedure was followed as for the small scale preparations for cell lysis; the cultures were decanted into 250ml centrifuge bottles, centrifuged at 5000 r.p.m., 4°C, in the Sorvall GSA rotor, and the supernatant poured off. The lysis procedure was carried out as above, using 10ml solution I, 20ml solution II, and 15ml solution III. The lysate was then transferred to 50ml sterile centrifuge tubes (Falcon), and centrifuged at 4000 r.p.m., 4°C, for 15 minutes in the

Sorvall RC3B centrifuge. The supernatant was divided between two Falcon tubes and the DNA precipitated by adding 0.6 volumes of isopropanol and incubating at room temperature for 15 minutes. The DNA was pelleted by centrifugation in the Sorvall RC3B at 4°C, 4000 r.p.m., for 15 minutes, and the supernatant decanted off. The pellet was air dried briefly and resuspended in 5ml sterile water. The plasmid DNA was further purified by centrifugation to equilibrium in a caesium chloride density gradient. 6g caesium chloride and 100µl of 10mg/ml ethidium bromide were added, and the solution was transferred to "Quickseal" ultracentrifuge tubes which were topped up with liquid paraffin, balanced, and sealed. Ultracentrifugation was carried out at 20°C, 48000 r.p.m., for 16 hours in the Ti70 rotor of a Beckman L8-70 ultracentrifuge. The plasmid band could be easily viewed under uv illumination; sometimes an upper band of contaminating chromosomal DNA was also visible. After puncturing the top of the tube, the plasmid band was harvested by piercing the tube just below the band with a 19 guage needle attached to a 1ml syringe. The DNA was then drawn into the syringe, and transferred to an Eppendorf tube. The ethidium bromide was removed from the DNA by extraction with an equal volume of butanol; this was repeated until the butanol layer was no longer pink. The plasmid DNA was then diluted two-fold with sterile water and divided between an appropriate number of Eppendorf tubes for precipitation with 2 volumes of ethanol. After incubation at -20°C overnight, the DNA was recovered by centrifugation, washed in 70% ethanol, and dried under vacuum. It was resuspended in sterile water and the concentration determined by optical density measurement at 260nm, assuming that an optical density of 1.0 represents a concentration of 50µg/ml.

2.2.3: Enzyme reactions of DNA manipulation

(i): Restriction enzyme digestion

Plasmid DNA was cleaved at specific sequences using restriction endonucleases, for the subcloning of fragments into new vectors. Restriction enzyme digestions were carried out in 10mM Tris.Cl pH 7.5, 10mM magnesium chloride and 1mM dithiothreitol, plus 0mM, 50mM or 100mM sodium chloride as recommended by the manufacturer for each restriction enzyme.

Typical reactions contained 10 μ g of DNA, 10 μ l 10X concentrated buffer, and 50 units of restriction enzyme (a 5-fold excess) in a total volume of 100 μ l. Digestions were carried out at 37°C for 1-2 hours after which an aliquot of the reaction mix was analysed by agarose gel electrophoresis to determine whether the reaction had gone to completion. On occasions when partial digestion was required, the digestion time and conditions were determined empirically.

(ii): Phosphatase treatment of vectors

When subcloning fragments into vectors, the vectors were generally treated with calf intestinal alkaline phosphatase after restriction enzyme digestion, to remove the 5' terminal phosphate group and thus prevent self-ligation of the vector molecules. The reaction was generally carried out in restriction buffer to which 0.5 μ l of phosphatase (10 units) was added per μ g DNA per 10 μ l reaction volume. After incubating at 37°C for 30 minutes, the enzyme was destroyed by heating at 70°C for 10 minutes, and the DNA purified by phenol and chloroform extraction followed by ethanol precipitation.

(iii): Ligation

Ligation of DNA fragments using bacteriophage T4 ligase was carried out in 20 μ l reactions containing 1mM ATP, 20mM dithiothreitol, 50mM Tris.Cl pH 7.4, 10mM magnesium chloride and 2 units of T4 ligase. Typically 100ng of vector DNA was used, with a 3 fold molar excess of insert fragment. Control ligations containing only the vector were routinely carried out to determine the frequency of self-ligation of vector molecules. Ligation of fragments with cohesive ends was done at 16°C for 16 hours while blunt-ended fragments were ligated at 25°C for 16 hours. Ligation of synthetic linker molecules to DNA fragments was achieved using a 30-50 fold molar excess of linker to fragment; for example, 100nM of 5' ends of fragment to 3-5 μ M of linker.

(iv): Addition of 5' terminal phosphate groups using T4 polynucleotide kinase

Two types of reaction were carried out using polynucleotide kinase; 5' end labelling of oligonucleotides

(for use as hybridisation probes) and DNA size markers with [γ - 32 P]ATP, and addition of unlabelled phosphate to the 5' end of oligonucleotides before ligation. Phosphatased DNA fragments or oligonucleotides (1-50 pmol) were typically kinased in a 20 μ l reaction containing 50mM Tris.Cl pH 8.5, 5mM dithiothreitol, 0.1mM spermidine, 10mM magnesium chloride, 0.1mM EDTA and 10 units T4 polynucleotide kinase. Either 40 μ Ci of [γ - 32 P]ATP was added, or 0.5mM unlabelled ATP, as appropriate. The reaction was incubated at 37°C for 30 minutes, then the protein was removed by extraction with phenol and chloroform, followed by ethanol precipitation.

Oligonucleotides to be used as hybridisation probes were separated from unincorporated [γ - 32 P]ATP by Sephadex G50 chromatography as follows. A 1ml disposable syringe was plugged with polymer wool and a 0.9ml column of G50 (pre-equilibrated with TNES) was prepared. The syringe was then suspended over an Eppendorf tube in a 15ml Falcon centrifuge tube. The column was pre-spun at 2000 r.p.m. for 4 minutes in a bench top centrifuge, then 100 μ l of TNES was applied to the column and it was spun as before, to wash the column. The sample was applied in a volume of 100 μ l, and the column spun exactly as before, collecting the 100 μ l of effluent containing the labelled oligonucleotide in a fresh Eppendorf tube. The unincorporated nucleotides are retained by the column which was then discarded into the appropriate radioactive decay bin.

2.2.4: Agarose gel electrophoresis

Horizontal submerged gel electrophoresis was carried out according to McDonnell et al (1977). This method is based on the principle that over a limited range of fragment sizes, molecules of linear duplex DNA migrate through gel matrices at rates inversely proportional to the log of their molecular weights. The size range for effective resolution depends on the agarose concentration, and for most purposes a 1% gel was used. DNA can readily be visualised by staining with the intercalating dye, ethidium bromide, which fluoresces under exposure to ultraviolet light. Agarose gel electrophoresis was used analytically and preparatively, the latter for purification of fragments after restriction enzyme digestion,

for subsequent ligation. The following stock solutions were used:

50XTAE: 2M Tris.acetate, 0.05M EDTA, made up by dissolving 242g Tris base, 57.1ml glacial acetic acid, and 18.6g EDTA in 1l water.

Ficoll Loading Buffer: 35% (w/v) Ficoll 70, 1.5% (w/v) EDTA, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol, and 0.1% (w/v) orange G.

For a 100ml, 1% (w/v) agarose gel, 1g of agarose was added to 100ml of 1X TAE containing 0.5µg/ml ethidium bromide, and dissolved by boiling. The agarose solution was then cooled to 60°C and poured into the gel apparatus on a level surface. Normally a 14-well gel comb was used with 5mm x 1mm slot size. The agarose was allowed to set and the gel placed in the electrophoresis tank which contained 1l of 1X TAE and 0.5µg/ml ethidium bromide. 5µl of Ficoll loading buffer was added to 10µl of DNA sample, and the samples were loaded into the wells. Electrophoresis was typically carried out at 2V/cm for 16 hours. The DNA bands were visualised under uv illumination and could be photographed with a Polaroid MP4 land-camera using type 57 film. For recovery of DNA fragments for cloning, long wavelength uv light (366nm) was used, to minimise mutagenesis, but for non-preparative purposes, 254nm uv was used.

2.2.5: Recovery of DNA from agarose

Restriction fragments separated by agarose gel electrophoresis were recovered from the gel by glass extraction using a "Geneclean" kit. The DNA band of interest was excised from the agarose gel with a scalpel, weighed, and 2.5 volumes of 6M sodium iodide solution was then added. It was then incubated for 5 minutes at 50°C to dissolve the agarose. 5µl of the "glassmilk" suspension was added with mixing and the tube incubated on ice for 15 minutes to allow the DNA to bind to the "glassmilk". The "glassmilk"/DNA complex was pelleted by centrifugation for 5 seconds in the Eppendorf centrifuge, and the supernatant removed. The pellet was then washed 3 times with 200µl "NEW" solution (supplied with the kit; sodium chloride, Tris pH 7-8.3, EDTA, ethanol, water), gently resuspending the pellet with the pipette tip each time. The DNA was eluted from the

"glassmilk" at 50°C with 2 washes of 10µl of sterile water. An aliquot of the eluted DNA was analysed by gel electrophoresis to estimate the percentage recovery. In general, DNA recovered by this method could be used directly in ligation or other reactions.

2.2.6: Preparation of competent cells (*E.coli* LM1035)

For general cloning purposes, *E.coli* LM1035 was used as host strain. A single colony of *E. coli* LM1035 from a freshly-streaked plate was transferred to 10ml of L broth and grown overnight with vigourous shaking. 2ml of this culture were then transferred to 200ml of L broth in a 1l flask, and this culture was allowed to grow to an optical density at 600nm of 0.2. After this point all procedures were carried out at 0-4°C. The cells were transferred to a pre-chilled 250ml centrifuge bottle and recovered by centrifugation at 5000 r.p.m. for 10 minutes in the Sorvall GSA rotor at 4°C. The pellet was very gently resuspended in 50ml ice-cold 0.1M magnesium chloride. The bacteria were then recentrifuged at 4000 r.p.m. for 10 minutes, the supernatant removed, and the pellet resuspended in 10ml ice-cold calcium chloride (0.1M). 90ml cold 0.1M calcium chloride was then added, and the suspension was kept on ice for 20 minutes. Finally the bacteria were centrifuged at 4000 r.p.m. for 10 minutes, and the pellet was resuspended in 4ml freezing mix (85% 0.1M calcium chloride, 15% (v/v) glycerol). Aliquots of 400µl were quick-frozen on dry ice and stored at -70°C.

2.2.7: Transformation of bacteria

Competent *E. coli* LM1035 were usually transformed, either with 1/4 of a ligation mix or 10ng plasmid DNA. The cells were thawed on ice, and 100µl aliquotted into cold Eppendorf tubes. The DNA was added and they were incubated on ice for 15 minutes, at 37°C for 10 minutes, then on ice once more for 10 minutes. 700µl L-broth was added to the transformation mix which was further incubated at 37°C for 30 minutes to allow expression of the antibiotic resistance marker. 9/10 and 1/10 of the cells were plated out on LB-agar plates containing the appropriate antibiotic, which were incubated at 37°C for 16-20 hours.

2.2.8: Site-directed mutagenesis

(i): Outline of the method

Site-directed mutagenesis was carried out using the "gapped duplex" method of Kramer et al (1982, 1984). This uses an oligonucleotide to direct mutations at specific sites in DNA which has been cloned into an M13 bacteriophage vector. The principles of the method are as follows. The target DNA is subcloned into M13mp8, which contains an *amber* mutation that allows it to grow only in suppressor strains of *E. coli*, eg: JM101. The single stranded recombinant DNA ((+) strand) is annealed to double stranded (replicative form) DNA from M13mp18, which is wild-type for the *amber* mutation, and has been linearised by restriction enzyme digestion. The annealed molecule (the gapped duplex) is therefore double stranded at the M13 region and single stranded at the insert. The mutagenic oligonucleotide, which is homologous to the insert except at the site of the mutation, is then annealed to the gapped duplex and the (-) strand is filled in using Klenow polymerase and T4 ligase. The DNA is transformed into a *mut L* repair deficient strain and plated out with a wild type strain which does not support the growth of the bacteriophage derived from the *amber*-containing (+) strand. This procedure therefore selects for the mutated strand.

(ii): Transformation with M13mp8 recombinants

Recombinant M13mp8, containing the target sequence for mutagenesis, was prepared using the standard DNA manipulation techniques described above. *E. coli* JM101 were made competent for transformation as follows. A single colony from a minimal medium plate was picked into 10ml of 2XTY medium and incubated at 37°C with vigorous shaking for 16-20 hours. 0.2ml of this culture was diluted into 20ml 2XTY and grown to an optical density at 600nm of 0.6 (approximately 2 hours). The cells were centrifuged at 2000 r.p.m. at 4°C in the Sorvall RC3B centrifuge, for 5 minutes. The supernatant was removed and the cells gently resuspended in 10ml of ice-cold 50mM calcium chloride, then placed on ice for 20 minutes. The cells were centrifuged again, this time at 1000 r.p.m. for 5 minutes at 4°C, and then gently resuspended in 2ml of ice-cold 50mM calcium chloride. They were kept on ice

until use, and were still competent 24 hours later.

For the transformation, 0.2ml of competent *E. coli* JM101 were placed in ice-cold 15ml Falcon centrifuge tubes and 5 μ l (20ng) of ligation mix or control DNA was added. They were placed on ice for 40 minutes, then heat shocked at 42°C for 3 minutes and replaced on ice. Meanwhile, 25 μ l of 20mg/ml IPTG, 25 μ l of 2% X-Gal, 4ml molten 0.7% H-top agar and 100 μ l of an overnight culture of JM101 were mixed and kept at 47°C. This mixture was then added to the transformation mix and poured onto prewarmed 1.5% 2XTY plates, allowed to set, and incubated at 37°C for 16-20 hours.

Recombinant plaques were picked with a Pasteur pipette into 10ml 2XTY medium which contained 0.1ml of an overnight culture of JM101 grown from a single colony. The cultures were grown for 6 hours with vigorous shaking at 37°C, then the bacteria were harvested by centrifugation at 5000 r.p.m. for 10 minutes in the Sorvall RC3B. The supernatant was retained at 4°C for subsequent preparation of single stranded bacteriophage, and the pellet was used to make double stranded RF as described for small scale plasmid preparations. These were then analysed using suitable restriction enzyme digests and agarose gel electrophoresis to determine the orientation of the inserts.

(iii): Preparation of single stranded M13mp8 recombinants

Single stranded bacteriophage DNA was prepared from culture supernatants by PEG precipitation as follows. 5ml of supernatant was added to 1ml of a mixture of 2.5M sodium chloride, 20% (w/v) PEG 6000, and left at room temperature for 15 minutes. The precipitates were recovered by centrifugation at 5000 r.p.m., at 4°C, in the Sorvall RC3B centrifuge. The supernatant was aspirated off and the tubes were respun to remove last traces of supernatant. The white bacteriophage pellets were resuspended in 500 μ l TE, transferred to Eppendorf tubes, and the DNA was purified by phenol and chloroform extraction and ethanol precipitation. The pellets were resuspended in 50 μ l sterile water, examined by agarose gel electrophoresis, and quantitated by optical density reading at 260nm, assuming an OD of 1.0 represents a concentration of 37 μ g/ml.

(iv): Mutagenesis

The following stock solutions were used:-

10x hybridisation buffer: 100mM Tris.Cl pH 8.0, 100mM MgCl₂

10x nucleotide mix: 250μM each dNTP, 250μM rATP, 50mM dithiothreitol.

Mutagenic oligonucleotides were treated with T4 polynucleotide kinase to attach a phosphate moiety to their 5' end, to enable them to ligate. 400ng of oligonucleotide (approximately 40 pmole), was kinased in a 20μl reaction as described above, and resuspended in 40μl of sterile water, at 1pmole/μl.

0.2 pmole of single stranded recombinant M13mp8 DNA was hybridised in 0.4 ml Eppendorf tubes to an equimolar amount of the double stranded M13mp8 gap fragment (linearised at the *Eco* RI and *Bgl* II sites) in 10μl of hybridisation buffer. The mixture was heated to 100°C for 4 minutes, and allowed to cool slowly to 65°C or below over a period of at least 20 minutes. 1 pmole of kinased oligonucleotide was added to the mix, which was then heated to 65°C for 15 minutes and cooled slowly to room temperature. The duplex mixture was filled in by addition of 4μl of nucleotide mix, 4μl T4 ligase, 1μl Klenow polymerase, and sterile water to a final volume of 40μl. This was incubated for at least 45 minutes at room temperature, and the reaction was stopped by addition of 2μl of 0.25M EDTA and heating to 65°C for 10 minutes. The ligation mix could be stored at -20°C until it was required, at which time it was transformed into competent *E. coli* HB2154 (*mut* L, *sup* 0) and plated out into HB2151 (*sup* 0), as described above for JM101.

(v): *in situ* hybridisation screening

To distinguish plaques containing the mutants, *in situ* hybridisation screening (as described by Grunstein and Hogness, 1975) was performed using the mutagenic oligonucleotides as probes. The T_m of the mutant/mutant duplex is higher than that for the mutant/parental duplex as the latter contains mis-matched base pairs; this was used as the basis for distinguishing between mutant and parental plaques. Duplicate lifts using "Hybond N" filters were taken from the plates; a filter was placed on the plate for 2-3

minutes, and the filter and plate were marked to allow the filter to be orientated subsequently. The second filter was placed on the plate for 10 minutes, and both filters were air dried and baked at 80°C for 2 hours.

10 pmole of the oligonucleotide used for mutagenesis was kinased in a 10µl reaction containing 30µCi of [γ -³²P]ATP, and recovered by chromatography on a G50 column as described in Section 2.2.3(iv). The plaque lifts were prehybridised for 2-3 hours in 6x SSC, 10x Denhardt's solution, and 0.1% (w/v) SDS at 37°C, then placed in a Perspex chamber and hybridised to the labelled probe in 15ml of buffer for 16-20 hours at 37°C. The T_m of the mutant/mutant duplex was estimated according to the relationship: $T_m = (4 \times \text{number of G-C base pairs}) + (2 \times \text{number of A-T base pairs})$ (Suggs *et al*, 1981; Wallace *et al*, 1981). The filters were initially washed in several changes of 6XSSC at room temperature, and then the washing temperature was increased up to the $T_m - 5^\circ\text{C}$. The filters were then exposed to X-ray film overnight. Plaques which hybridised to the probe were distinguished by placing the X-ray film over the agar plates using the marks on the filters to orientate the film. Plaques giving a positive signal were grown up as described in section (ii), single stranded bacteriophage DNA was prepared and analysed by chain-termination sequencing as described below.

(vi): Nucleotide sequencing

Chain termination sequencing (Sanger *et al*, 1977; Messing *et al*, 1981) was carried out using the "Sequenase" kit according to the manufacturer's instructions. Equimolar amounts (0.5pmole) of single-stranded template and oligonucleotide sequencing primer were annealed in a volume of 10µl of "Sequenase" buffer (40mM Tris.Cl pH7.5, 10mM magnesium chloride, 50mM sodium chloride). The mixture was heated to 65°C for 2 minutes and allowed to cool slowly to room temperature over a period of 30 minutes. 5X concentrated labelling mix containing 7.5µM each dGTP, dCTP, and dTTP was diluted 5 fold with sterile water, and the "Sequenase" enzyme was diluted immediately before use, 1:8 in ice-cold TE. 1µl of 0.1M dithiothreitol, 2µl of diluted labelling mix, 0.5µl of [α -³⁵S]dATP (1000Ci/mmol), and 2µl of

diluted "Sequenase" were then added to the annealed primer/template. This labelling mixture was then added immediately to the termination mixes instead of incubating at room temperature as described in the protocol. The termination mixes consisted of 2.5 μ l of 80 μ M each deoxynucleoside triphosphate and 8 μ M of the appropriate dideoxynucleoside triphosphate. 3.5 μ l of labelling mix was added to each termination mix and they were incubated at 37°C for 5 minutes. 4 μ l of stop solution (95% (v/v) formamide, 20mM EDTA, 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol) was added to each tube and the samples were stored at -20°C until used.

The sequencing reactions were analysed by polyacrylamide/urea gel electrophoresis. The following stock solutions were used:-

5x TBE: 54g Tris base, 27.5g boric acid, 3.72g EDTA.2H₂O, dissolved in 1l water. When diluted to 1x TBE, the pH was 8.3.

Acrylamide solution: 40% (w/v) acrylamide/bisacrylamide (19:1); 38g acrylamide and 2g N-N'-methylenebisacrylamide dissolved in sterile water, made up to a final volume of 100ml, and sterilised by filtration through a 0.2 μ m "Nalgene" filtration unit. The solution was stored at 4°C for up to one month.

The gel solution was prepared by dissolving 424g urea in water with 200ml 5x TBE, and 150ml acrylamide solution, making the final volume to 1l. This solution was filtered through a 0.2 μ m "Nalgene" filtration unit and stored at 4°C for up to one month. Before use, 65ml sequencing gel solution was allowed to reach room temperature. 240 μ l of 10% (w/v) ammonium persulphate and 90 μ l TEMED were added, and the gel was immediately poured between 40x20x0.4cm glass plates separated by 40x1x0.04cm Plastikard spacers. The top glass plate was notched to allow insertion of the slot former, which had 20 0.5cm slots. Before use, the top glass plate was "siliconised" by treating with dimethyldichlorosilane solution, to facilitate separation of the plates after electrophoresis. When the gel had polymerised, the slot-former was removed and the gel clamped to the electrophoresis tank, which was filled with 1x TBE. The samples were heated to 80°C for 2 minutes and immediately loaded onto the gel;

3 μ l of each sample was used. The samples were run into the gel at 2000V for 3 minutes, then the gels were run at 20-25mA for 1.5-2 hours, depending on the distance of the sequence being read from the primer. After electrophoresis, the top glass plate was removed and the gel was soaked for 45 minutes in 10% (v/v) acetic acid, 10% (v/v) methanol, to remove the urea. They were then dried down under vacuum at 80°C before being exposed to X-ray film.

2.3: RNA methods

2.3.1: Removal of ribonuclease activity from glassware and solutions

All buffer solutions and glassware to be used with RNA were treated with diethylpyrocarbonate (DEPC) before use. DEPC carboxymethylates ribonuclease, (as well as other proteins and nucleic acids), thereby inactivating it. Buffer solutions were made up as normal, and then DEPC was added to a final concentration of 0.1% (v/v). The solution was shaken vigourously to dissolve the DEPC, and incubated for one to two hours at 37°C. The DEPC was destroyed by autoclaving. Litre bottles of deionised water were treated in the same way, and this was used to make up solutions which could not be DEPC treated themselves, for example those containing Tris.Cl, which inactivates the DEPC. Such solutions were made up in DEPC water and then sterilised by autoclaving, or by filtration through 0.2 μ m filters. General glassware was treated by incubating in 0.1% DEPC in water, and then autoclaving. "Corex" tubes were scrubbed with detergent, rinsed in deionised water and coated with dimethyldichlorosilane solution before being rinsed in deionised water again, air dried, and baked at 180°C for two hours to sterilise them.

In all the following methods, DEPC treated water and solutions were used throughout. All manipulations were carried out wearing gloves and using sterile Eppendorf tubes and Gilson tips. Separate stocks of those reagents which could not be DEPC treated (e.g. nucleotides, enzymes, etc.), were designated to be used for RNA work only.

2.3.2: Isolation of RNA

(i): Total RNA

Total RNA was prepared from mammalian cells according to the method of Cathala et al (1983). Cells were harvested by centrifugation at 1500 r.p.m. in a bench top centrifuge at room temperature for 5 minutes. They were then washed with isotonic saline (140mM sodium chloride, 10mM Tris.Cl pH8, 1.5mM magnesium chloride), and recentrifuged. The cell pellet was lysed in a solution containing 5M guanidinium thiocyanate (Fluka Ltd., Glossop, Derbyshire), 10mM EDTA, 50mM Tris.Cl pH7.5, and 8% (v/v) 2-mercaptoethanol. The 2-mercaptoethanol was not added until just before use. 0.5ml of this solution was used per 10^7 cells, and the cells were vortexed to ensure complete lysis. At this stage the lysates were frequently stored at -70°C for convenience. The lysates were sheared by passing them through a 19 guage needle 10 times and then through a 23 guage needle 20 times, to ensure that the DNA was all low molecular weight and did not precipitate at the next stage.

The RNA was precipitated by adding 5 volumes of 4M lithium chloride, and if RNA was being prepared from 10^7 cells or less, the lysate was generally split between an appropriate number of Eppendorf tubes. For larger numbers of cells, sterile centrifuge tubes (Falcon) were used. For greater than 10^8 cells, 7 volumes of 4M lithium chloride were added. The samples were left at 4°C for 20 hours to precipitate, and then centrifuged to recover the RNA. The small scale preparations were spun for 15 minutes in the Eppendorf centrifuge; large scale preparations were transferred to sterile siliconised "Corex" tubes and centrifuge at 10000 r.p.m. for 30 minutes in the Sorvall SS-34 rotor. In both cases, the supernatant was carefully removed, leaving a clearly visible white pellet. This pellet was washed by resuspending it in 3M lithium chloride, and pipetting it up and down until an even suspension was obtained. The centrifugation step was then repeated and the supernatant carefully removed as before. The pellet was resuspended in a small volume of TE containing 0.1% (w/v) SDS; usually $400\mu\text{l}$ for the small scale preparations or up to

1ml for the large scale preparations. It was then divided as appropriate between Eppendorf tubes. The pellet was dissolved by freezing the suspension on dry ice and vortexing it as it thawed, until the lumps were dispersed. To remove any remaining protein, the solution was extracted with equal volumes of phenol/chloroform (1:1; v/v) until no interface was visible, removing the aqueous layer to a clean tube each time. Finally the solution was extracted with an equal volume of chloroform, and the aqueous layer was transferred to a fresh tube. Sodium acetate was added to a final concentration of 0.3M, followed by 2 volumes of ethanol, and the samples were precipitated overnight at -20°C . The RNA was then recovered by centrifugation in the Eppendorf centrifuge, washed in 70% (v/v) ethanol, and dried briefly under vacuum. The small scale preparations were resuspended in 20 μl DEPC treated water, the large scale ones in 50-100 μl , and they were treated with deoxyribonuclease as described below.

(ii): Cytoplasmic RNA

Cytoplasmic and nuclear RNA were fractionated essentially as described by Favaloro et al (1981), with some modifications. The cells were harvested and washed as described for total RNA; the cell pellet was lysed in 1ml of 140mM sodium chloride, 10mM Tris.Cl pH8.6, 1.5mM magnesium chloride, and 0.5% (v/v) Nonidet P40 (added just before use) per 10^7 cells. 10mM vanadyl ribonucleoside complex (BRL) was sometimes added at this stage, as a ribonuclease inhibitor, but was not found to be essential. The cells were vortexed to lyse them, and placed on ice for 5 minutes. The lysates were underlayered with an equal volume of lysis buffer containing 1% (w/v) NP40 and 24% (w/v) sucrose, and centrifuged at 5000 r.p.m. in the Sorvall RC3B for 15 minutes to pellet the nuclei. The cytoplasmic layer was then carefully removed to a fresh tube, the sucrose layer discarded and the nuclear pellet drained. The nuclear pellet was lysed in guanidinium thiocyanate solution and stored at -70°C until nuclear RNA was prepared as described below in section (iii).

Cytoplasmic lysates (which could also be stored at -70°C), were treated with proteinase K by addition of an equal

volume of 200mM Tris.Cl pH7.5, 25mM EDTA, 300mM sodium chloride, 2% (w/v) SDS and 400µg/ml proteinase K (added just before use). Samples were incubated in this buffer at 37°C for 30 minutes, then extracted with an equal volume of phenol followed by an equal volume of chloroform, removing the aqueous layer to a fresh tube each time. Two volumes of ethanol were then added to the aqueous phase, the samples precipitated and recovered as for the total RNA preparations, and treated with deoxyribonuclease as described below (section (iv)).

(iii): Nuclear RNA

RNA was prepared from the nuclei exactly as described for the small scale total RNA preparations, taking extra care to properly shear the DNA before the lithium chloride step, as the relative concentration of DNA to RNA is much higher in these preparations.

(iv): Deoxyribonuclease treatment of RNA preparations

Quite often, when RNA was examined by agarose gel electrophoresis, it was found that a small amount of DNA contamination was present. Therefore all the RNA preparations were routinely treated with ribonuclease-free deoxyribonuclease. 10mM Tris.Cl pH7.5 and 6mM magnesium chloride were added to the RNA sample, followed by an excess (eg 50 units in a 100µl reaction volume) of deoxyribonuclease (Pharmacia). Samples were incubated at 37°C for 30 minutes to 1 hour, then extracted twice with equal volumes of phenol/chloroform (1:1) and the RNA precipitated and recovered as described for total RNA preparations.

(v): Optical density measurement of RNA

The concentration of RNA was determined by optical density measurement of suitable dilutions in a quartz cuvette at 260nm, using a Beckmann DU-8 spectrophotometer, assuming an O.D. of 1.0 represents a concentration of 37µg/ml.

2.3.3: Isolation of polyadenylated RNA

Polyadenylated RNA was selected by two rounds of hybridisation to oligo(dT) cellulose (Pharmacia). This was done essentially as described by Aviv and Leder, (1972),

using a batch method in Eppendorf tubes.

The following stock solutions were made up in DEPC treated water:-

2x binding buffer: 1.0M potassium chloride, 20mM Tris.Cl pH 7.6, 20mM magnesium chloride. 0.2% (v/v) sarkosyl was added just before use.

Elution buffer: 10mM Tris.Cl pH7.5, 1mM EDTA. 0.05% (v/v) sarkosyl was added just before use.

Oligo(dT) cellulose (type 7 from Pharmacia) was equilibrated in water, washed twice in elution buffer, and twice in 1x binding buffer. It was resuspended at an approximate concentration of 1g per 10ml binding buffer and stored at 4°C until use.

Total RNA was heated to 65°C for 5 minutes then chilled rapidly on ice. It was then added to an equal volume of 2x binding buffer and mixed with oligo(dT) cellulose; normally 30mg oligo(dT) cellulose was used per 50µg total RNA. Samples were mixed well and left at room temperature for 5 minutes, mixed again and then centrifuged in the Eppendorf centrifuge for 5 minutes. The supernatant was removed to a clean tube and kept at -70°C. The oligo(dT) cellulose was washed 5 times with 1ml binding buffer, and the optical density of the washes was monitored at 260nm; no further decrease in the optical density was observed after the fourth wash, indicating that the unbound material had been removed. The polyadenylated RNA was eluted by washing 4 times with 800µl of elution buffer, and precipitated by addition of sodium acetate to a final concentration of 0.3M, and 2 volumes of ethanol. After precipitation for 20 hours at -20°C, the polyadenylated RNA was recovered by centrifugation in "Corex" tubes at 10000 r.p.m., 4°C, in the Sorvall SS-34 rotor, washed in 70% (v/v) ethanol, dried briefly under vacuum, and resuspended in DEPC treated water. The binding and elution procedure was then repeated. The RNA was quantitated by optical density measurement as for total RNA.

The specific activity of ³H-labelled polyadenylated RNA was determined by placing an aliquot of known volume in a scintillation vial with 10 volumes of an aqueous scintillant (Emulsifier Safe from Packard), and counting on the Packard 1500 liquid scintillation counter. The counter was calibrated with a series of quench standards to enable d.p.m.

to be determined from c.p.m. Specific activity in d.p.m./ μg was calculated by dividing the number of d.p.m./ml by the concentration of RNA in $\mu\text{g}/\text{ml}$.

2.3.4: Agarose gel electrophoresis of RNA

RNA was resolved by electrophoresis on agarose gels containing 6M formaldehyde, as described by Lehrach et al, (1977). The following stock solutions were used:-

10x gel buffer: 0.2M MOPS pH 7.0, 0.05M sodium acetate, 5mM EDTA.

Loading dyes: 50% (v/v) sterile glycerol, 1mM EDTA, 0.4% (w/v) bromophenol blue, 0.4% (w/v) xylene cyanol.

Before each use, the electrophoresis apparatus was soaked for two hours in 0.5M sodium hydroxide, 1% (w/v) SDS, to remove any contaminating ribonuclease, then rinsed thoroughly in deionised water. 150ml gel solution was made up as follows. 1.5g agarose was dissolved in 102.4ml sterile deionised water by boiling. It was allowed to cool to 60°C, then 32.6ml of 37% formaldehyde (Fisons Ltd., Loughborough.) and 15ml 10x gel buffer was added, and the gel was poured.

Samples were denatured by addition of 2 μl 10X gel buffer, 3.5 μl 37% formaldehyde, and 10 μl of deionised formamide in a total volume of 25 μl . They were heated to 55°C for 15 minutes, then chilled rapidly on ice before adding 2 μl loading dye. Gels were run submerged in running buffer (675ml deionised water, 225ml 37% formaldehyde, 100ml 10X gel buffer per litre), at 10V/cm, until the bromophenol blue dye was 2/3 of the way down the gel.

For visualisation of the RNA by ethidium bromide staining, gels were rinsed in deionised water several times at room temperature, then stained in 1 $\mu\text{g}/\text{ml}$ ethidium bromide overnight. They were destained in water and photographed under uv illumination.

2.3.5: Northern blotting

(i): Transfer of RNA to "Hybond-N"

RNA resolved on agarose gels was transferred to "Hybond-N" (Amersham International) essentially as described by Thomas (1980) for nitrocellulose. After electrophoresis, gels were rinsed 6 times for 5 minutes each in deionised water. They were then placed on a "wick" of Whatmann 17

paper soaked in 10X SSC, the ends of the paper dipping into a reservoir of 10X SSC. This was achieved by supporting the wick, with the gel on top, on a glass plate resting on a shallow tray containing the buffer. Care was taken to avoid the inclusion of air bubbles between the gel and the paper. A sheet of "Hybond-N" was placed on top of the gel, again being careful to remove any air bubbles. It was not necessary to pre-wet the Hybond. Two sheets of 3MM paper, wetted in 2X SSC, were placed on top of the Hybond, followed by two dry sheets of 3MM. These were covered with a 10cm thick layer of paper towels, a glass plate, and a weight. Transfer was allowed to proceed for 12-24 hours. The filter was baked at 80°C for two hours and stored at room temperature until it was hybridised. At this stage, marker tracks containing the BRL 0.24-9.5kb RNA ladder were cut off from the rest of the filter with a scalpel to visualise the marker bands. The strip of filter was fixed in 7.5% (v/v) acetic acid for 15 minutes, then stained in 0.04% (w/v) methylene blue, 0.5M sodium acetate, for 15 minutes, destained as necessary in water, then allowed to dry.

(ii): Hybridisation with SP6 probes

Filters were prehybridised for 1-2 hours at 52°C in 50% (v/v) formamide, 5X SSC, 8x Denhardt's solution, 50mM disodium hydrogen orthophosphate (pH6.5), 0.1% (w/v) SDS, 0.25mg/ml salmon sperm DNA, and 0.5mg/ml yeast RNA. They were then transferred to a heavy duty polythene bag containing 10-20 mls of the same buffer, and the appropriate ³²P-labelled RNA probe, generated as described in Section 2.3.6, was added. The hybridisation was allowed to proceed for 16-24 hours at 52°C. Non-hybridised molecules were washed off by incubating the filter at 65°C in 2X SSC, 0.1% (w/v) SDS, 3 times for 30 minutes each, then at 65°C in 0.2X SSC, 0.1% (w/v) SDS for 30 minutes. This hybridisation protocol was suitable for all probes which were completely homologous to the RNA sequence of interest. In addition, some of the longer probes were washed for 30 minutes in 0.1XSSC, 0.1% (w/v) SDS, at 80°C. This removed non-specific binding to ribosomal RNA.

The filters were sealed in thin polythene bags and exposed to Fuji X-ray film. If it was necessary to reprobe

the filter later with a new probe, the old probe was stripped off by boiling the filter in 0.1X TE, 0.1% SDS for 20 minutes. The filter was re-exposed to X-ray film to check that all of the previous signal had been removed, and then pre-hybridised and hybridised as before.

(iii): Quantitation of hybridisation signal.

Two methods were used to quantitate the hybridisation signal from Northern blots, liquid scintillation counting and densitometric scanning. For the first, the positions of the radioactive bands on the filter were determined by using radioactive ink to make crosses on paper surrounding the filter which would give a signal on the autoradiograph. These were then used to accurately position the autoradiograph on the filter, and the appropriate bands were cut out using a scalpel. The gel slices were placed in scintillation vials (Packard) and 5ml of aqueous scintillant ("Emulsifier Safe") was added. The vials were counted in the Packard 1500 liquid scintillation counter.

Densitometric scanning was carried out using an LKB Ultrosan II laser densitometer on a series of timed exposures of autoradiographs, to determine the linear response region of the film for each band to be quantitated. The signal intensity in terms of peak area was plotted against exposure time for each band, and the slope of the linear portion of the graph was taken for comparison of hybridisation signals between different samples on the same gel.

2.3.6: Slot blotting

RNA samples were loaded onto nitrocellulose using a Schleicher and Schüll slot-blot apparatus, which uses vacuum suction to apply the samples to the filter. Samples in 100 μ l of DEPC treated water were denatured by adding 150 μ l each of 6.15M formaldehyde and 10X SSC, and heating to 65°C for 15 minutes. They were then chilled quickly on ice. The nitrocellulose was presoaked in 10X SSC and the slot blot apparatus assembled according to the manufacturer's instructions. The sample wells were washed with 400 μ l 10X SSC before application of the samples, and the samples were applied under gentle suction. The wells were then washed

again with 10X SSC. The apparatus was dismantled carefully and the nitrocellulose baked at 80°C for 2 hours. The filters were stored at room temperature until use, then hybridised to ³²P-labelled probes as described for Northern blots.

2.3.7: *in vitro* transcription using SP6 polymerase

(i): Linearisation of SP6 plasmid templates

Plasmids containing the SP6 promoter for generation of RNA probes (pSP64 and pSP65 containing the desired insert) were linearised downstream of the probe sequence by restriction enzyme digestion. Generally it was convenient to use a restriction enzyme site in the polylinker sequence, eg *Hind* III for pSP65 and *Eco* RI for pSP64. Typically, 20µg of plasmid was digested in a 100µl reaction containing >50 units of enzyme. This was made possible by the use of high concentration restriction enzymes. After incubation at 37°C for two hours, an aliquot was examined by agarose gel electrophoresis to demonstrate that the reaction had gone to completion, ie that a single linear band was visible. The DNA was purified by extraction with equal volumes of phenol followed by chloroform, removing the aqueous phase to a clean tube each time. It was precipitated by addition of sodium acetate to a final concentration of 0.3M, and two volumes of ethanol. After precipitation on dry ice for 10 minutes, or at -20°C for 16-20 hours, it was recovered by centrifugation for 15 minutes in the Eppendorf centrifuge, washed in 70% (v/v) ethanol, and dried briefly under vacuum. The DNA was generally resuspended in 20µl of water, i.e. at 1µg/µl.

(ii): Transcription reaction

In vitro transcription reactions were carried out essentially as described by Green et al (1983), using two variations on the basic method, depending on the subsequent use of the probe. The basic reaction mix contained 500µM all four ribonucleoside triphosphates, 40mM Tris.Cl pH 7.5, 6mM magnesium chloride, 2mM spermidine, 10mM dithiothreitol, 1 unit/µl "RNasin" (Promega Biotech), 100µg/ml linearised plasmid template, 1 unit/µl SP6 polymerase (BCL).

Probes for hybridisation to Northern blots were generated in 20µl reactions containing 100 or 50µCi of [α -

^{32}P]GTP and 10 μM unlabelled GTP; this generates a probe with a specific activity of greater than 10^8 c.p.m./ μg . Probes to be used in ribonuclease protection experiments are not required to be of high specific activity, but it is important that they are uniformly full length, and larger amounts of probe are generally required. Therefore in these reactions the unlabelled GTP was kept at 500 μM , and in addition, an extra 1mM ATP was added. The K_m of SP6 polymerase is significantly higher for ATP than for the other nucleotides, so this ensured that it was not at limiting concentration (Melton et al, 1984). Labelled GTP was added as appropriate and the reactions were carried out in 50 μl volume.

All reactions were incubated at 40°C for two hours and then treated with 1 unit/ μl of deoxyribonuclease (ribonuclease free from Pharmacia), at 37°C for 20 minutes. The RNA probe was recovered by extraction with an equal volume of phenol/chloroform (1:1; v/v). Subsequent purification of the probe again depended on its intended use; probes for Northern blots were separated from unincorporated nucleotides by Sephadex G100 chromatography in TNES, whereas probes for ribonuclease protection experiments were precipitated several times from ethanol. These two methods are now described in detail.

(iii): Sephadex G100 chromatography

G100 was equilibrated in TNES and used to form a 1ml column in a sterile 1ml syringe, the end of which was blocked with polymer wool. The syringe was suspended over an Eppendorf tube, and the column washed with 1ml TNES. 50 μg yeast tRNA (Boehringer-Mannheim Ltd.) in TNES was applied to the column to block any non-specific RNA binding sites, followed by a further 1ml wash with TNES. The probe sample was applied to the column in a volume of 200 μl , and the flow-through discarded. 200 μl washes were collected; the incorporated radioactivity was found in the first 600 μl , the unincorporated nucleotides remaining on the column.

(iv): Removal of unincorporated nucleotides by ethanol precipitation

Ribonuclease protection probes were precipitated by

addition of ammonium acetate to a final concentration of 2M, followed by 2 volumes of ethanol. After incubation on dry ice for 10 minutes the probes were recovered by centrifugation for 15 minutes in the Eppendorf centrifuge, dried briefly under vacuum, and resuspended in 100 μ l DEPC treated water. The precipitation procedure was repeated three times in all; this was found to remove greater than 90% of the unincorporated nucleotides.

(v): Quantitation of probes

Unlabelled probes were quantitated by optical density measurement of suitable dilutions in a quartz cuvette at 260nm, using a Beckman DU-8 spectrophotometer. It was assumed that an A_{260} reading of 1.0 is equivalent to 37 μ g/ml of RNA.

The 32 P-labelled probes were quantitated by measuring the level of incorporation of the radioactive nucleotide. This was achieved by applying duplicate spots of 5 μ l of a 1/10 dilution of the sample onto Whatmann DE81 paper. One duplicate set was allowed to dry, and the other was washed (without allowing it to dry), six times for 5 minutes each in 0.5M disodium hydrogen orthophosphate, pH6.5, at room temperature. The papers were then rinsed in water and in ethanol for 1 minute each, and allowed to dry. They were placed in scintillation vials and counted in the Packard 1500 scintillation counter using "Emulsifier Safe" scintillant. The washed papers represent the incorporated radioactivity, the unwashed the total radioactivity. It is possible to calculate the number of moles of RNA synthesised from the concentration of GTP in the reaction and the number of guanosine nucleotides in the probe sequence, as illustrated by the following example.

The probe pHGHPROBE (see Chapter 3) contains 62 guanosine residues, and the amount of unlabelled GTP in the reaction is 25nmoles. In a typical reaction, 1.6×10^7 c.p.m. were incorporated into RNA out of a total of 3.6×10^7 in the reaction.

The amount of labelled GTP in the reaction is negligible, so the specific activity of the GTP in the reaction is

$$3.6 \times 10^7 / 25 = 1.44 \times 10^6 \text{ c.p.m./nmole}$$

Therefore the number of nmole GTP incorporated into RNA is

$$1.6 \times 10^7 / 1.44 \times 10^6 = 11.1 \text{ nmole.}$$

Therefore the number of nmole RNA made is

$$11.1 / 62 = 0.179 \text{ nmole.}$$

2.3.8: Ribonuclease protection analysis

(i): Ribonuclease digestion

Probes generated using SP6 polymerase were hybridised to total or cytoplasmic RNA according to the method of Hay et al, (1977). Appropriate amounts of RNA and probe, depending on the experiment, were mixed in a volume of 30 μ l of buffer containing 10mM sodium chloride, 10mM Tris.Cl pH7.5, and 1mM EDTA. The mixture was denatured by addition of 9 volumes of dimethyl sulphoxide and incubation at 45°C for 30 minutes. Sodium chloride, Tris.Cl pH7.5 and EDTA were then added to give final concentrations of 30mM, 10mM, and 1.5mM respectively, and the dimethyl sulphoxide concentration was reduced to 63% (v/v). The RNA and probe were allowed to hybridise for 20 hours at 37°C, and then precipitated by addition of sodium acetate to a final concentration of 0.3M, and two volumes of ethanol. If small amounts of RNA were being used, (less than 10 μ g), then tRNA from *E. coli* (Boehringer-Mannheim Ltd.) was added as carrier. After precipitation on dry ice for 10 minutes, the RNA hybrids were pelleted by centrifugation for 15 minutes in an Eppendorf centrifuge, and dried briefly under vacuum.

Ribonuclease digestion was carried out essentially as described by Zinn et al (1983). The pellets were resuspended in 350 μ l of ribonuclease buffer (10mM Tris.Cl pH7.5, 5mM EDTA and 300mM sodium chloride), and ribonuclease A and T₁ were added to an appropriate concentration depending on the amount of RNA and the probe being used. The ribonuclease digestion conditions were determined for each set of experiments by titration of the amount of ribonuclease and also varying the temperature of digestion. Typical reaction conditions were 10 μ g/ml ribonuclease A, and 0.5 μ g/ml T₁, at a temperature of 30°C. After digestion, 50 μ g of proteinase K and 20 μ l of 10% (w/v) SDS were added and the samples were incubated at 37°C for 15 minutes to allow the ribonuclease to be digested.

They were then extracted with an equal volume of phenol/chloroform (1:1; v/v), and the aqueous phase was transferred to a fresh tube. The protected fragments were precipitated by addition of 10 μ g glycogen as carrier and 1ml ethanol. After incubation on dry ice for 10 minutes, they were recovered by centrifugation in the Eppendorf centrifuge for 15 minutes, washed in 70% (v/v) ethanol, recentrifuged, and dried briefly under vacuum. These pellets were then analysed by polyacrylamide/urea gel electrophoresis, as described below.

(ii): Polyacrylamide gel electrophoresis

Analysis of protected fragments from ribonuclease protection experiments was done on 1mm thick polyacrylamide gels (5% (w/v) or 3.5% (w/v)), containing 8M urea. The acrylamide and TBE stock solutions were prepared as described for sequencing gels (Section 2.2.8(vi)).

Formamide dyes: 8ml deionised formamide, 1ml 10X TBE, 0.5ml 1% (w/v) xylene cyanol, 0.5ml 1% (w/v) bromophenol blue (total volume 10ml).

5% (w/v) ammonium persulphate: 0.5g dissolved in 10ml sterile deionised water. This solution was made up fresh for each gel.

100ml of 5% (w/v) gel solution was prepared by dissolving 40g urea in 10ml TBE, 12.5ml 40% (w/v) acrylamide, 1.5ml ammonium persulphate and 47.5ml water. For 3.5% (w/v) gel solution, 8.75ml 40% (w/v) acrylamide and 51.25ml water were used.

To prepare a gel, the gel solution was dissolved, then filtered through a "Nalgene" 0.2 μ m filter, and allowed to reach room temperature. 30 μ l of TEMED was added, and the gels were immediately poured between two 40x20x0.3 cm glass plates, separated by 40x1x0.1cm spacers. The top gel plate was siliconised, and notched at one end, where a 0.1cm thick 20-well gel comb was inserted to form the slots. When the gel had polymerised, it was clamped into a vertical electrophoresis apparatus ready for loading, the reservoirs were filled with 1X TBE and the gel comb removed. The slots were washed out using a syringe before the samples were loaded.

Samples were dissolved in 10 or 15 μ l formamide dyes,

heated to 95°C for 5 minutes and cooled rapidly on ice before loading. Electrophoresis was carried out in 1X TBE at 750-1000 volts, until the bromophenol blue dye was at the bottom of the gel. The glass plates were then separated, and the gel was either treated for fluorography (gels containing ³H), or transferred to Whatmann 3MM paper, covered in Saranwrap, and dried down under vacuum at 80°C (gels containing ³²P).

(iii): Fluorography

In order to visualise bands labelled with ³H, gels were subjected to fluorography as follows. Polyacrylamide gels were soaked in deionised water to remove the urea. They were then treated with "Amplify" (Amersham International) for 30-45 minutes at room temperature with gentle agitation. Subsequently they were transferred to Whatmann 3MM paper, covered in Saranwrap, and dried down under vacuum at 60°C.

(iv): Autoradiography

Gels were covered in Saranwrap and exposed to Fuji X-ray film at -70°C. To enhance sensitivity for gels containing ³H-labelled RNA, the films were preflashed before exposure.

(v): Quantitation of radioactivity in gel slices

To locate the bands which were to be cut from an acrylamide gel, radioactive ink was used to make crosses on the gel which would give a signal on the autoradiograph. These were then used to orientate the autoradiograph on the gel, and the appropriate bands were cut out using a scalpel. The gel slices were placed in glass scintillation vials (Packard), and 0.3ml water and 1ml "Protosol" tissue solubiliser (New England Nuclear) were added. The vials were mixed well to form an emulsion, and incubated at 37°C for 16-20 hours, with gentle shaking. 5ml of aqueous scintillant ("Emulsifier Safe") was added and the vials were incubated at 4°C for at least 20 hours to allow chemiluminescence to die down. They were then counted in the Packard 1500 liquid scintillation counter. Appropriate gel slices from regions of no signal were used to measure background, and a standard consisting of a ³H-SP6 probe of known specific activity was used to monitor the efficiency of recovery of counts; this

was routinely 20% and did not vary much from gel to gel.

2.4: Protein Methods

2.4.1: Western blotting

Cell culture supernatants and lysates from transfected cells expressing the immunoglobulin μ heavy chain gene were resolved by SDS-PAGE under denaturing conditions (Laemmli, 1970), and immunological analysis was carried out by Western blotting (Burnette, 1981; Towbin et al, 1979; Lin and Kasamatsu, 1983), using an anti μ heavy chain antibody. The following stock solutions were used for the protein gels:-

"Lower Tris" (4X): 1.5M Tris.Cl pH8.8, 0.4% (w/v) SDS.

"Upper Tris" (4X): 0.5M Tris.Cl pH6.8, 0.4% (w/v) SDS.

Acrylamide solution: 30% (w/v) acrylamide, 0.8% (w/v) N,N', methylenebisacrylamide. 150g acrylamide and 4g bisacrylamide were dissolved in water and made up to 500ml, then filtered through a 0.2 μ m "Nalgene" filtration unit. Stored at 4°C.

4x Tank buffer: 0.1M Tris base, 0.8M glycine.

10% acrylamide gels were made up as follows. 10ml "lower Tris", 13.3ml acrylamide solution and 16.12ml sterile water were mixed, filtered through a 0.2 μ m Nalgene filter unit and allowed to warm to room temperature. 30 μ l of TEMED and 200 μ l fresh 10% (w/v) ammonium persulphate were then added and the gel poured between glass plates separated by 1mm spacers. A layer of water-saturated butanol was applied to the top of the gel while it was setting, to exclude air. An upper stacking gel was made by mixing 12.7ml sterile water, 2ml acrylamide solution, 5ml "upper Tris" and filtering as above. 30 μ l TEMED and 100 μ l 10% (w/v) ammonium persulphate were added and the stacking gel was layered above the main gel after the butanol had been poured off. A slot former with 0.5cm slots was inserted in the stacking gel. Aliquots of 5×10^5 cells were normally loaded in each slot; the cells were washed in isotonic saline prior to loading and the cell pellet was resuspended in an equal volume of 2X sample buffer containing 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 3% (w/v) SDS, 25mM Tris.Cl pH 6.8, and 0.1% (w/v) bromophenol blue. 15 μ l aliquots of cell culture supernatants were mixed with 15 μ l of 2X sample buffer before

loading. The samples were heated to 90°C for 4 minutes before loading onto the gel, which was run in 1X tank buffer containing 0.1% (w/v) SDS, in a vertical electrophoresis apparatus at 200 volts for 3 hours or at 40 volts for 15 hours.

After electrophoresis, the proteins were transferred to nitrocellulose, in a buffer consisting of 25mM Tris.Cl pH8.3, 192mM glycine, and 20% (w/v) methanol. The gel was placed on a piece of 3MM paper soaked in buffer and a piece of nitrocellulose soaked in the same buffer was layered on top of the gel, being careful to exclude any air bubbles. A further piece of 3MM paper was placed on top and the resulting sandwich was inserted between the "Scotch Brite" pads of the Western blot apparatus. The apparatus was assembled, filled with running buffer and run at 0.1 amps, 30 volts, for 16-20 hours.

The apparatus was dismantled and the nitrocellulose treated with blocking buffer (20mM Tris.Cl pH7.6, 0.9% (w/v) sodium chloride, 0.05% (v/v) Triton X-100, 0.5% (w/v) casein), for 30 minutes with two changes of buffer. The filter was then placed on a glass plate in a humid chamber, consisting of a sandwich box containing tissues soaked in water. 1ml of a 1/100 dilution in blocking buffer of affinity purified goat anti-mouse IgM (Tago, catalogue no. 4142) was applied to the filter on the protein side, being careful that it was spread over all parts of the filter. The filter was placed protein side down on the plate and incubated in the humidity chamber for 1 hour at room temperature. The filter was washed in low salt buffer (20mM Tris.Cl pH7.6, 0.9% (w/v) sodium chloride, 0.05% (v/v) Triton X-100), 4 times for 15 minutes each with agitation at room temperature. The filter was then returned to the humidity chamber and incubated with 5ml of a 1/200 dilution of rabbit anti-goat IgG (Cappel, catalogue no. 0606-0082) as before. The wash was repeated and the filter was finally incubated with 4 μ Ci iodinated protein A (labelled with Bolton and Hunter reagent, obtained from Amersham International) for 1 hour. It was then washed in high salt buffer (20mM Tris.Cl pH7.6, 1M sodium chloride, 0.4% (w/v) N-lauroyl sarcosine) 4 times for 15 minutes each as before. The filter was then placed between two pieces of Saranwrap and exposed to X-ray

film.

2.4.2: Antibody assays

Enzyme-linked immunoabsorbant assays (ELISA) were used to detect the presence of NP-binding antibody in cell culture supernatants (Engvall and Perlmann, 1971, 1972). 96 well Nunc flat-bottomed plates were coated overnight at 4°C with 100µl per well of NP/BSA (1:1) conjugate at 1µg/ml in coating buffer (0.015M sodium carbonate, 0.035M sodium hydrogen carbonate pH9.6, 0.02% (w/v) sodium azide). The coating buffer was discarded and the plates washed extensively under the tap, excess liquid being removed by banging the plates on paper towels on the bench. The non-specific binding sites were blocked for 1 hour at room temperature with 100µl per well of coating buffer containing 0.5% (w/v) casein hammarsten. The plates were washed as before and 100µl of test samples and standards were applied to the plates in sample conjugate buffer (0.1M Tris.Cl pH7.0, 0.1m sodium chloride, 0.02% (v/v) Tween 20 and 0.2% (w/v) casein). Purified antibody from B1-8 cells, (kindly provided by M. Neuberger) was diluted to produce a standard curve. Samples were incubated for 1 hour at room temperature, then the plates were washed as before and bound material was detected using 100µl affinity purified peroxidase conjugated goat anti-mouse IgM (Cappel, catalogue no. 3611-3181) diluted 1/1000 in sample-conjugate buffer.

After the 1 hour incubation at room temperature, the plates were washed once more and then the amount of peroxidase activity in each well was determined by measuring the change in A_{450} after incubation with the substrate TMB (3,3',5,5'-tetramethyl benzidine, from Miles) and stopping of the reaction with sulphuric acid. This was done as follows. Substrate buffer was prepared by titrating 0.1M sodium acetate with 0.1M citric acid until a pH of 6.0 was reached. Immediately before use, TMB was added to a final concentration of 0.1mg/ml, and hydrogen peroxide to 0.004% (v/v). 100µl of substrate was added to each well and after 20-30 minutes at room temperature, the peroxidase activity caused the solution to turn blue. The reaction was stopped when the colour had developed sufficiently, by addition of 50µl of 2.5M sulphuric acid to each well, which caused the

colour to change from blue to yellow. The intensity of colour was read immediately at A_{450} with a Dynatech MR600 microplate reader.

2.5: Cell culture methods

2.5.1: Materials

Cell lines WEHI231 (Gutman et al, 1981), J558L (Oi et al, 1983), JW122 (Neuberger, 1983) and MXW231 (Raschke et al, 1979) were kind gifts from M. Neuberger, MRC. Laboratory of Molecular biology, Hills Rd., Cambridge.

CBMG cells (Pavlakis and Hamer, 1983a) were a kind gift from G. Pavlakis, National Institutes of Health, Bethesda, Maryland, USA.

2.5.2: General procedures

All mammalian cell lines were grown in DMEM containing 10% (v/v) heat inactivated foetal calf serum, 2mM glutamine, 50 units/ml penicillin, 50 μ g/ml streptomycin. WEHI231 cells also had 50 μ M 2-mercaptoethanol added to their growth medium. Cells were generally passaged by diluting them 1/10 into fresh growth medium.

CBMG18 cells, which are anchorage dependent, were passaged as follows. The old media was poured off and the cells were washed briefly in trypsin (0.5g trypsin, 0.2g EDTA per litre). An appropriate amount of trypsin was then added to the cells, e.g. 3ml for a 75cm² flask, and they were incubated at 37°C for 3-5 minutes. The cells could easily be detached from the flask by gentle agitation, and they were resuspended by addition of 9mls growth medium containing serum, to inactivate the trypsin. The cells were dispersed by pipetting them up and down, and diluted 1/10 for continued growth. Cells were counted using a Neubauer Improved haemocytometer, 0.0025mm² x 0.1mm deep.

Long term storage of cell lines was in liquid nitrogen. Cells were counted, pelleted by centrifugation at 1500 r.p.m. in a bench top centrifuge at room temperature, and resuspended at 10⁷ cells per ml in growth medium containing 10% DMSO. They were aliquoted at 1ml per freezing vial and frozen by incubation in an insulating container at -70°C for

16-20 hours. They were then transferred to liquid nitrogen. Cells were thawed by immersing the base of the vial in a 37°C waterbath until the medium had melted, then carefully transferring the cells with a sterile pasteur pipette into 10ml of prewarmed growth medium. Anchorage dependent cells were put directly in a 75cm² flask and allowed to settle overnight, the medium being changed the next day. Suspension cells were centrifuged at 1500 r.p.m. as above and resuspended in fresh growth medium.

2.5.3: Cloning by limiting dilution

Cells were counted and diluted to 10⁵/ml in growth medium. They were then serially diluted to 10³/ml and 10/ml, 1/100 each time, taking care to keep the cells fully suspended. 0.2ml, 0.1ml, and 0.05ml of the 10/ml suspension were aliquoted into 48 wells each of a 96 well tissue culture plate, and all wells were made up to 0.2ml final volume. After the cells had settled, the number of cells per well was scored, and wells with one cell per well, or two cells which had just divided, were scored as clones. Only wells containing cells at the most dilute level were scored as clones, the rest were discarded.

2.5.4: Cell labelling and pulse chase experiments

(i) CBMG18 cells

Pulse chase experiments were carried out on CBMG18 cells as follows. Cells were trypsinised and split 1/10 into 10 25cm² flasks and allowed to reach a cell density of 5x10⁶ cells per flask, then were given fresh growth medium. 24 hours later the medium was removed, the cells were washed once in growth medium and 1ml of labelling medium was added to each flask. The labelling medium was growth medium containing 200μCi/ml [5',6'-³H]uridine. The cells were incubated in labelling medium for two hours, then the medium was removed and the cells were washed twice in chase medium containing 5mM each uridine and cytidine, then 5ml of chase medium was added to each flask. At suitable time points thereafter, cells were harvested by trypsinisation and RNA was prepared from them as described in the RNA methods section.

For the experiment using glucosamine, the cells were pre-incubated for 1 hour in medium containing 20mM glucosamine before the labelling, and incubated for the first hour of the chase in chase medium plus 20mM glucosamine, as described by Guyette *et al*, (1979).

(ii) WEHI1231 and MXW231 Cells

To ensure that the cells were in exponential growth, 24 hours before the experiment was carried out the cells were counted, centrifuged at 1500 r.p.m. in the bench-top centrifuge, and resuspended at 10^5 /ml. The next day, 10^8 cells were harvested and resuspended in 20ml labelling medium (growth medium containing 200 μ Ci/ml [5',6',³H]uridine, and dialysed foetal calf serum). The cells were labelled for two hours and then recovered by centrifugation and washed with chase medium (growth medium containing 5mM each uridine and cytidine). They were resuspended in 250ml chase medium, and 25ml of cells were harvested at each time-point during the chase. RNA was prepared from the cells as described in the RNA methods section. Medium was added to the cells as necessary to maintain approximately the same cell density during the chase.

2.5.5 Generation of stable cell lines

B cell lines were transfected by electroporation (Neumann *et al*, 1982) using the Biorad electroporation apparatus. The DNA to be transfected was linearised by digestion with an appropriate restriction enzyme, at a site in a non-essential region of the vector. 40 μ g of DNA was digested as described in Section 2.2.3, in a volume of 200 μ l, using high concentration restriction enzyme. Before use, an aliquot of the digest was checked for complete digestion by agarose gel electrophoresis.

Typically, 10^7 cells were electroporated with 40 μ g linear DNA. The cells were harvested by centrifugation and washed in PBS, then recentrifuged. They were resuspended in 0.8ml PBS, and aliquoted into a sterile electroporation cuvette. 0.2ml of linear DNA in restriction buffer or 0.2ml PBS (for the mock transfection) was added and mixed carefully by pipetting up and down. The cells and DNA were incubated

on ice for 5 minutes and then placed in the electroporation apparatus. They were given two pulses at 1500 volts, $3\mu\text{F}$, then replaced on ice for 5 minutes. They were transferred to a sterile centrifuge tube containing growth medium, and plated out neat, and at 1/10 and 1/100 dilutions in 96 well plates, with 0.1ml per well.

The following day the appropriate selective media were added. Vectors containing the bacterial *gpt* gene (Mulligan and Berg, 1981) were selected for using mycophenolic acid; this blocks the formation of xanthine monophosphate from inosine monophosphate, thereby preventing the synthesis of guanosine monophosphate. This can be overcome by the addition of xanthine to the growth medium if the bacterial enzyme guanine-hypoxanthine phosphoribosyl transferase (*gpt*) is expressed in the cells (mammalian cells are not normally able to utilise xanthine). Vectors containing the neomycin resistance gene (Southern and Berg, 1982) confer resistance to the antibiotic G418, as the *neo^R* gene codes for a phosphotransferase which inactivates the G418. The selective media for these two genes were as follows:

gpt selection: 2ml HT (50X hypoxanthine plus thymidine, from Gibco), 2ml xanthine (12.5 mg/ml in 0.2M sodium hydroxide), 2ml mycophenolic acid (0.25 mg/ml in 0.1M sodium hydroxide), and 0.6ml sterile 1M hydrochloric acid (to restore the pH), per 100ml medium.

The above provides 1X selection; normally selection was added gradually over two days, 50 μl of 1.5X added on the day following the transfection, and 50 μl of 2X added the day after that. The subsequent growth of the cells was in 1X selection.

neo selection: 1ml G418 (100 mg/ml, obtained from Sigma Chemical Co. as Geneticin) per 100ml medium.

The above is 1X selection; 100 μl of 2X selection was added the day after the transfection (i.e. final concentration = 1X). Thereafter the cells were grown in 1X selection.

After 1-2 weeks, colonies began to appear on the transfection plates. Wells containing single colonies were grown up and analysed for expression of the transfected gene.

2.5.6: Analysis of transfectants containing the μ gene under the control of the heat shock promoter

Colonies of cells selected as described above were grown up and tested by heat-shocking as follows. 10ml of cells (approximately 5×10^6), were placed in a 25cm² flask, gassed with 95% CO₂ and incubated, standing upright, in a waterbath at 42.5°C for 1 hour. They were then transferred to a 37°C incubator for a further hour, and RNA was prepared from the cells as described in Section 2.3.2. RNA was also prepared from control cells which had not been incubated at 42.5°C. The RNA was analysed by slot-blotting and/or Northern blotting with appropriate probes, as described in Sections 2.3.5 and 2.3.6.

2.5.7: Measurement of RNA decay using the heat shock promoter

To ensure that cells were in exponential growth, they were seeded at 10^5 /ml 24 hours before the experiment was carried out. 4×10^7 cells were then centrifuged at 1500 r.p.m. in the bench-top centrifuge, and resuspended in 40ml of growth medium that had been prewarmed to 42.5°C. The flask was gassed with 95% CO₂ and placed upright in a waterbath at 42.5°C for 1 hour. Meanwhile, 5×10^6 of the remaining cells were harvested and RNA was prepared from them as described in the RNA Methods section, for the time zero control. After 1 hour at 42.5°C, the cells were diluted to 320ml with medium at 37.5°C, and placed in the 37°C incubator. At suitable time points thereafter, 40ml of cells were harvested and RNA was prepared from them; either total RNA or nuclear plus cytoplasmic, depending on the experiment. The RNA was analysed by Northern blotting as described in Section 2.3.5. Aliquots of 5×10^5 cells were set aside for analysis of their protein content by Western blotting (see Section 2.4.1).

2.5.8: Growth of cells in the presence of tunicamycin after heat shock induction.

10^7 cells in exponential growth were harvested by centrifugation, resuspended in small flasks containing 10ml of medium at 42.5°C, and gassed with 95% CO₂. The flasks were placed in a water bath at 42.5°C for one hour, then the

cells were diluted into 80ml of medium at 37°C. This was divided into two flasks containing 40ml each, and 40µl of 10mg/ml tunicamycin was added to one flask, before continued incubation at 37°C. Samples were removed for analysis by Western blotting at suitable time points thereafter.

2.5.9: Specific activity of the UTP pool

The specific activity of the UTP pool was measured according to the method of Brock and Shapiro (1983b). This method uses h.p.l.c. to measure the concentration of nucleotides in cell lysates. Aliquots of cell lysates were incubated with 0.5M perchloric acid at 0°C for 20 minutes. The precipitate was removed by centrifugation in the Eppendorf centrifuge for 5 minutes, and the supernatant was neutralised by addition of 3M potassium hydroxide. The resulting potassium chlorate precipitate was also removed by centrifugation in the same way, and 200µl aliquots of the supernatant were run on a Partisil-10-SAX column (Thames Chromatography), using the Hewlett-Packard HP1090 Liquid Chromatograph and HP79994A software. The column was pre-equilibrated in 0.1M ammonium phosphate, 15% (v/v) acetonitrile at a flow rate of 0.1ml per minute overnight. The flow rate was increased to 1.5ml per minute, then the 200µl sample injected and the column washed for 10 minutes. The nucleotides were then eluted by an ammonium phosphate gradient of 0.1M to 0.5M in 15% (v/v) acetonitrile over the next 15 minutes, then the column was washed by increasing the ammonium phosphate concentration to 1M over the next 3 minutes and keeping it at 1M for 3 minutes. The nucleotides were monitored at 262nm on a Hewlett Packard 1046A fluorescence detector, and the UTP peak was identified by comparison of the retention time with UTP standards; the identity of the peak was further confirmed by examining its peak spectrum. A series of dilutions of UTP standards were run on the column and these were quantitated by integration of the peak area to construct a calibration curve. Samples from suitable time points during a pulse chase experiment were run on the column and the UTP peak was collected and subjected to liquid scintillation counting to determine its specific activity.

CHAPTER 3
DEVELOPMENT OF PULSE-CHASE METHOD AND RIBONUCLEASE
PROTECTION ASSAY

3.1: Introduction

In order to develop a method for measuring mRNA half-lives in mammalian cells, a model system, consisting of a cell line which had previously been extensively studied, was used. The cell line chosen, known as CBMG, are C127 cells which have been transfected with a bovine papilloma virus (BPV) based vector containing a recombinant human growth hormone (hGH) gene (Pavlakis and Hamer, 1983a). The parent C127 cells are a fibroblast cell line derived from a mouse mammary tumour (Lowy *et al*, 1978), and grow in an anchorage-dependent, contact-inhibited manner. Upon transformation with BPV, the morphology of the cells changes, they lose contact inhibition, are able to grow in low serum concentrations and to a high cell density, and form tumours in nude mice (Meishke, 1979; Lowy *et al*, 1980; Dvoretzky *et al*, 1980). A 5.4kb *Hind* III-*Bam* HI fragment consisting of sixty nine percent of the BPV genome has been shown to be sufficient for transformation (Lowy *et al*, 1980), and also allows replication of the DNA to multiple copies per cell (Antmann *et al*, 1980; Law *et al*, 1981; Lancaster, 1981; Pavlakis and Hamer, 1983a).

The form of the replicated DNA, (episomal or integrated into the chromosome), depends on the exact form of the transfected vector, and seems to reflect the interaction between the BPV genes and the inserted sequences (for review see Stephens and Hentschel, 1987; also Sarver *et al*, 1982; Law *et al*, 1983; Allshire and Bostock, 1986). Vectors based on the 69% transforming fragment of the genome, for example, are missing an enhancer element which is thought to play a role in the episomality of the genome (Lusky and Botchan, 1985, 1986). Such vectors are much more prone to integration and rearrangement than those containing the whole genome (Zinn *et al*, 1982; Mitrani-Rosenbaum *et al*, 1983; Denniston *et al*, 1984). This system has been widely used to express foreign genes in fibroblast cells (Stephens and Hentschel, 1987), and CBMG cells have been extensively characterised at

Celltech, both biochemically and genetically. For this reason, CBMG cells were chosen to be used as a model system.

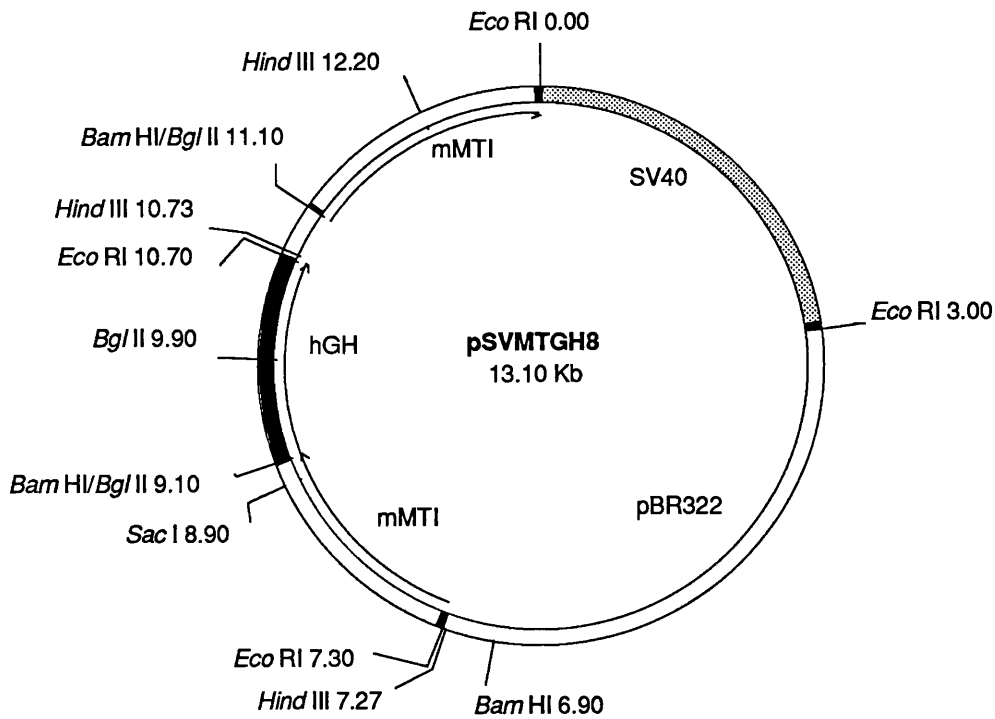
The vector used to transfect C127 cells contained the 69% transforming region of BPV, and an hGH "minigene" under the control of the mouse metallothionein-I promoter. The construction of the hGH "minigene" is described in detail by Pavlakis and Hamer, (1983a,b); briefly, the genomic sequences (DeNoto *et al*, 1981) between the *Pvu* II site in exon 2 and the *Bgl* II site in exon 5 were replaced with the corresponding fragment of an hGH cDNA clone (Martial *et al*, 1979). The "minigene" was then subcloned on a *Bam* HI fragment into the *Bgl* II site in the 5' untranslated region of the mouse metallothionein-I gene to form pSVMTGH8, shown in Fig. 3.1A (Pavlakis and Hamer, 1983a,b). The resulting hybrid gene was excised from pSVMTGH8 on a *Hind* III fragment and inserted into pBPV69TD, which contains the 69% *Bam* HI-*Hind* III fragment of BPV1. This produced two plasmids which differed in the relative orientations of the metallothionein and BPV1 promoters. The majority of the remaining bacterial plasmid sequences were excised by digestion with *Bam* HI, and the linear molecules were religated before transfection. The relevant version for this work is BPVMG7, shown in Fig. 3.1B, which contains the metallothionein and BPV1 promoters in the same orientation.

C127 cell transformants were selected on the basis of altered morphology and analysed for their ability to produce hGH mRNA and protein. The copy number and restriction map of the transfected hGH gene have been determined, by Southern blot analysis, for one of the clones isolated by Pavlakis and Hamer, CBMG7-4 (Bebbington, unpublished). It was found that the cells contained approximately 600 copies of the hGH gene in an unrearranged state, and, in addition, unpredicted single copy restriction fragments were observed. These extra single copy fragments could have resulted from integration of the tandem array of vector sequences into the genome. This hypothesis was confirmed by *in situ* hybridisation to metaphase chromosome spreads which showed that the tandem array was integrated into a single site in the chromosome (Bebbington and Cox, unpublished).

hGH protein and mRNA production by the cells has also been analysed.

FIGURE 3.1

A



B

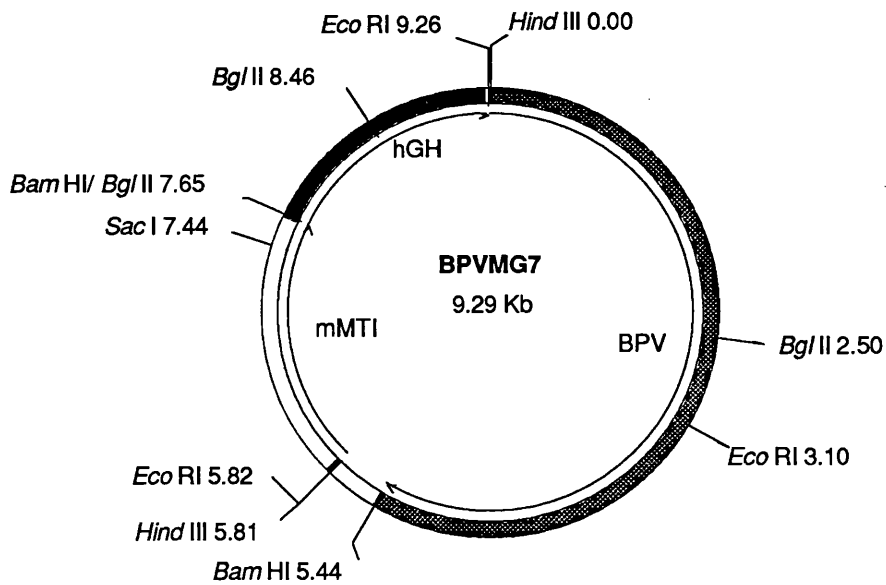


Figure 3.1: Plasmids containing the hGH "minigene".

(A) shows pSVMTGH8 which contains the hGH "minigene" inserted at the *Bgl* II site in the mouse metallothionein 5' untranslated region.

(B) shows BPVMG7; the BPV based vector containing the hGH transcription unit, which was used to transfect C127 cells. The construction of these plasmids is outlined in the text.

Note that the plasmid maps shown here and in subsequent Figures are not necessarily to scale.

hGH is secreted at a rate of $2-6 \times 10^8$ molecules per cell per day as determined by immunoradiometric assay (Pavalakis and Hamer, 1983a) and the hGH mRNA has been shown by Northern blotting (Thomas, 1980) to be of the correct size and by primer extension to initiate at the normal site from the metallothionein-I promoter (Cox, unpublished; Pavlakis and Hamer, 1983a), although additional minor start sites were also observed (Cox, unpublished). It is of interest, therefore, to determine whether this high level of expression is mainly due to the high copy number of the gene, or whether the mRNA is exceptionally stable.

As the first stage in developing a method for measuring mRNA half-life, a ribonuclease protection assay was developed for detecting hGH transcripts, using RNA probes synthesised *in vitro*. These probes were generated using the polymerase from bacteriophage SP6 (Butler and Chamberlin, 1982), which shows stringent promoter specificity and can be used to transcribe any sequence placed downstream of bacteriophage SP6 promoter in an appropriate plasmid. The plasmid is linearised by restriction enzyme digestion 3' to the sequence of interest, and run-off transcripts are generated *in vitro* using purified SP6 polymerase. These probes have been used extensively in ribonuclease protection assays for detection and mapping of specific RNA (e.g. Zinn *et al*, 1983; Melton *et al*, 1984; Mason *et al*, 1988, Brewer and Ross, 1988). Initial experiments focussed on the establishment of suitable conditions for the hybridisation and ribonuclease digestion, using ^{32}P -labelled SP6 probes. The system was then adapted for use in conjunction with a pulse-chase protocol, by labelling CBMG cells with [^3H]uridine and hybridising the RNA isolated from them to an unlabelled SP6 generated probe. Pulse chase experiments were then carried out on the CBMG cells and a method was investigated for improving the chase conditions. The problems associated with pulse-chase experiments in mammalian cells, and the mechanisms of regulation of hGH mRNA levels in CBMG cells are discussed.

3.2: Results

3.2.1: Ribonuclease Protection Assay

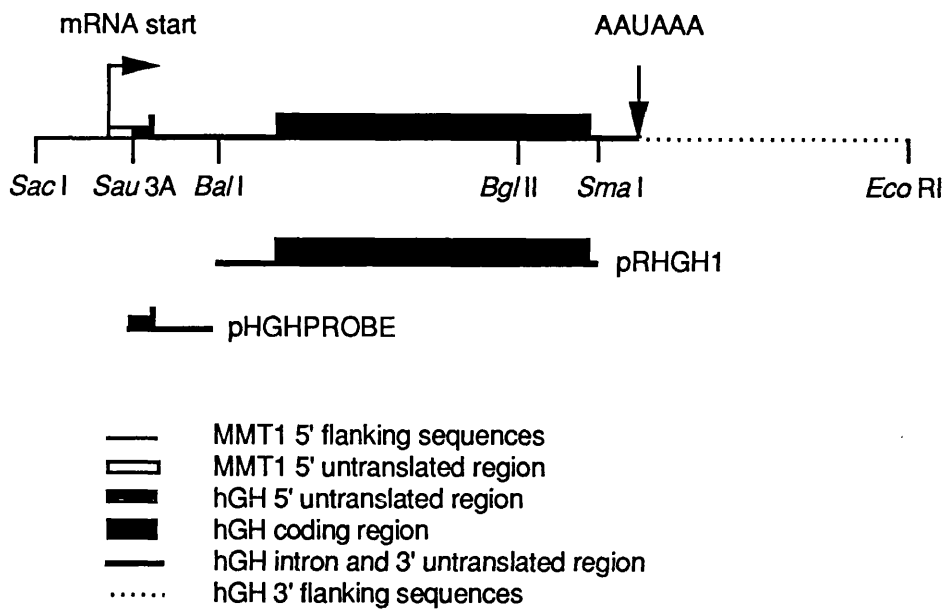
Preliminary experiments to optimise the ribonuclease protection assay were carried out using the plasmid pHGHPROBE (Cockett, unpublished). This contains the 205bp *Sau* 3A-*Bal* I hGH fragment from the hGH "minigene" inserted into pSP65 using the *Bam* HI and *Sma* I sites in the polylinker (indicated in Fig. 3.2). The hGH sequences were derived from pSVMTGH8, (Fig. 3.1A), an intermediate in the construction of BPVMG7 (Fig. 3.1B) (Pavlakakis and Hamer, 1983b). The plasmid pHGHPROBE was linearised at the *Bam* HI site downstream of the insert, and used to generate ³²P-labelled run-off transcripts with SP6 polymerase, essentially as described by Green *et al*, (1983) (see Section 2.3.7). This template generates an RNA probe whose total length is 231 nucleotides, (including 26 nucleotides of pSP65 polylinker 5' to the hGH sequence), of which 71 nucleotides will hybridise to correctly spliced hGH mRNA in the 5' untranslated region (see Fig. 3.2A).

In vitro transcription reactions were carried out as described in Section 2.3.7 to generate probes for ribonuclease protection experiments. Typically 50-90% of the radioactive nucleotide ([α -³²P]GTP) was incorporated into RNA probe, resulting in a specific activity of 1-5x10⁶ cpm/ μ g. The relatively low specific activity was due to the inclusion of 25nmol unlabelled GTP in the reaction, in order not to limit the amount of RNA which could be produced. In the ribonuclease protection experiments described below, 10⁶ cpm were routinely hybridised to 10 μ g total RNA isolated from CBMG cells. Based on previous estimates of the content of hGH mRNA in these cells, (0.01-0.1% of total RNA; Cox, unpublished), this represents at least a 50-fold molar excess of probe. To develop the ribonuclease protection assay, initial experiments were carried out to investigate the effect of various ribonuclease digestion conditions.

Six hybridisation reactions of 10 μ g cytoplasmic CBMG RNA and 10⁶ cpm probe were set up using the hybridisation method of Hay *et al* (1977), and these were digested with a range of amounts of ribonuclease for 30 minutes at 30°C (Zinn *et al*, 1983). The resulting protected fragments were resolved by polyacrylamide/urea gel electrophoresis. These procedures

FIGURE 3.2

A



B

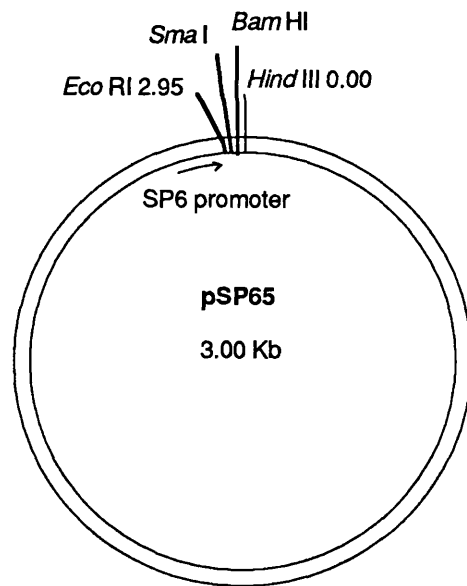


Figure 3.2: hGH probes used for ribonuclease protection experiments.

(A) shows the hybrid mMT1-hGH gene contained in pSVMTGH8. The position of the start of the mRNA, and the AAUAAA polyadenylation signal are shown. The restriction enzyme sites used to subclone the fragments to be used as probes are marked, and the positions of the two probes are indicated below the figure.

(B) shows pSP65 with the positions of the *Sma* I and *Bam* HI sites into which the probe fragments identified in (A) were cloned.

are described in detail in Section 2.3.8. The results of autoradiography of the gel are shown in Fig. 3.3, where the positions of the full length probe and protected fragments are marked with arrows. Their rate of migration in the gel is as predicted relative to the molecular weight markers (lanes M1 and M2). It is evident that at lower ribonuclease concentrations, (lanes 4 and 5), some incompletely digested probe molecules remain, and at higher concentrations the signal from the protected fragment is slightly reduced (lane 1). It was therefore concluded that $4\mu\text{g/ml}$ ribonuclease A and $0.2\mu\text{g/ml}$ ribonuclease T_1 were the optimum digestion conditions for this amount of RNA, as the minimum amount of extra bands are present under these conditions, and the protected fragment remains intact (lane 2). The amount of radioactivity present in the undigested sample (lane 6), where 1/10 the amount of probe was loaded, compared to the digested ones suggests that, as expected, the probe is in a large excess; this was formally tested in later experiments.

In order to use the ribonuclease protection assay to detect pulse-labelled RNA, an unlabelled probe must be hybridised to RNA prepared from cells labelled in culture with [^3H]uridine. To determine whether any probe-dependent signal could be detected using this system, CBMG cells were labelled at $5 \times 10^6/\text{ml}$ with $200\mu\text{Ci/ml}$ [^3H]uridine for two hours, and total polyadenylated RNA prepared, using the methods described in Section 2.5.4 and 2.3.3 respectively. A specific activity of 2×10^5 cpm/ μg was achieved for polyadenylated RNA with this protocol, which is comparable to that obtained by Aviv *et al* (1976), and Lowenhaupt and Lingrel (1978), using a similar method. The polyadenylated RNA was then hybridised to unlabelled SP6 probe generated from pHGHPROBE. After ribonuclease digestion and polyacrylamide gel electrophoresis, the gel was treated with fluorographic reagent as described in Section 2.3.8, to allow detection of ^3H -labelled bands. Control hybridisations using a ^{32}P -probe with $2.5\mu\text{g}$ polyadenylated RNA and $10\mu\text{g}$ total RNA were also run on the gel, to mark the position of the protected band and show the enrichment obtained by using polyadenylated RNA (Fig. 3.4; lanes 3 and 4 respectively; see arrow). No signal could be detected with the unlabelled

FIGURE 3.3

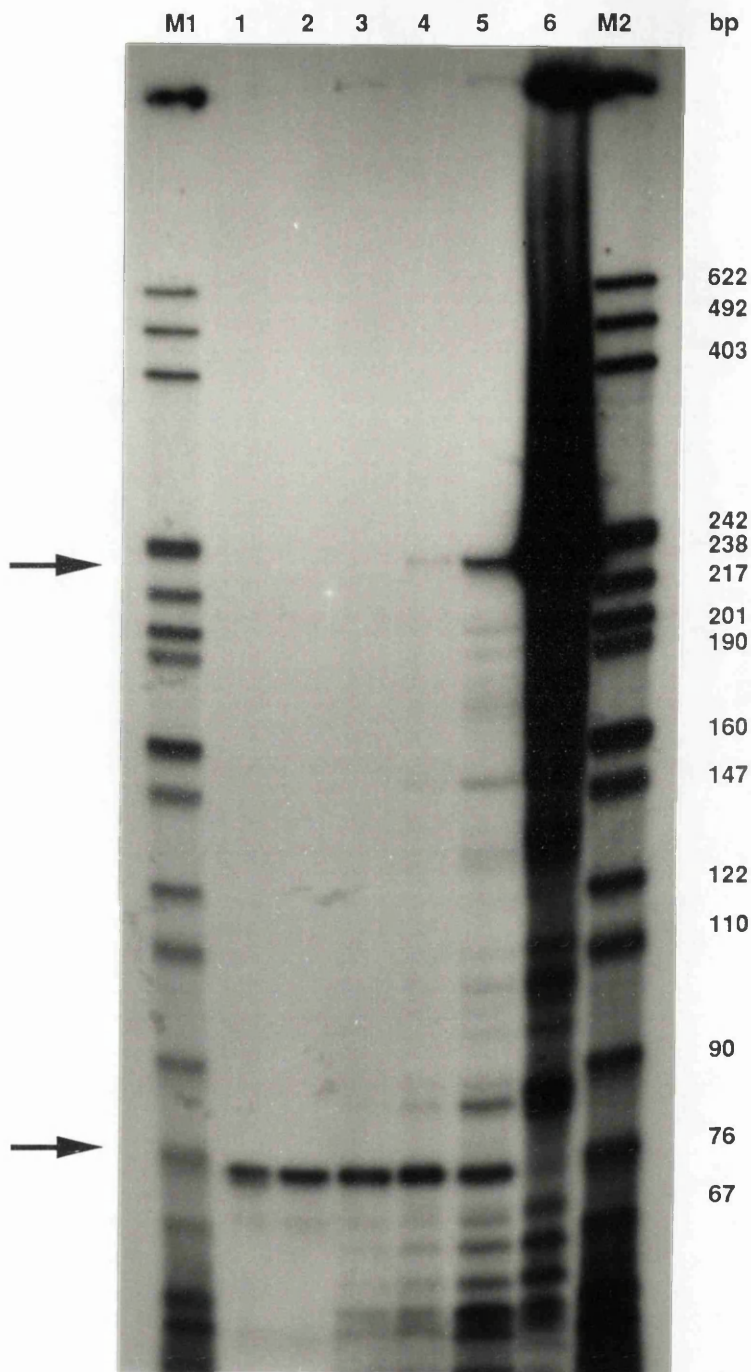


Figure 3.3: Ribonuclease protection experiment with different amounts of ribonuclease.

A ribonuclease protection experiment was carried out as described in the text using total RNA from CBMG cells and a labelled probe generated from pHGHPROBE, with the following amounts of ribonucleases A and T1 respectively: 10 μ g/ml A, 0.5 μ g/ml T1 (lane 1); 4 μ g/ml A, 0.2 μ g/ml T1 (lane 2); 2 μ g/ml A, 0.1 μ g/ml T1 (lane 3); 1 μ g/ml A, 0.05 μ g/ml T1 (lane 4); 0.4 μ g/ml A, 0.02 μ g/ml T1 (lane 5). Lane 6 contains a sample of undigested probe, 1/10 the amount used in the hybridisations. Lanes M1 and M2 contain different loadings of molecular weight markers generated by *Hpa* II digestion of pAT153 and end-labelling of the resulting fragments using T4 polynucleotide kinase.

probe, however, from the ^3H -labelled polyadenylated RNA, after 1 week's exposure of the autoradiograph (Fig. 3.4; lane 2). This was not unexpected, given the ten fold difference in specific activities of the ^{32}P -labelled probe and the ^3H -labelled RNA, and the fact that the tritium signal is much harder to detect, even when fluorography is used. The enrichment obtained by using polyadenylated RNA is insufficient to compensate for these effects (Fig. 3.4; lanes 3 and 4). The smear of undigested ^3H -RNA gives some indication of the low intensity of the tritium signal. In an attempt to overcome this problem, a longer probe was constructed so that more labelled nucleotides would be contained in the protected fragment. In addition, the hybridisation and ribonuclease digestion system was tested with larger amounts of total RNA, to obviate the need to purify polyadenylated RNA. The construction of the new probe is described first.

The plasmid pSVMTGH8 (Fig 3.1A) was digested with the enzymes *Bal* I and *Sma* I to yield a 774 base pair fragment from the hGH gene (shown in Fig 3.2A) which was isolated from a preparative agarose gel. This fragment was ligated to pSP65 DNA (Fig 3.2B) which had been linearised with *Sma* I and treated with calf intestinal phosphatase. After transformation into *E. coli* LM1035, mini-plasmid preparations were analysed by restriction enzyme digestion and a colony was isolated which contained the insert in the "antisense" orientation, ie, the orientation such that transcripts complementary to mRNA would be generated from the SP6 promoter. Plasmid DNA was prepared from these cells and linearised at the *Hind* III site to generate SP6 transcripts. This template (pRHGH1) produces a probe 834 nucleotides long (including polylinker sequences) which protects a 650 nucleotide fragment of hGH mRNA (indicated in Fig. 3.2A). In the following experiment it was used in the ribonuclease protection assay, with larger amounts of CBMG RNA.

25 and 50 μg of total CBMG RNA were hybridised to the new probe, and digestions were carried out to confirm the optimum conditions determined in the first experiment; the results are shown in Fig. 3.5. The probe gives rise to a protected fragment which migrates just above the 657

FIGURE 3.4

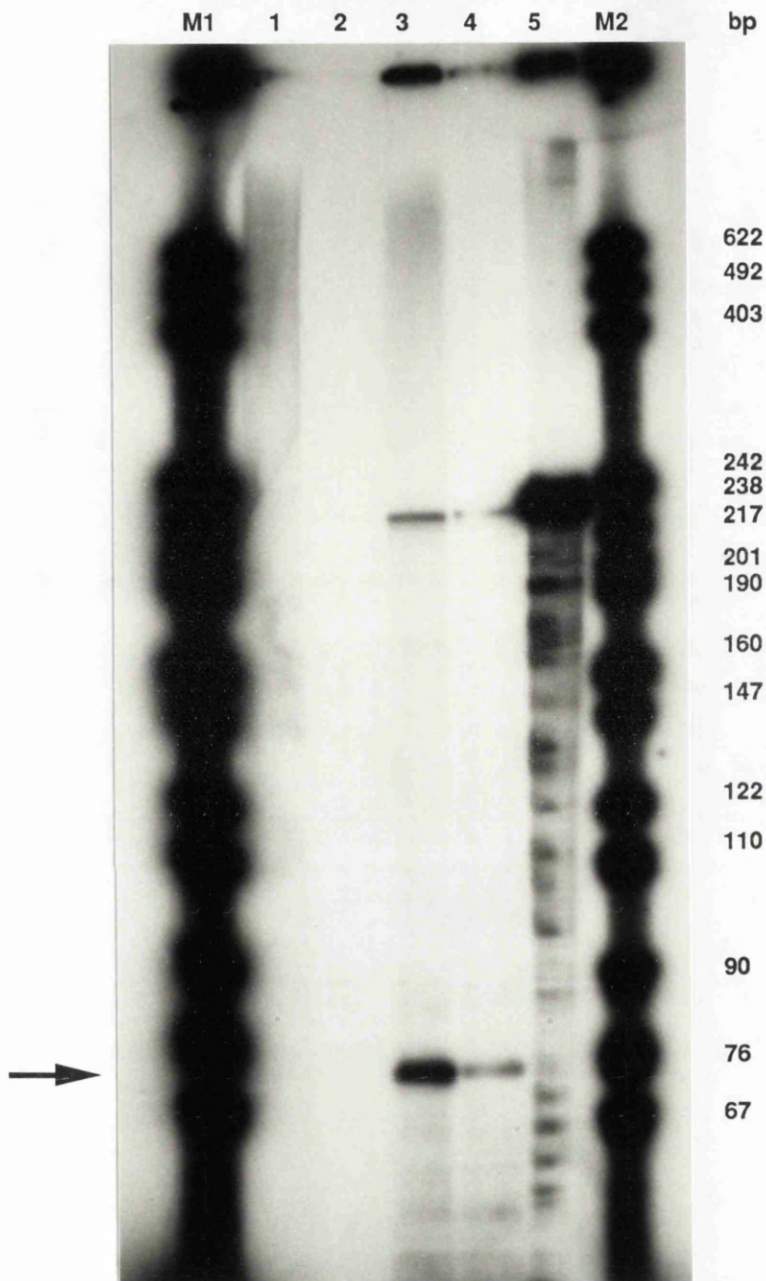


Figure 3.4: Tritiated poly(A)+ RNA hybridised to an unlabelled probe.

A ribonuclease protection experiment was carried out as described in the text, using tritiated poly(A)+ RNA from CBMG cells with an unlabelled probe generated from pHGHPROBE. Lane 1 contains 2.5 μ g tritiated poly(A)+ RNA, undigested. Lane 2 contains 2.5 μ g tritiated poly(A)+ RNA after ribonuclease protection. Controls consisting of 2.5 μ g unlabelled poly(A)+ RNA, and 10 μ g unlabelled total RNA, after ribonuclease protection with labelled probe, are shown in lanes 3 and 4 respectively. Lane 5 contains undigested labelled probe, and lanes M1 and M2 contain different loadings of molecular weight markers as described in the legend to Fig. 3.3.

FIGURE 3.5

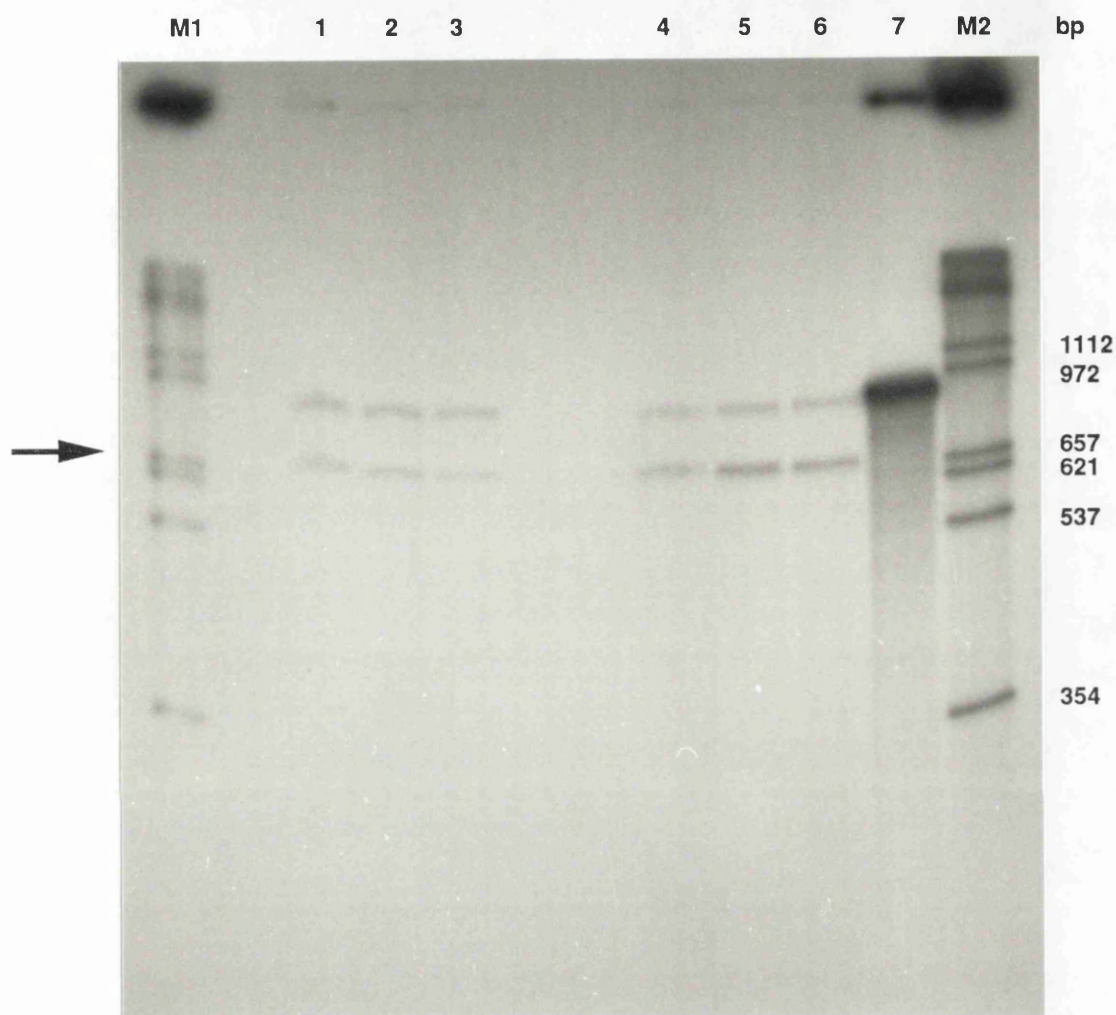


Figure 3.5: Ribonuclease protection with a longer probe.

A ribonuclease protection experiment was carried out using 25 μ g (lanes 1-3) and 50 μ g (lanes 4-6) total RNA from CBMG cells, with a labelled probe generated from pRHGH1. The following ribonuclease digestion conditions were used: 4 μ g/ml ribonuclease A, 0.2 μ g/ml ribonuclease T1, 30 minutes (lanes 1 and 4); 4 μ g/ml A, 0.2 μ g/ml T1, 60 minutes (lanes 2 and 5); 10 μ g/ml A, 0.5 μ g/ml T1, 30 minutes (lanes 3 and 6). All digestions were at 30°C. Lane 7 contains a sample of undigested probe, and lanes M1 and M2 contain different loadings of molecular weight markers generated by *Cla* I digestion of bacteriophage λ DNA, and end-labelling of the resulting fragments using T4 polynucleotide kinase.

nucleotides (lanes 1-3, 4-6, arrow), which is higher than expected. The discrepancy may be explained by slight differences in the rates of migration between RNA and DNA molecules. With these amounts of RNA, some undigested probe remains in all tracks, (lanes 1-3, 4-6), but otherwise the digestions are complete, and it is clearly possible to increase the ribonuclease concentrations to at least 10 μ g/ml ribonuclease A and 0.5 μ g/ml ribonuclease T₁ without any detectable loss of protected fragment signal (lane 6). The slightly lower signal in lane 4 may be due to loading error. It was thought that any further optimisation of the digestion conditions, if necessary, would be carried out using ³H-RNA with an unlabelled probe, as the signal to noise ratio will be different if the cellular RNA is labelled instead of the probe.

To determine whether a protected fragment could be detected from ³H-labelled RNA using the pRHGH1 probe, CBMG cells were labelled with [³H]uridine as before. Cytoplasmic RNA was extracted from the cells, and increasing amounts were hybridised to 700ng of unlabelled probe. This represents approximately a 10 fold excess of probe for 100 μ g total RNA. Samples were treated with 10 μ g/ml ribonuclease A and 0.5 μ g/ml ribonuclease T₁, and the acrylamide gel was treated with fluorographic reagent as before. Fig. 3.6 shows a protected band of the expected size was visible in all samples, (lanes 1-3, 5; arrow) and not present in the control hybridisation which contained no probe (lane 4). This demonstrates that the method is sensitive enough to detect hGH mRNA in a sample of total cytoplasmic RNA labelled with tritium. In addition to the expected protected band, there is a faint high molecular weight band which increases in intensity as the RNA loading increases, and is also present in the control track with no probe (lanes 1-5). This band is most likely to be due to contamination of the RNA sample with DNA; DNA of cells labelled with [³H]uridine becomes weakly labelled with tritium via several biosynthetic pathways involving the interconversion of nucleoside phosphates and deoxynucleoside phosphates (Thelander and Reichard, 1979). In later experiments, when ribonuclease-free deoxyribonuclease became available, the RNA preparations could be safely treated with

FIGURE 3.6



Figure 3.6: Ribonuclease protection with increasing amounts of tritiated total RNA

Ribonuclease protection was carried out as described in the text using tritiated total RNA from CBMG cells and an unlabelled probe generated from pRHGH1. The following amounts of labelled total RNA were used: 10 μ g (lane 1); 25 μ g (lane 2); 50 μ g (lane 3); 85 μ g (lane 5). Lane 4 shows the result of ribonuclease protection with 50 μ g labelled total RNA when no probe was added to the hybridisation mix. Lanes M1 and M2 contain different loadings of molecular weight markers generated as described in the legend to Fig. 3.5.

deoxyribonuclease, and this removed the high molecular weight band, supporting the hypothesis that it was DNA (e.g. Fig. 3.13). A smaller band of approximately 250 bases is visible in the track containing the highest concentration of RNA. Several possibilities exist for the origin of this band. It could be caused by some secondary structure phenomenon in the mRNA, or result from protection of the mRNA by shorter probe molecules arising from early termination of the SP6 polymerase. Such "drop-off" products are seen in some probe preparations, but it is not possible to correlate them with the amount of the extra band, as they are only detectable in the ^{32}P -labelled probes. It is also possible that this band represents a stable degradation intermediate of hGH mRNA, but this is unlikely as the pulse-chase experiments described below show that it decays with the same kinetics as the main band.

The autoradiograph shown in Fig. 3.6 was scanned with a densitometer to determine whether the signal response was linear over the range of RNA amounts used, as would be expected if the probe were in excess. The results are shown in Fig. 3.7; it can be seen that the response is indeed linear over this range of RNA amounts. To confirm this result, the experiment was repeated, and the results are shown in Fig. 3.8. This time the radioactivity in protected fragments was quantitated by cutting out the bands from the gel, treating them with solubiliser, and measuring the amount of radioactivity by liquid scintillation counting as described in Section 2.3.8. It can be seen that the graph of protected radioactivity against amount of RNA hybridised is linear up to approximately 100 μg , confirming the previous result.

3.2.2: Pulse Chase Experiments with CBMG Cells

To demonstrate that it was possible to measure the half-life of a particular messenger RNA using the ribonuclease protection method, flasks of confluent CBMG cells were labelled with [^3H]uridine as before for 2 hours, then washed and chased using conditions similar to those described by others, described in Section 2.5.4 (Aviv *et al*, 1976; Lowenhaupt and Lingrel, 1978; Paek and Axel, 1987). The CBMG

FIGURE 3.7

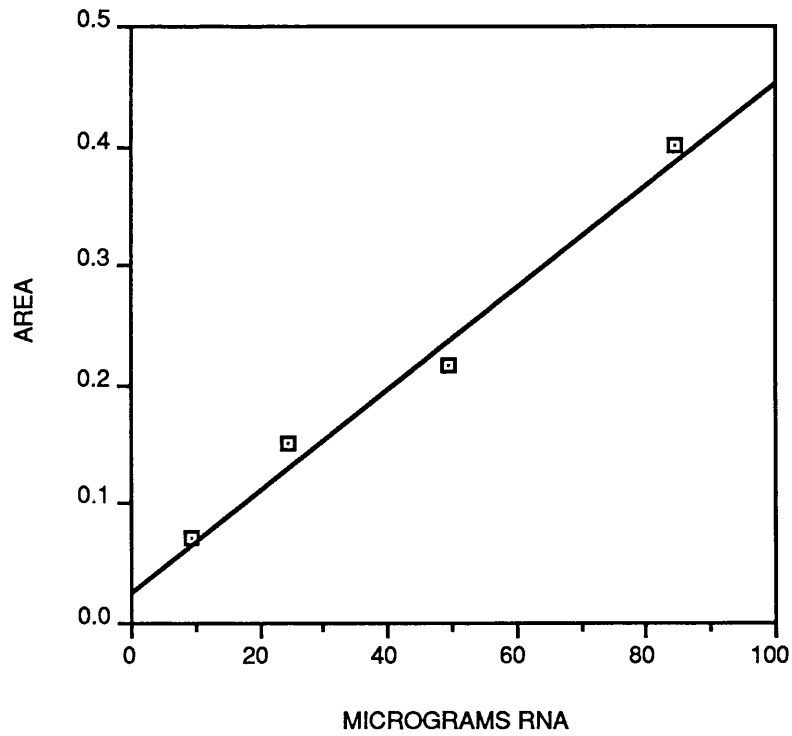


Figure 3.7: Peak area vs. RNA input for gel shown in Fig. 3.6

The autoradiograph of Fig. 3.6 was scanned with a densitometer, and the area under the peak from the protected hGH fragment was plotted against the amount of RNA in the hybridisation. R-squared for the line of best fit was 0.983.

FIGURE 3.8

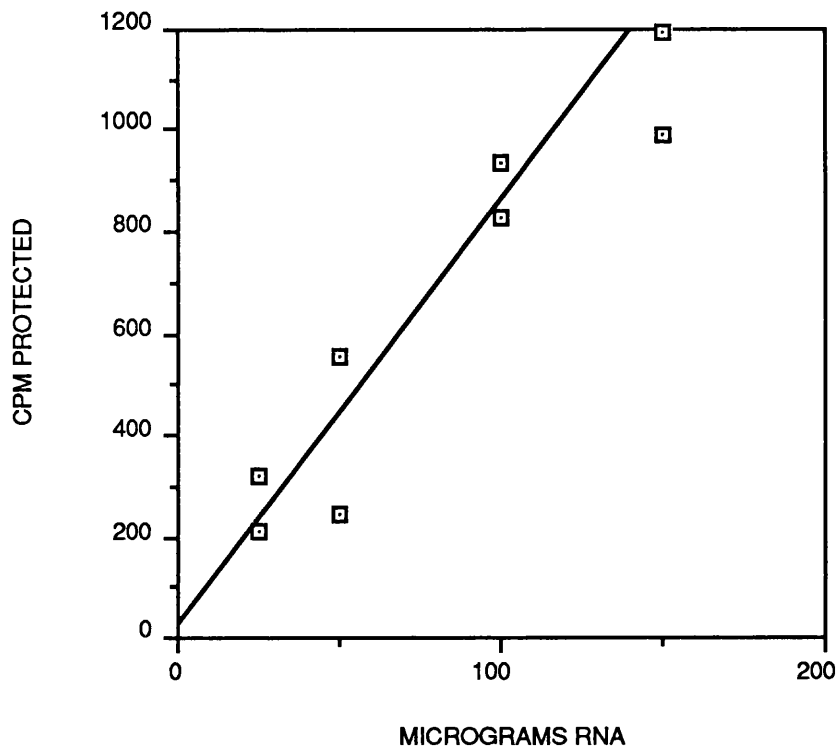


Figure 3.8: Protected cpm vs. RNA input for increasing amounts of tritiated total RNA.

The hGH-specific bands from a gel similar to that shown in Fig. 3.6 were cut out from the gel and the radioactivity solubilised and quantitated as described in the text. The number of cpm in each protected band were plotted against the amount of tritiated total RNA in the hybridisation. R-squared for the line of best fit was 0.860, ignoring the last point.

cells do not divide at the cell densities used in these experiments; the viable cell number in this experiment remained between 3.4 and 4.6×10^6 per flask throughout. Total cytoplasmic RNA was extracted from the cells at suitable time points during the chase, $50 \mu\text{g}$ of each RNA preparation was hybridised to 700ng unlabelled hGH probe, and ribonuclease protection and gel electrophoresis carried out as before. The results are shown in Fig. 3.9, and a control hybridisation of the labelled RNA to a ^{32}P -labelled probe is shown in Fig. 3.10, which shows that similar amounts of RNA were included in each hybridisation (lanes 1-9). The ^3H -labelled protected fragment can be seen to decay during the chase, and the 250 nucleotide band, only weakly visible in this gel, decays with similar kinetics (Fig. 3.9A, lanes 1-9). Fig. 3.9B shows the bottom of the gel, where it can be seen that at late times during the chase, lower molecular weight decay products appear (lanes 4-7). These migrate just above the nucleotide front, but could represent larger partially degraded mRNA molecules. Such fragments would not be distinguished from full length molecules in a filter-binding assay.

The decay of the main band was quantitated by liquid scintillation counting of the protected fragments as described above, and the amount of radioactivity is plotted against time during the chase in Fig. 3.11. Linear regression analysis of the \log_e dpm values was used to calculate the slope of the line, and a half-life value was calculated using the relationship $T_{1/2} = \log_e 2 / \text{slope}$ (see Appendix). This yields a half-life value of 10.8 hours. The graph shown in Fig. 3.11 illustrates that labelled nucleotide continues to be incorporated into the hGH mRNA during the early part of the chase, and for this reason, time points before 4.5 hours were ignored when calculating the half-life value. The continued incorporation is due to the large size of the ribonucleotide precursor pool in mammalian cells, and this effect has been reported in the literature (Warner *et al.* 1966; Schimke, 1970; Scholtissek, 1971; Cowan and Milstein, 1974; Levis and Penmann, 1977; Brock and Shapiro, 1983a,b). It has also been reported that the size of the precursor pool can be artificially reduced by treatment of

FIGURE 3.9

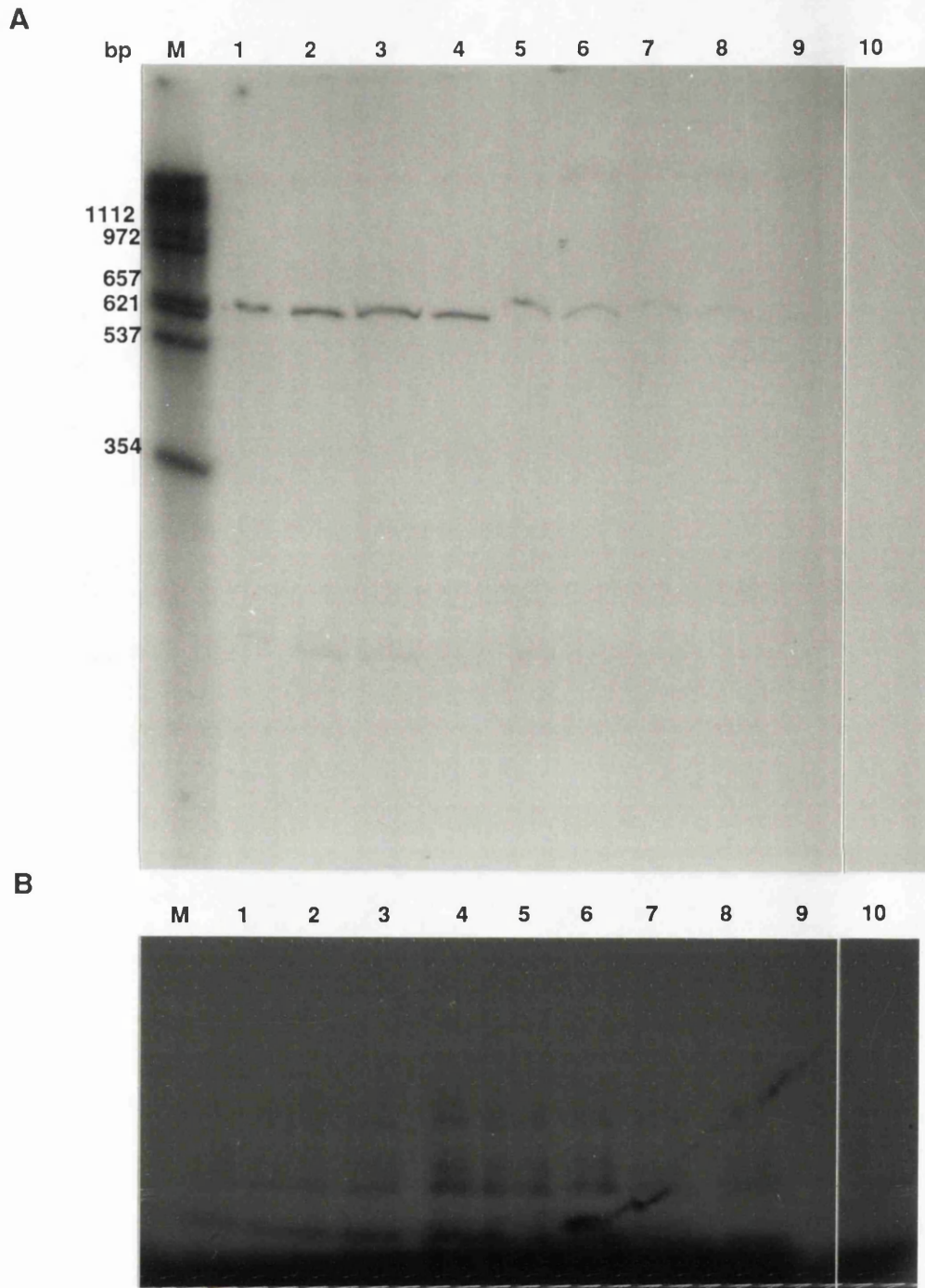


Figure 3.9: Pulse-chase analysis of hGH mRNA in CBMG cells.

A pulse-chase experiment was carried out as described in the text, and 50 μ g total RNA isolated at various time points during the chase was used in the ribonuclease protection assay with the pRHGH1 probe. (A) shows the top part of the resulting autoradiograph; lanes 1-9 correspond to 0, 1, 2.25, 6, 10, 18, 26, 34, and 44.25 hours chase respectively. Lane 10 shows the ribonuclease protection of 50 μ g labelled total RNA with no probe added to the hybridisation mix. Lane M contains λ /C/a I molecular weight markers generated as described in the legend to Fig 3.5. (B) shows the bottom part of the same autoradiograph; the lane numbers correspond to those in (A).

FIGURE 3.10

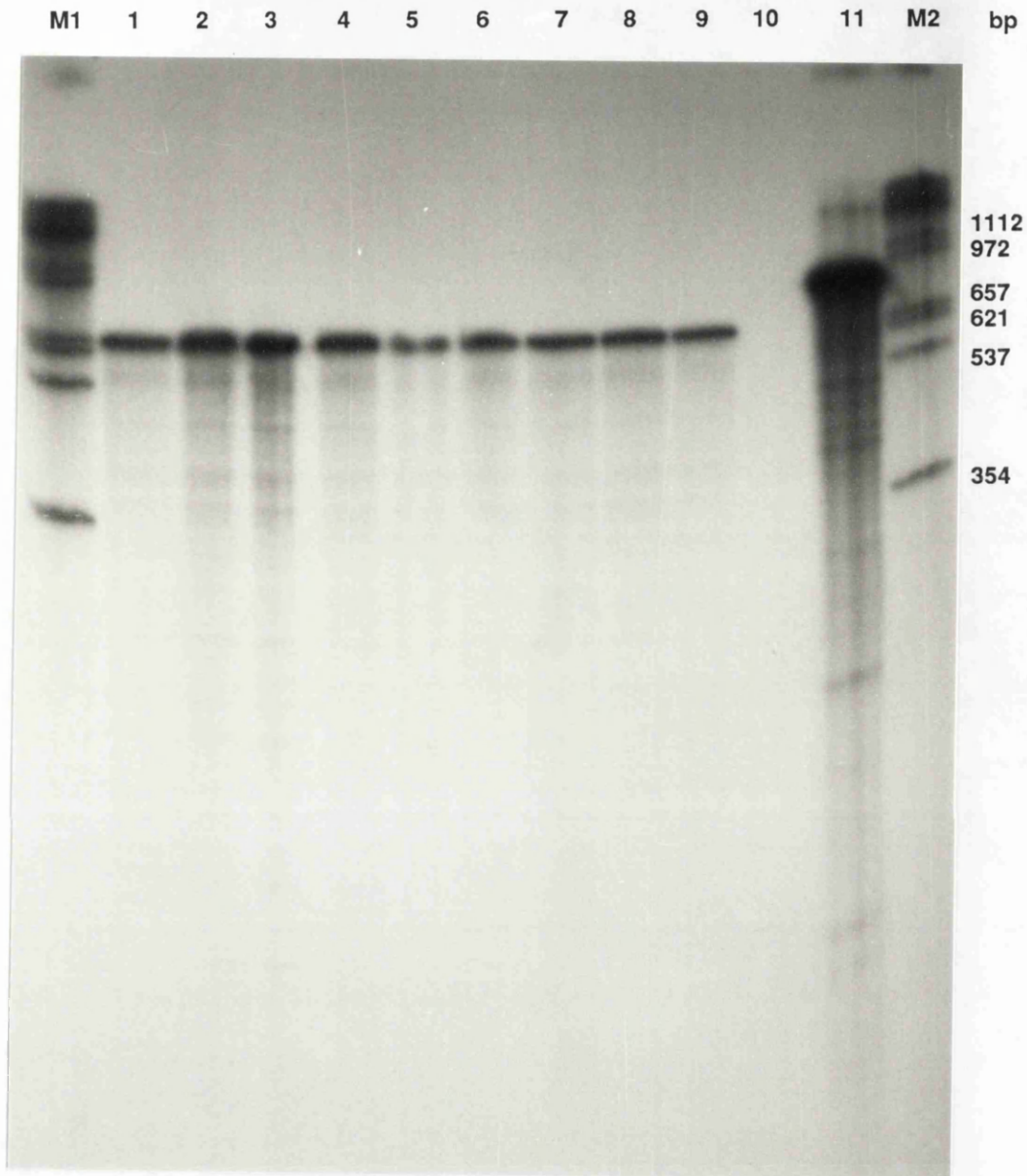


Figure 3.10: Ribonuclease protection of pulse-chase samples using a labelled probe.

The tritiated total RNA samples from the experiment shown in Fig. 3.9 were also hybridised to a labelled probe to confirm that approximately equal amounts of hGH mRNA were available to hybridise in each sample. Lanes 1-9 correspond to 0, 1, 2.25, 6, 10, 18, 26, 34, 44.25 hours chase respectively. Lane 10 shows the result of ribonuclease protection with the same probe with yeast tRNA in the hybridisation mix. Lane 11 contains a sample of undigested probe, and lanes M1 and M2 contain different loadings of λ /Cla I markers generated as described in the legend to Fig. 3.5.

FIGURE 3.11

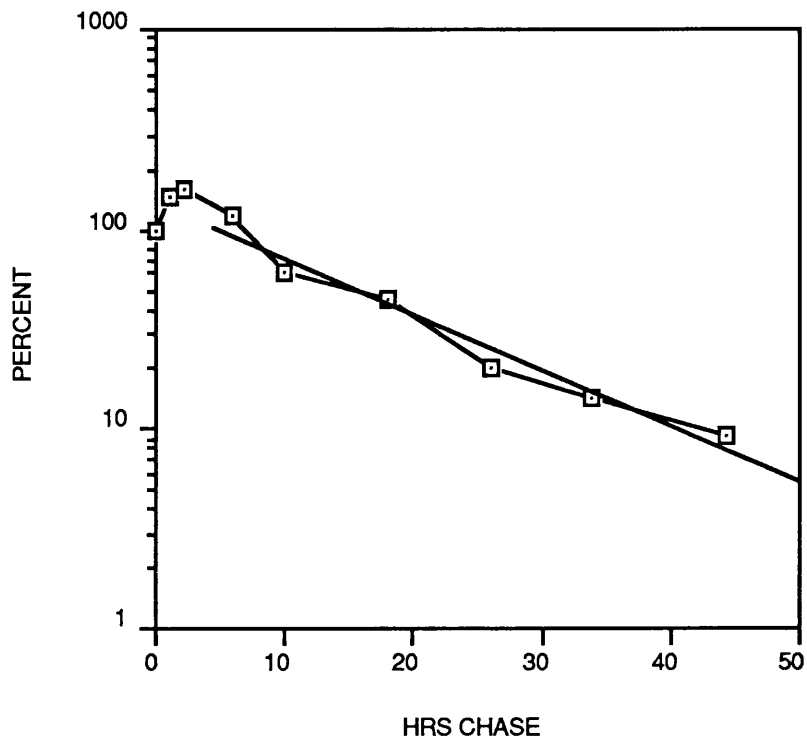


Figure 3.11: Decay of hGH mRNA in CBMG cells.

The protected fragments from the gel shown in Fig. 3.9 were cut out and quantified as described in the text. The no. of dpm for each sample was expressed as a percentage of the zero time point and plotted against the time during the chase. The no. of protected dpm (after subtraction of background) at time zero was 1926, and at time 44.25 hours, 181. Background dpm = 51. For linear regression analysis time points after 4.5 hours were used; R-squared for the line of best fit was 0.962.

the cells with glucosamine, which sequesters RNA precursors in the form of N-acetyl hexosamines (Scholtissek, 1971; Wertz, 1975; Levis and Penman, 1977). Therefore further experiments were carried out to determine whether glucosamine improved the chase conditions in these cells.

Cells were pretreated for one hour with glucosamine, and incubated with glucosamine for the first hour of the chase, as described by Guyette *et al*, (1979). This method minimises any possible toxic effects of the glucosamine that might result from a longer exposure of the cells to the drug. The specific activity of total RNA with and without the glucosamine treatment is shown in Fig. 3.12; it can be seen that chase conditions are similar in each case. The decay of hGH mRNA in the presence and absence of glucosamine is shown in Fig. 3.13. The small bands observed at the bottom of the gel in Fig. 3.9B are also seen here (Fig. 3.13B). The rate of decay of the hGH band was quantified as before and the results are shown in Fig. 3.14. Half-lives of 8.8 hours without glucosamine and 13.3 hours with glucosamine were obtained. Fig. 3.15 shows all three decay graphs plotted on the same axis, and Table 3.1 shows the half-lives and confidence intervals for each determination. These data indicate that all three half-lives are very similar, and that the glucosamine treatment does not have any significant effect on the half-life of hGH mRNA in CBMG cells, when time points after 4.5 hours are used. Owing to the variability of the individual data points in a single experiment, it is not possible to determine conclusively whether glucosamine does have an effect during the early part of the chase.

3.3: Discussion

The work presented in this chapter describes the development of a ribonuclease protection assay for use in detecting ³H-labelled transcripts in pulse-chase experiments. It was possible to detect a specific hGH band in ³H-labelled RNA using a probe which protected 774 nucleotides of hGH mRNA. This system has been used in a quantitative manner to measure the rate of decay of hGH mRNA in pulse-chase experiments, and has the advantage that any stable

FIGURE 3.12

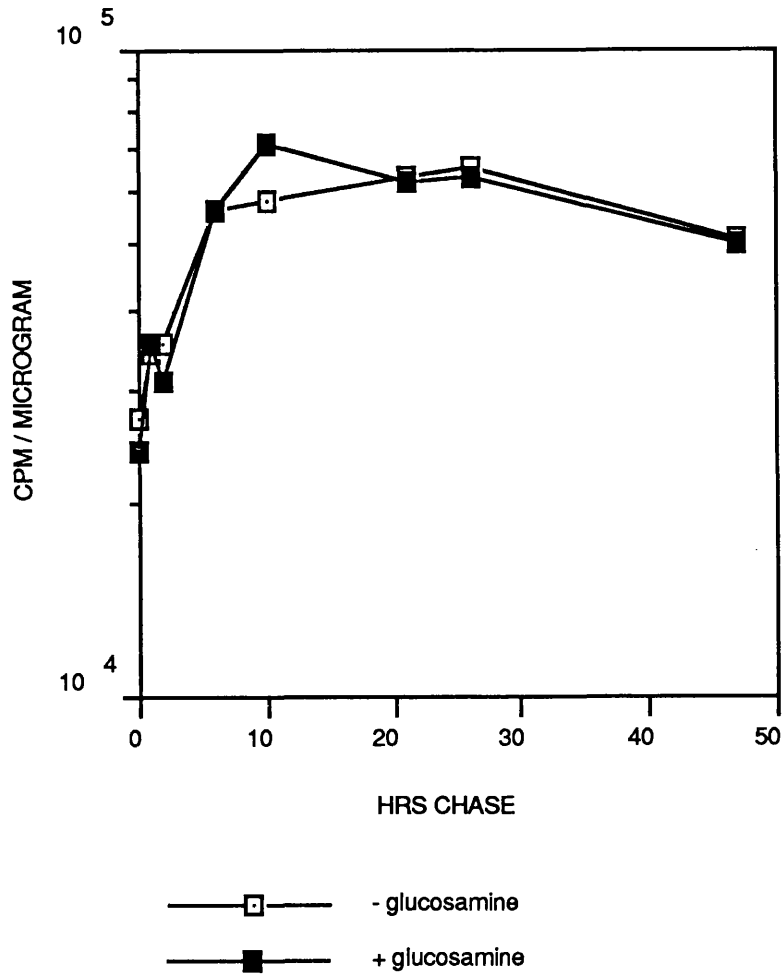


Figure 3.12: Decay of total mRNA in CBMG cells, in the presence and absence of glucosamine.

A pulse-chase experiment was carried out on CBMG cells in the presence and absence of glucosamine, and the specific activity of total RNA was determined at various time points during the chase as described in the text. The number of cpm/ μ g were plotted against time during the chase.

FIGURE 3.13

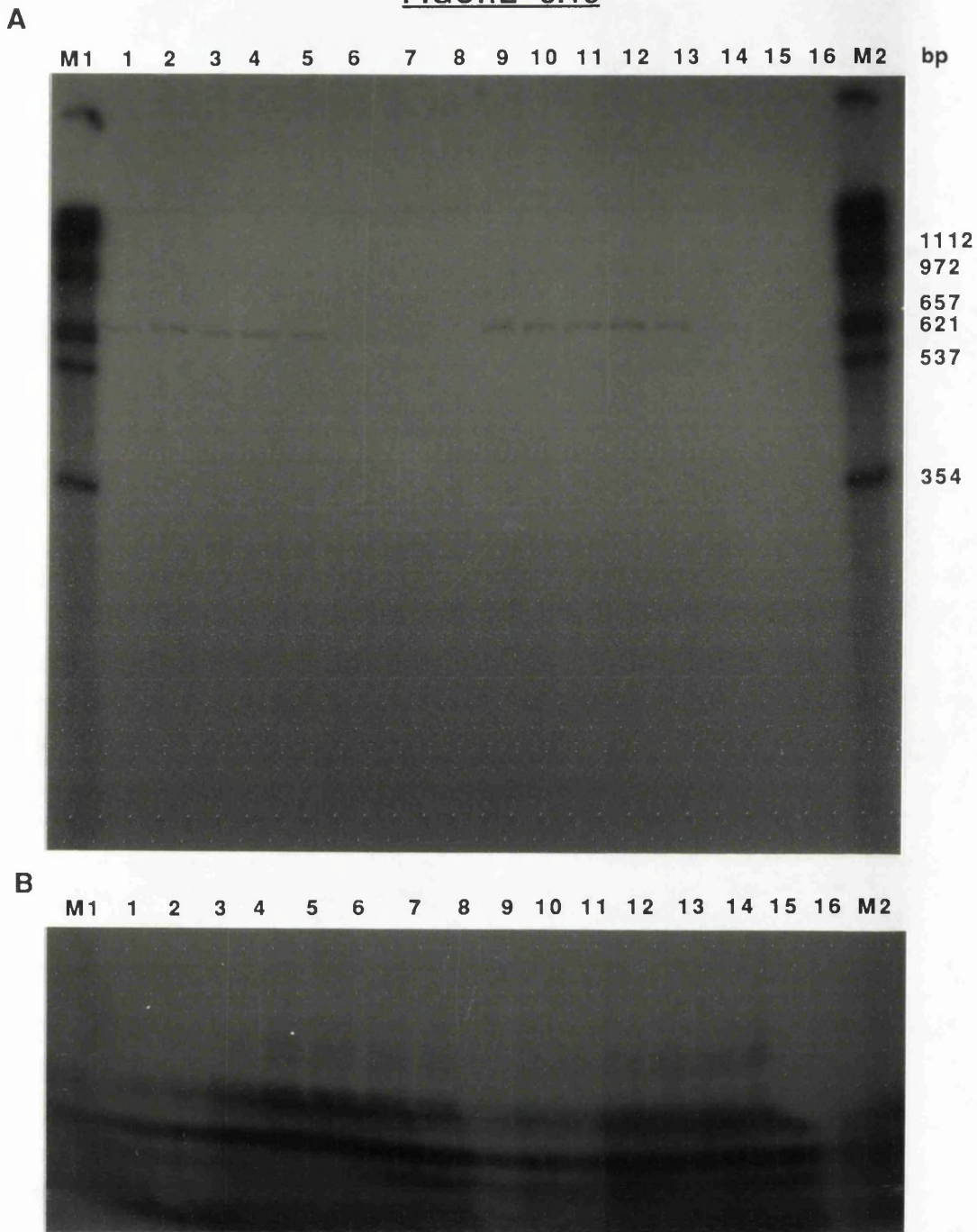


Figure 3.13: Pulse-chase experiment to test the effect of glucosamine on the chase conditions.

Pulse-chase analysis was carried out on CBMG cells, in the presence and absence of glucosamine, as described in the text. 50 μ g samples of total RNA isolated at various time points during the chase were used in the ribonuclease protection assay with the pRHGH1 probe. (A) shows the top part of the resulting autoradiograph; lanes 1-8 contain samples from the chase in the presence of glucosamine, lanes 9-16 contain samples from the chase in the absence of glucosamine. The chase time points in hours were as follows: 0 (lanes 1 and 9); 1 (lanes 2 and 10); 2 (lanes 3 and 11); 6 (lanes 4 and 12); 10 (lanes 5 and 13); 21 (lanes 6 and 14); 26 (lanes 7 and 15); 47 (lanes 8 and 16). Lanes M1 and M2 contain different loadings of λ Cla I markers generated as described in the legend to Fig. 3.5. (B) shows the bottom part of the same autoradiograph; the lane numbers correspond to those in (A).

FIGURE 3.14

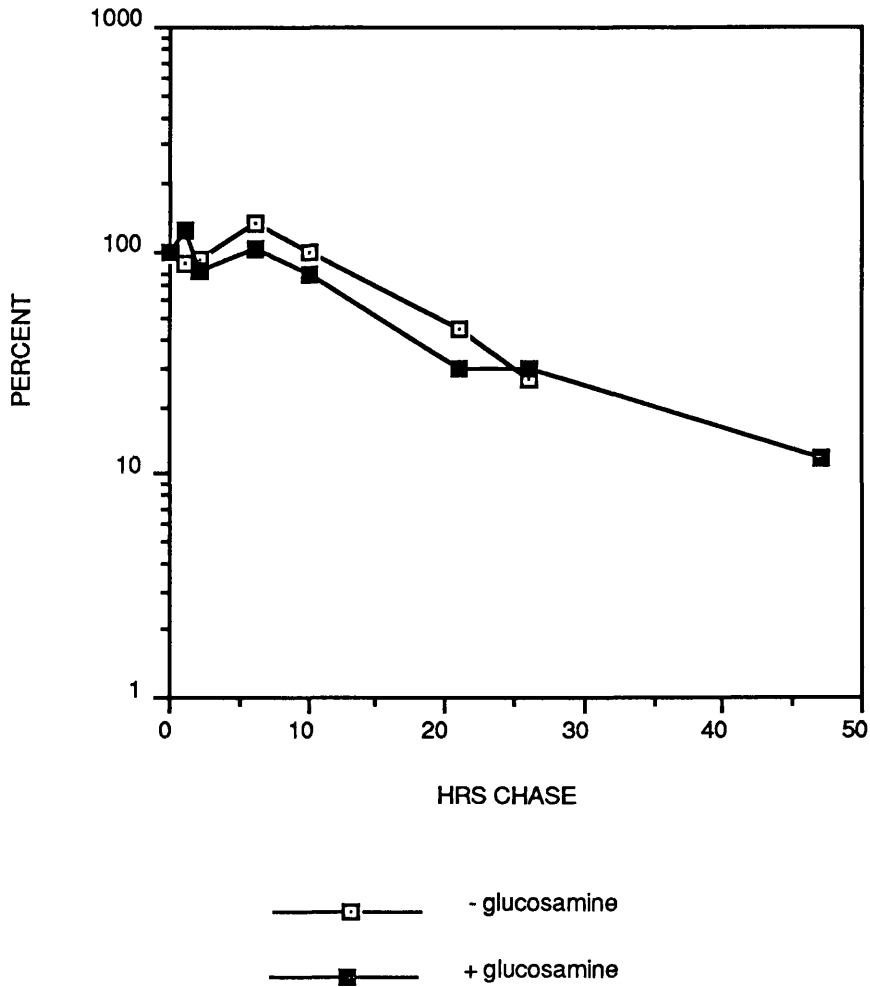


Figure 3.14: Decay of hGH mRNA in CBMG cells, in the presence and absence of glucosamine.

The protected bands from the gel shown in Fig. 3.13 were cut out and quantified as before, expressed as a percentage of the time zero value, and plotted against time during the chase. The initial dpm were 866 and 925 for the experiments with and without glucosamine, respectively, and the final dpm were 103 (47 hour time point) and 245 (26 hour time point) respectively. For linear regression analysis, time points after 4.5 hours were used, and the R-squared values were 0.956 (with glucosamine) and 0.994 (without glucosamine).

FIGURE 3.15

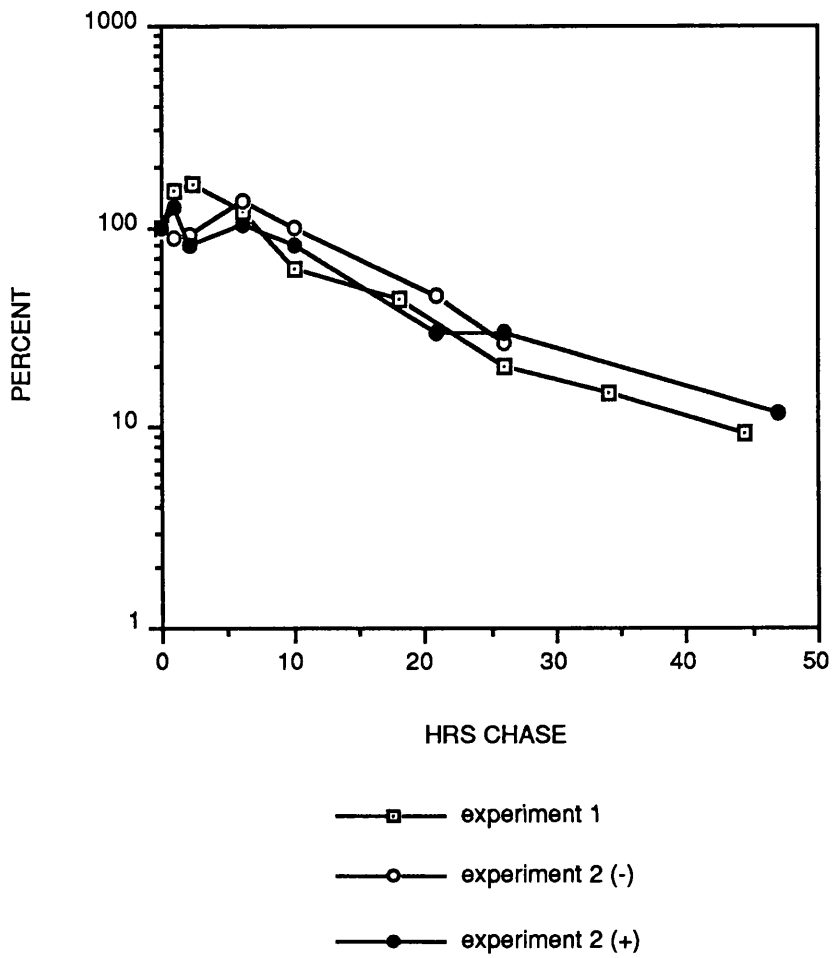


Figure 3.15: Summary graph

The graphs of Figs. 3.11 and 3.14 have been combined on the same axes; (-) and (+) indicate the absence and presence of glucosamine respectively.

Table 3.1

<u>Experiment</u>	<u>Half-life</u>	<u>95% confidence interval</u>
1	10.8 hours	8.5-14.9 hours
2 (no glucosamine)	8.8 hours	7.1-11.4 hours
2 (+ glucosamine)	13.3 hours	9.6-21.9 hours

Table 3.1: The half-life of hGH mRNA in CBMG cells

The half-life values for the two experiments described in this Chapter are shown. The graph for experiment 1 is shown in Fig. 3.12, and those for experiment 2 are shown in Fig. 3.15. The half-lives were calculated using linear regression analysis of the \log_e dpm values plotted against time to determine the slope of the regression line, ignoring time points before 4.5 hours. The value of the t statistic for all three lines was significant to at least the 1% level. The half-lives were calculated from the relationship $T_{1/2} = \log_e 2 / \text{slope}$. The confidence intervals (CI) were calculated using the equation: $\log_e(95\% \text{ CI}) = \text{slope} \pm t s$, where t is the value of the t statistic at n-2 degrees of freedom for 95% confidence, and s is the standard error of the slope.

degradation intermediates can be distinguished by the gel electrophoresis step; most pulse-chase methods involve hybridisation to immobilised DNA probes which does not allow this distinction (e.g. Guyette *et al*, 1979; Brock and Shapiro, 1983b; Paek and Axel, 1987).

As mentioned in Chapter 1, there are several factors which may affect the measured half-life of an mRNA species when using a pulse-chase protocol, and these must be taken into consideration when drawing conclusions from these experiments. Firstly, the large size of the ribonucleotide precursor pool, and the fact that the phosphorylated precursors cannot be chased out of the cells, means that perfect chase conditions (where no further labelled nucleotides are incorporated after the start of the chase), cannot be achieved (Warner *et al*, 1966; Schimke, 1970; Scholtissek, 1971; Cowan and Milstein, 1974; Levis and Penmann, 1977). This is clearly the case with the CBMG cells, as incorporation into hGH mRNA continues for at least the first four hours of the chase. Without knowing the absolute rate of transcription of the hGH gene and the specific activity of the UTP pool during the chase, it is impossible to accurately correct for this error. However, to minimise

its effects, time points from the early part of the chase were ignored when calculating the half-life.

An alternative approach, reported in the literature, involves the use of glucosamine to mimic perfect chase conditions by artificially reducing the size of the nucleotide pool (Scholtissek, 1971; Murphy and Attardi, 1973; Wertz, 1975; Levis and Penmann, 1977; Guyette *et al*, 1979;). No dramatic effect on the chase conditions could be detected in this experiment, and if anything the hGH mRNA had a longer half-life after the glucosamine treatment. It would be necessary to repeat the experiment several times, concentrating on the early time points, to determine whether glucosamine yields any real improvement on the chase in this cell type. Use of time points after 4.5 hours, when the decay is exponential, yielded reasonably reproducible results, so there seems to be no advantage in the use of glucosamine, as it may have toxic effects. It is likely that chase conditions, and the degree of effectiveness of the glucosamine treatment, vary between cell types, as the size and rates of metabolism of nucleotide pools are unlikely to be identical between different cells. For example, the glucosamine chase was not found to be completely effective in mammary gland explant cultures (Guyette *et al*, 1979). Schneider *Drosophila* cells, on the other hand, seem to chase quite effectively (Levis and Penman, 1977).

Another factor which will affect the measured half-life of an mRNA species is the rate of cell division during the chase, as cell division effectively dilutes out the labelled molecules. This is not a significant factor in these experiments, as CBMG cells at the cell densities used remain at a constant viability with a very low level of cell turnover. To rigorously control for cell division, a stable molecule could be monitored under the same chase conditions, as described in later Chapters for pulse-chase experiments in B cell lines.

Since this work was carried out, others have used a pulse chase method to measure the half-life of hGH mRNA in murine fibroblast cells (Paek and Axel, 1987). These workers obtained a value of 9 hours for the half-life of hGH mRNA, (in a similar cell type), which agrees well with the result described above. Paek and Axel were investigating the

mechanism of regulation of hGH by dexamethasone, using mouse L cells transfected with various hGH gene constructs. They observed a 2-3 fold induction of the level of hGH mRNA with dexamethasone, using an hGH cDNA driven by a thymidine kinase promoter. A 2-5 fold induction was obtained when a genomic hGH gene driven by its own promoter was used. This indicates that a large part of the induction is a result of post-transcriptional regulation, and Paek and Axel showed that dexamethasone stabilises a proportion of the hGH mRNA, and that this stabilisation is associated with an increase in the length of the hGH mRNA poly(A) tail. There are several interesting points to note with regard to their work, the experiments of Pavlakis and Hamer (1983a), and the work described here.

Pavlakis and Hamer observed that the expression of the hGH gene under the control of the mMTI promoter was induced by heavy metals, but not by dexamethasone, under conditions where the endogenous mMT genes remained responsive to both agents. Similar results with the mMTI promoter have been obtained by others (Searle *et al*, 1984; Yagle *et al*, 1985), indicating that the glucocorticoid-response element may lay outside the promoter region used in these experiments. However, the first intron of the hGH gene, present in BPVMG7, does contain a glucocorticoid-response element (Moore *et al*, 1985; Slater *et al*, 1985), and so expression of the gene should be transcriptionally regulated by glucocorticoids. In addition, from the work of Paek and Axel and others (Diamond and Goodman, 1985), the hGH mRNA would be expected to be regulated at the level of mRNA stability. Therefore it is initially puzzling as to why Pavlakis and Hamer observed no response to glucocorticoid, but it can be explained in the light of the work by Paek and Axel.

In the expression system used by Paek and Axel, the expression levels of hGH mRNA are very low, and the total induction observed is quite small, around 2-5 fold, even in the presence of the hGH promoter. Therefore, given the high copy number of hGH in CBMG cells, it is quite likely that any induction (either transcriptional or post-transcriptional), would be masked by the high basal levels of mRNA. In addition, the mRNA stabilisation observed by Paek and Axel occurred after a lag of 12-24 hours, and Pavlakis and Hamer

only describe an observation at a single time point of 8 hours, so even if this effect was occurring, they would not have detected it.

With regard to the measurement of hGH mRNA stability, the experiments described in this Chapter were carried out in the presence of serum, which may contain some glucocorticoid, so it is possible that some stabilisation effect is acting on the hGH mRNA in CBMG cells. Paek and Axel observed that only 25% of the hGH mRNA was stabilised by the hormone, and that 75% retained a half-life close to that of hGH mRNA in uninduced cells. The stabilisation effect therefore does not become apparent until after 20-25 hours of the chase, and in my experiments there are insufficient time points after this time to be able to conclude whether or not a proportion of the RNA was being stabilised. Again, it is possible that cytoplasmic factors responsible for stabilisation would be titrated out by the high levels of hGH mRNA, so a larger proportion of the RNA would retain the shorter half-life. This would make it even less likely that such an effect would be detected.

As mentioned in the introduction to this Chapter, it was of interest to note whether hGH mRNA had an exceptionally long half-life in these cells, in the light of its high level expression. Clearly the half-life of 8-13 hours (at least for the majority of hGH mRNA molecules) is not significantly different from the average rate of decay of a large proportion of mammalian mRNAs (Singer and Penman, 1973; Perry and Kelley, 1973; Abelson *et al*, 1974; Lowenhaupt and Lingrel, 1978; Guyette *et al*, 1979) indicating that the high copy number is important for the high expression level of hGH mRNA.

In conclusion, then, a method has been developed which can be used to detect labelled hGH mRNA in pulse-chase experiments, and this method has been used to determine the half-life of hGH mRNA. As expected, the chase conditions are not perfect, so this method is not suitable for measuring very short mRNA half-lives, as too high a percentage error would be introduced into the result. Nevertheless, the method yields reproducible results for a half-life of several hours, and gave results which agree with those obtained by others (Paek and Axel, 1987). It was therefore concluded

that this method was suitable to compare the half-lives of immunoglobulin mRNA in different B cell lines. This work is described in the following Chapter.

CHAPTER 4

THE HALF-LIFE OF μ mRNA IN TWO B CELL LINES**4.1: Introduction**

As discussed in Chapter 1, when B cells terminally differentiate, they switch from producing mainly mRNA encoding the membrane form of IgM (μ_m) to mRNA encoding the secreted form (μ_s), with a concomitant large increase in the cellular content of immunoglobulin mRNA (Perry and Kelley, 1979; Alt et al, 1980; Rogers et al, 1980; Lamson and Koshland, 1984; Yuan and Tucker, 1984b; Mather et al, 1984; Berger, 1986). The secretion of large amounts of specific immunoglobulin into the bloodstream plays a crucial role in the immune response in mammals, and it is of interest to know the mechanism by which the increase in immunoglobulin mRNA levels is brought about. The measured increase in transcription rate of immunoglobulin genes accompanying B cell differentiation is insufficient to fully account for the increase in steady state μ mRNA levels (Perry and Kelley, 1979; Mather et al, 1984; Kelley and Perry, 1986; Gerster et al, 1986; Mason et al, 1988; Jäck and Wabl, 1988; Yuan and Dang, 1989). Therefore it seems likely that post-transcriptional processes are involved in the regulation mechanism. These may involve regulation of nuclear mRNA processing or degradation, transport of mRNA to the cytoplasm, or rate of cytoplasmic degradation (Darnell, 1982). Control of mRNA stability is emerging as an important mechanism for regulating gene expression in a variety of systems (see references in Section 1.3). Therefore experiments were undertaken, using the pulse-chase method described in Chapter 3, to determine whether differences in the rate of degradation of μ mRNA could account for the increase in steady-state μ mRNA levels when B cells differentiate.

Immortalised B cell lines representing different stages of B cell differentiation were used as the model system in which to carry out these experiments. This approach has yielded useful results in many studies of immunoglobulin gene expression, including those on transcription and RNA processing (Perry and Kelley, 1979; Rogers et al, 1980; Alt

et al, 1980; Nelson et al, 1983; Mather et al, 1984; Kelley and Perry, 1986; Berger et al, 1986; Gerster et al, 1986; Jäck and Wabl, 1988; Mason, et al, 1988;). The use of cell lines is necessitated by the difficulty in obtaining sufficient quantities of pure populations of primary B cells to carry out experiments. This would be a particular problem for pulse-chase experiments to measure mRNA half-life, where large cell numbers are required to obtain measurable amounts of radioactivity incorporated into a specific mRNA. The results from experiments in cell lines where steady state μ mRNA levels and transcription rates have been measured, agree qualitatively with the more limited data available from primary B cells (Perry and Kelley, 1979; Lamson and Koshland, 1984; Mather et al, 1984; Yuan and Tucker, 1984a, 1984b; Kelley and Perry, 1986; Berger, 1986), thus making it reasonable to assume that measurements of the rates of decay of μ mRNA in cell lines might also broadly reflect the situation *in vivo*.

In order to study the rate of decay of μ mRNA in B cell lines, two murine cell lines representing the resting B cell and plasma cell stages of differentiation were chosen. WEHI231 is a B cell lymphoma that expresses monomeric IgM on its cell surface and does not secrete any detectable immunoglobulin, or make J chain (Warner et al, 1975; Raschke et al, 1979; Gutman et al, 1981; Lamson and Koshland, 1984). Therefore this cell line has the phenotype of a B cell arrested prior to the plasma cell stage of differentiation, and probably represents the immature B cell stage of development, as it does not possess membrane-bound IgD and has a high density of membrane-bound IgM (Gutman et al, 1981). MXW231 is a hybridoma formed by fusing WEHI231 with the IgG-producing plasmacytoma MPC11 (Rashke et al, 1979). Like other hybridomas, MXW231 shows characteristics of the plasma cell stage of differentiation, presumably because of the presence of *trans*-acting factors from the plasmacytoma parent (Eshhar et al, 1979; Alt et al, 1980; Rogers et al, 1980; Perry et al, 1981). It continues to produce secreted IgG from the MPC11 parent, and in addition produces and secretes pentameric IgM; the μ heavy chain being derived from the WEHI231 gene (Raschke et al, 1979).

These two cell lines provide the opportunity of studying

the expression of the same endogenous WEHI231 μ gene in two cellular environments, which mimic those of a B cell and a plasma cell. Both of these cell lines have been used by others in studies of B cell differentiation (Perry and Kelley, 1979; Alt et al, 1980; Lamson and Koshland, 1984; Mather et al, 1984; Kelley and Perry, 1986; Mason et al, 1988). In particular, Mason et al (1988) have shown that the steady state nuclear μ mRNA levels in MXW231 are only 1.6 fold higher than in WEHI231 (determined by densitometry of Northern blots), and that cytoplasmic μ mRNA levels differ by 15 fold. The ratio of the rates of transcription measured by nuclear run-off experiments was found to be up to 1.5 fold, accounting for the difference in steady-state nuclear RNA levels. Therefore in these cell lines there is approximately a ten fold difference in μ mRNA levels in the cytoplasm which can only be accounted for by differences in rates of late nuclear processing, transport to the cytoplasm, or cytoplasmic turnover.

This Chapter describes preliminary Northern blot experiments to confirm that the two cell lines produced the expected species of μ mRNA, the optimisation of the ribonuclease protection assay conditions to detect ^3H -labelled μ mRNA, and the pulse-chase experiments to compare the half-life of μ mRNA in the two cell lines. The data reveal a clear increase in μ mRNA stability between the resting B cell and plasma cell stages, which makes a significant contribution to the increased steady state level of μ mRNA.

4.2: Results

4.2.1: Construction of probes for Northern blot analysis and ribonuclease protection.

The nucleic acid probes used in the work described below were generated *in vitro* using the bacteriophage SP6 transcription system, in a similar manner to that described in Chapter 3 for the hGH probes. Sequences of interest were inserted downstream of the SP6 promoter in the vectors pSP64 and pSP65, and are illustrated in Fig. 4.1. All DNA manipulations were carried out as described in Section 2.2.

FIGURE 4.1

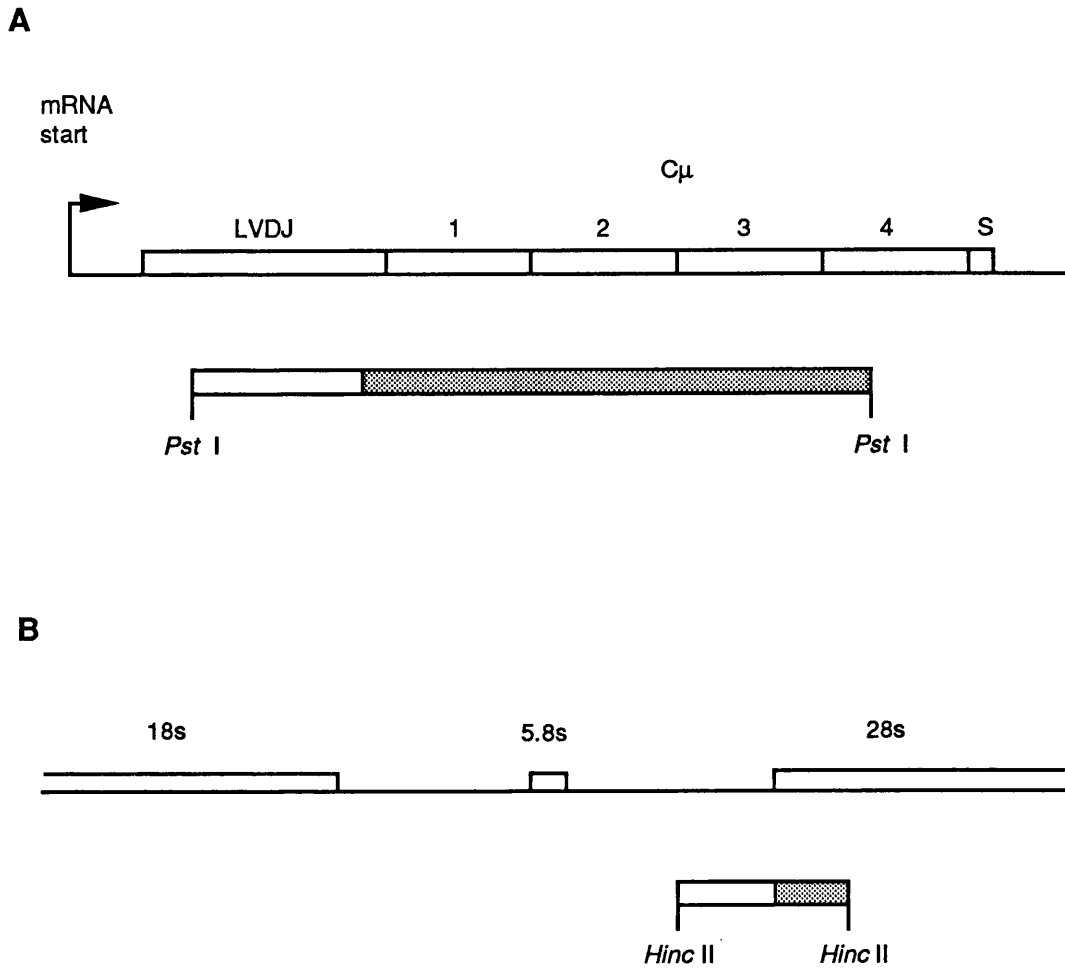


Figure 4.1: Probes used in Northern blot analysis and ribonuclease protection.

(A) shows μ mRNA coding for the secreted form of the protein with the coding region shown as open boxes and the 5' and 3' untranslated regions as a single line. L, V, D, and J indicate the leader, variable, diversity, and joining regions respectively. The numbers 1-4 refer to the constant region exons and s indicates the codons for the C-terminus of the secreted form of μ protein. The 1.4kb *Pst* I fragment inserted into pSP64 is shown underneath, with the 1.1kb region of homology shaded.

(B) shows part of the primary transcript for murine ribosomal RNA, with the 18S, 5.8S and 28S mature ribosomal RNAs shown as open boxes and the transcribed spacers as a single line. The 944bp *Hinc* II fragment inserted into pSP65 is shown underneath, with the region of homology to 28S rRNA shown shaded.

The probe for detection of μ heavy chain mRNA was constructed by isolating a 1.4kb *Pst* I fragment from the plasmid pNPI (Wood et al, 1984), which contains a cDNA encoding the μ chain with specificity for the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP). This cDNA was cloned from the hybridoma cell line B1-8 (Bothwell et al, 1981), which had been derived from immunisation of a C57Bl/6 mouse. The 1.4kb fragment was ligated to pSP64 DNA that had been digested with *Pst* I and treated with calf intestinal phosphatase. After transformation into *E. coli* LM1035, mini plasmid preparations from the resulting colonies were analysed by digestion with appropriate restriction enzymes to determine the orientation of the inserted *Pst* I fragment. A transformant was selected which contained the insert in an orientation such that transcription from the SP6 promoter would produce RNA complementary to mRNA. The resulting plasmid is pSP64 μ NP. The probe insert in pSP64 is shown in Fig. 4.1A, with the region which will hybridise to μ mRNA of any non-NP specificity shaded.

A probe for ribosomal RNA was constructed in a similar manner, and is shown in Fig. 4.1B. It consists of a 944bp *Hinc* II fragment from pMEB3 (Michot et al, 1982), inserted into the *Hinc* II site of pSP65. This fragment contains the first 358bp of the murine 28S rRNA sequence and 586bp of upstream transcribed spacer, and is also inserted in the antisense orientation in the resulting plasmid, pSP64rRNA. pSP64 μ NP was linearised at the *Eco* RI site, and pSP65rRNA was linearised at the *Hind* III site, for generation of RNA probes using SP6 polymerase.

4.2.2: Northern blots of RNA from WEHI231 and MXW231 cells.

To analyse the μ mRNA species produced in these cell types, total and cytoplasmic RNA preparations from cells in exponential growth were subjected to electrophoresis on formaldehyde/agarose gels. The RNA was then transferred to "Hybond-N" and hybridised to a high specific activity ³²P-labelled probe generated from pSP64 μ NP. These procedures are described in detail in Section 2.3. RNA from J558L cells was also loaded on the gel as a negative control. J558L cells are a heavy-chain loss variant of J558, an IgA-secreting myeloma, and do not contain any mRNA coding for heavy chain, but do

produce $\lambda 1$ light chain (Oi et al, 1983). The results of the hybridisation are shown in Fig. 4.2. It was necessary to wash the filters at 80°C in 0.1x SSC to remove non-specific binding of the probe to 28S ribosomal RNA, as demonstrated by removal of all signal from the J558L RNA (lane 5). Other workers have already shown that WEHI231 cells produce mRNA molecules of 2.4 and 2.7 kb, coding for both μ_s and μ_m polypeptides respectively, and MXW231 cells only produce mRNA encoding μ_s (Perry and Kelley, 1979; Alt et al, 1980; Lamson and Koshland, 1984; Mather et al, 1984; Kelley and Perry, 1986; Mason et al, 1988;). This is confirmed in Fig. 4.2 by the presence of a single band in this region in lanes 2 and 4 (MXW231 cell RNA) and a doublet in lanes 1 and 3 (WEHI231 cell RNA), the lower band of which comigrates with the band in MXW231. Consistent with the different stages of differentiation these two cell lines represent, the signal from MXW231 RNA is much more intense than that from WEHI231 RNA. Hybridisation to higher molecular weight material is also observed, particularly in the total RNA preparations (lanes 3 and 4). This signal may be due to precursor RNAs, which are only found in the nucleus, or some residual cross hybridisation to ribosomal RNA molecules.

To obtain an estimate of the relative levels of μ mRNA between the two cell lines, a range of amounts of total RNA from each was loaded onto formaldehyde/agarose gels and analysed by Northern blotting as before, and the results from one of two duplicate gels are shown in Fig. 4.3. The hybridisation signal from these experiments was quantified both by scanning suitable exposures of the autoradiographs using a densitometer (Fig. 4.4A), and by cutting out the bands from the hybridisation filter and determining the amount of radioactivity in them by liquid scintillation counting (Fig.4.4B). (These methods are described in more detail in Section 2.3.) Both methods indicate that the signal from WEHI231 shows a linear response up to 7.5 μ g, but the signal from MXW231 cells departs from linearity above 1-2 μ g. This indicates that this hybridisation was not quantitative above 1 μ g for MXW231 cells, perhaps due to an insufficient excess of probe in the hybridisation mix, or steric hindrance of the probe at the higher loadings of the

FIGURE 4.2

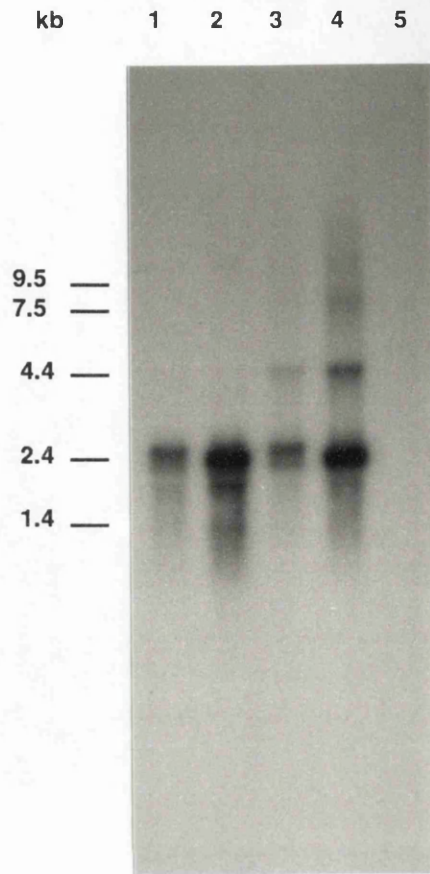


Figure 4.2: Northern Blot of RNA from WEHI231 and MXW231 cells

One of two duplicate Northern blots probed with the pSP64 μ NP probe. Lane 1; WEHI231 cytoplasmic RNA, lane 2; MXW231 cytoplasmic RNA, lane 3; WEHI231 total RNA, lane 4; MXW231 total RNA, lane 5; J558L cytoplasmic RNA. 2.5 μ g of each RNA was loaded onto the gel. Molecular weight markers were provided by the B.R.L. 9.5-0.24kb RNA ladder.

FIGURE 4.3

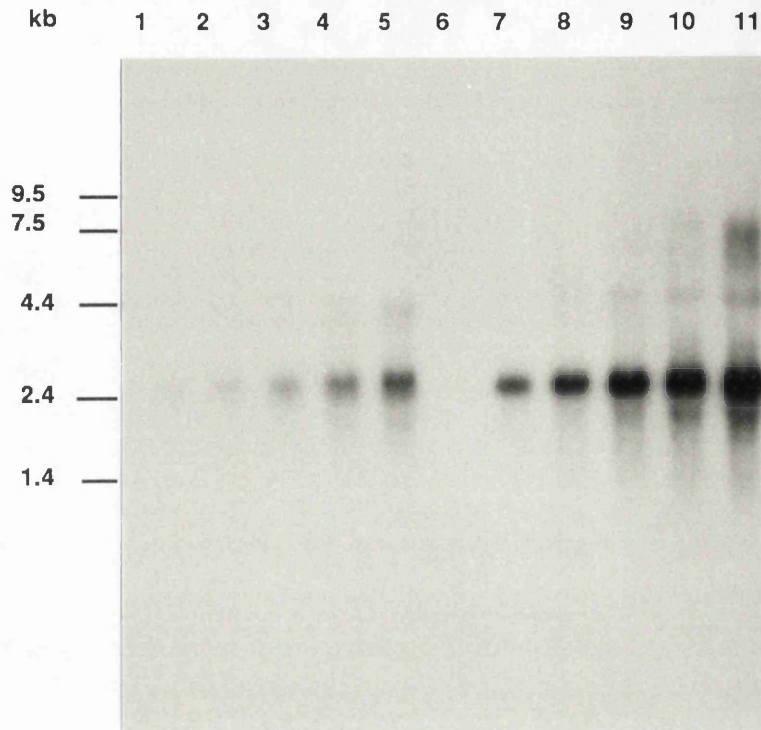
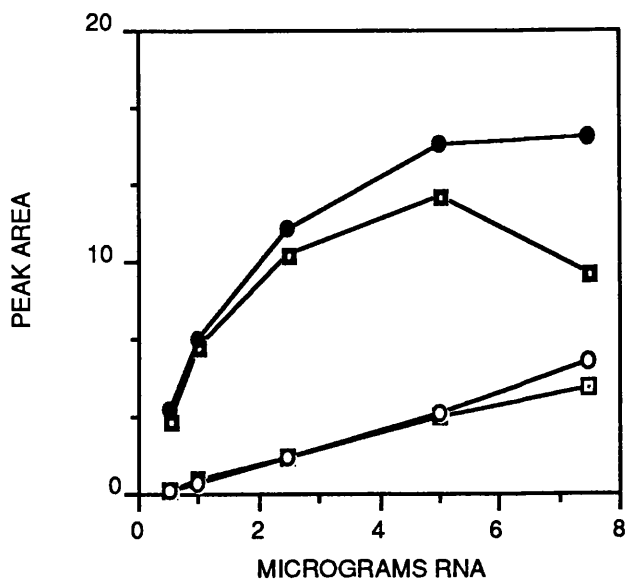


Figure 4.3: Northern Blot of a range of amounts of RNA from WEHI231 and MXW231 cells.

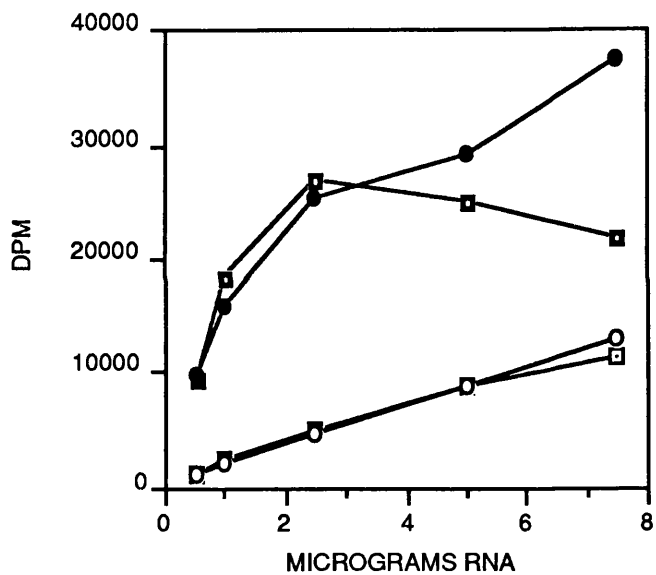
One of two duplicate Northern blots is shown with various amounts of total RNA from WEHI231 cells (lanes 1-5) and MXW231 cells (lanes 6-11), probed with the pSP64 μ NP probe. The amounts of RNA loaded were 0.5 μ g (lanes 1 and 7); 1.0 μ g (lanes 2 and 8); 2.5 μ g (lanes 3 and 9); 5 μ g (lanes 4 and 10); and 7.5 μ g (lanes 5 and 11). 2.5 μ g J558L RNA was loaded in lane 6. Molecular weight markers were provided by the 0.24-9.5kb B.R.L. RNA ladder.

FIGURE 4.4

A



B



○ — WEHI231/GEL 1 □ — WEHI231/GEL 2
● — MXW231/GEL 1 ■ — MXW231/GEL 2

Figure 4.4: Quantitation of Northern blots probed with pSP64 μ NP.

(A) One hour exposures of autoradiographs of duplicate Northern blots, one of which is shown in Fig. 4.3, were scanned with a densitometer and the peak area for the μ -specific peak in each track was plotted against the amount of total RNA loaded.

(B) The μ -specific band was cut out from each track of the same Northern blots as in (A), the radioactivity quantitated by liquid scintillation counting, and plotted against the amount of total RNA loaded. The first 2 points from each MXW231 graph were used when estimating the ratios shown in Table 4.1A, and for WEHI231 linear regression analysis was performed using all points. R-squared values for (A) were 0.996, 0.997, and for (B) were 0.999, 0.989.

gel. The experiment was repeated with a different range of amounts of RNA, and the result of scanning this gel is shown in Fig. 4.5, which departs slightly from linearity at high and low signal intensities due to the non linearity of the response of the X-ray film. The slopes of the linear regions of these graphs were used to estimate the relative levels of μ mRNA between the two cell lines, by dividing the slope of the line for WEHI231 into that for MXW231, with the results shown in Table 4.1A.

To control for any error in the concentration measurement of the total RNA used in these experiments, a Northern blot was carried out with a range of RNA amounts from 1 to 100ng, and probed with the rRNA probe (Fig. 4.6). The major band of 4.5kb corresponds to 28S ribosomal RNA (Hassouna *et al*, 1984). Densitometric scanning and liquid scintillation counting were used again to compare the amounts of rRNA between the two cell lines, and the results are shown in Fig. 4.7A and B respectively. The ratio of the slopes of the linear regions of the graphs in Fig. 4.7A agreed well with the ratio from Fig. 4.7B, both yielding a value of 1.2; i.e. there was 1.2 fold more rRNA in the WEHI231 sample than in the MXW231 sample. This factor was used to correct the ratios in Table 4.1A, giving the final estimates for the ratio of μ mRNA between the two cell lines in Table 4.1B.

The major source of variation between the ratio values for the different gels is most likely to be due to variations in the loading of individual slots, particularly for gels 1 and 2 (Fig. 4.4), where only two samples are available on the linear range for MXW231. The results do demonstrate good agreement between the liquid scintillation counting and densitometric scanning methods, however, indicating approximately an order of magnitude difference in μ mRNA levels between the two cell lines. Total polyadenylated RNA preparations from pulse-chase experiments were also analysed for their μ mRNA content and the results of Northern blots from these experiments are shown in Section 4.2.6. An average ratio of μ mRNA levels between the two cell lines obtained by densitometric scanning of these autoradiographs was found to be 9.1 fold, a figure which agrees well with the data shown here for total RNA.

FIGURE 4.5

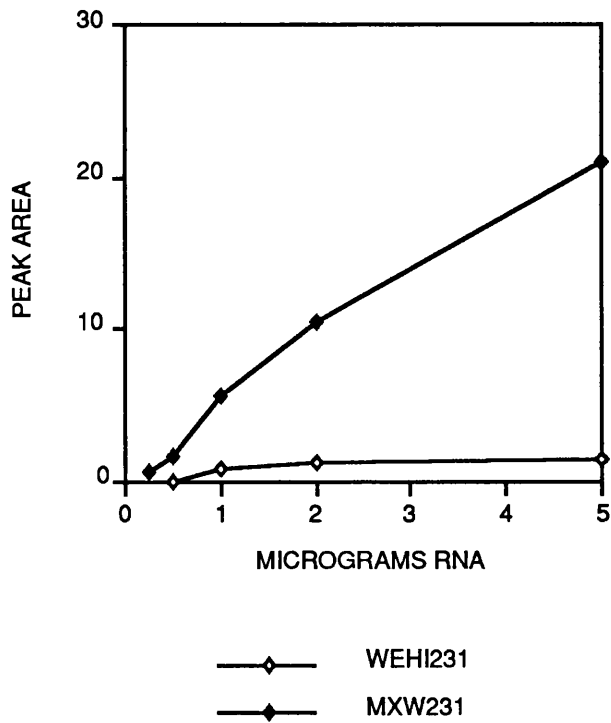
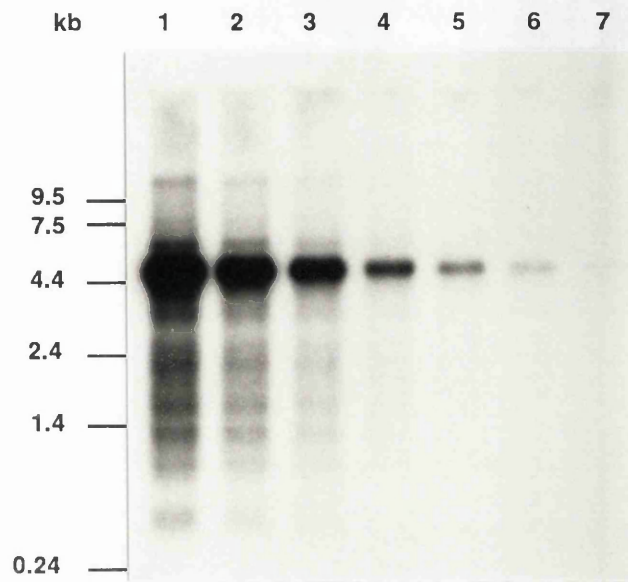


Figure 4.5: Quantitation of Northern blot probed with pSP64 μ NP.

A third Northern blot of a range of amounts of RNA from MXW231 and WEHI231 cells was probed with the μ NP probe, and the resulting autoradiograph was scanned with a densitometer. The peak area for the μ -specific peak was plotted against the amount of total RNA loaded. Points up to 2 μ g were used for linear regression analysis; R-squared was 0.991 for MXW231 and 0.919 for WEHI231.

FIGURE 4.6

A



B

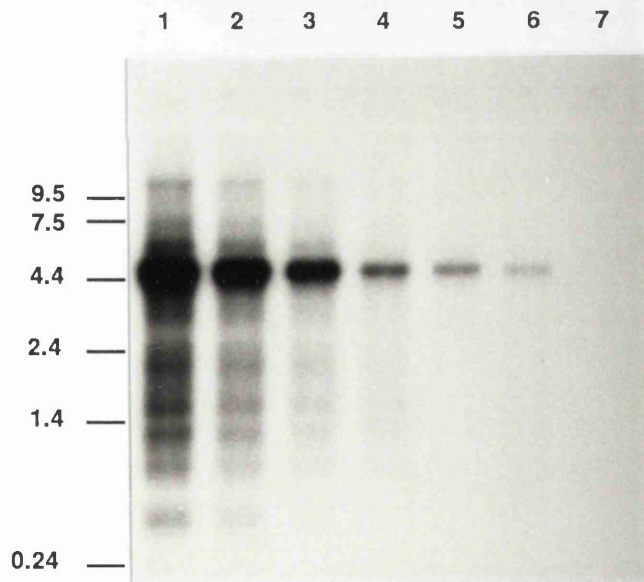


Figure 4.6: Northern blot probed with the pSP65rRNA probe

A Northern blot was carried out with a range of amounts of total RNA from (A) WEHI231 and (B) MXW231, and probed with the pSP65 rRNA probe. The amounts of RNA loaded were; 100ng (lane 1); 50ng (lane 2); 25ng (lanes3); 10ng (lane 4); 5ng (lane 5); 2.5ng (lane 6); and 1ng (lane 7). Molecular weight markers were provided by the B.R.L. 0.24-9.5 kb RNA ladder.

FIGURE 4.7

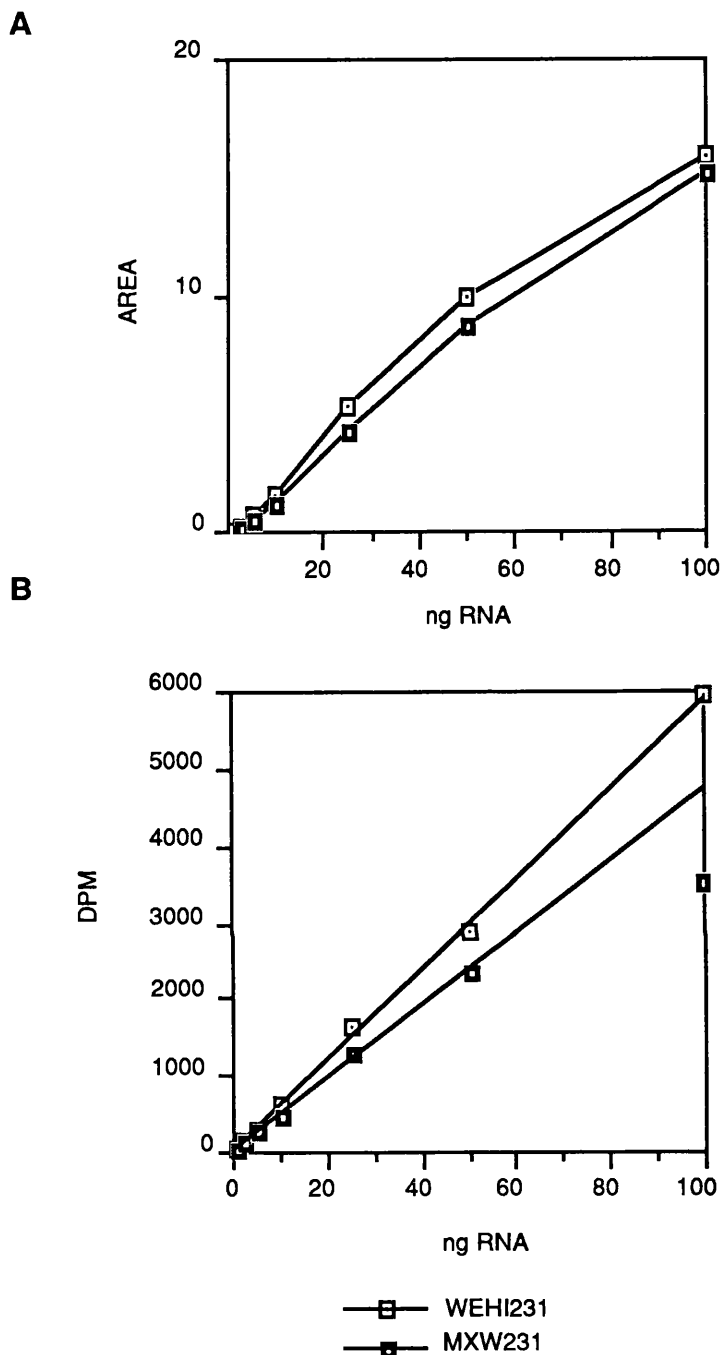


Figure 4.7: Quantitation of ribosomal RNA Northern blots

(A) The autoradiograph shown in Fig. 4.6 was scanned with a densitometer and the 28S rRNA-specific peak area for each track plotted against the amount of RNA loaded. Points up to 50ng were used for linear regression analysis; R-squared was 0.996 for WEHI231 and 0.998 for MXW231.

(B) The 28S rRNA-specific bands from the Northern blot shown in Fig. 4.6 were cut out and quantitated by liquid scintillation counting, and plotted against the amount of RNA loaded. Points up to 100ng for WEHI231, and up to 50ng for MXW231, were used for linear regression analysis; R-squared values were 0.999 and 0.997 respectively.

Table 4.1**A**

	<u>MXW231/WEHI231</u>		
	<u>Gel 1</u>	<u>Gel 2</u>	<u>Gel 3</u>
LSC	7.6	12.3	ND
densitometry	7.5	10.2	8.1

B

	<u>MXW231/WEHI231</u>		
	<u>Gel 1</u>	<u>Gel 2</u>	<u>Gel 3</u>
LSC	9.1	14.8	ND
densitometry	9.0	12.2	9.7

Table 4.1: Ratio of μ mRNA levels between WEHI231 and MXW231 cells**(A): Quantitation of μ NP signal.**

The slopes of the linear regions of the graphs shown in Figs. 4.4 (gels 1 and 2) and 4.5 (gel 3) were determined by linear regression analysis, and the slope ratio MXW231/WEHI231 for each gel is shown here. ND indicates not done.

(B): Correction of μ NP ratios by rRNA ratio.

The slopes of the linear regions of the graphs shown in Fig. 4.7 were determined by linear regression analysis, and the slope ratio WEHI231/MXW231 was found to be 1.2 in each case. This figure was used to correct the values shown in Table 4.1A.

4.2.3: Optimisation of ribonuclease protection assay.

To analyse labelled RNA obtained from pulse-chase experiments on the two cell lines, the ribonuclease protection assay described in Chapter 3 was used with the μ NP and rRNA probes shown in Fig. 4.1. The μ NP probe is predicted to protect 1.1kb of μ mRNA derived from the WEHI231 μ gene, and the rRNA probe should protect 358 nucleotides of 28S rRNA. The sizes of the protected species were confirmed by hybridising ^{32}P -labelled μ NP and rRNA probes generated with SP6 polymerase to unlabelled total RNA from the two cell lines. Fig. 4.8 shows that the major band protected by the rRNA probe migrates at a position consistent with the expected size of 358 nucleotides (lanes 1-6, middle arrow). In addition, a higher molecular weight band is present (lanes 1-6, top arrow), which migrates in the same position as full

FIGURE 4.8

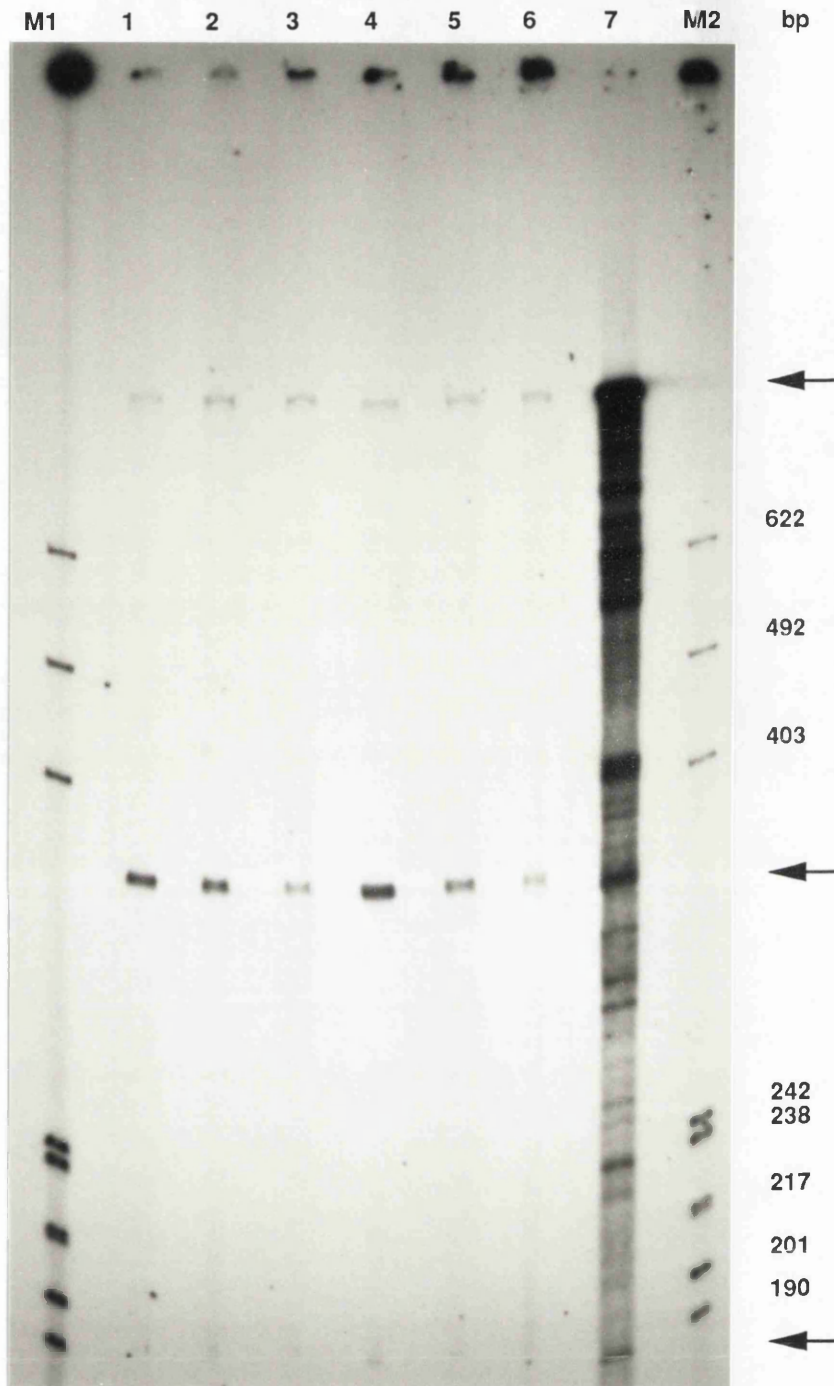


Figure 4.8: Ribonuclease protection using the rRNA probe.

A ribonuclease protection experiment was carried out in which total RNA from MXW231 and WEHI231 cells was hybridised to a labelled probe generated from pSP65rRNA. 10µg/ml ribonuclease A and 0.5µg/ml ribonuclease T1 were used, at 30°C for 30 minutes. A range of amounts of total RNA were used, as follows; 40ng (lanes 1 and 4); 20ng (lanes 2 and 5); 10ng (lanes 3 and 6). Lane 7 contains a sample of undigested probe and lanes M1 and M2 contain different loadings of molecular weight markers generated by *Hpa* II digestion of pAT153 and end-labelling of the resulting fragments with T4 polynucleotide kinase. Arrows mark the bands described in the text.

length probe (lane 7, top arrow). This could arise from incomplete digestion of the probe molecules (cf. Fig. 3.5), and/or from protection of the probe by an rRNA precursor molecule. It is likely in this case to be mainly due to undigested probe, because precursor molecules would be expected to be present at a low level in total RNA, and also the intensity of the band does not vary with the amount of RNA hybridised. Interestingly, a similar band was also observed in hybridisations containing unlabelled probe and labelled RNA (see Section 4.2.4 below), which is thought to represent a precursor molecule. A weak low molecular weight band of around 200 nucleotides is also present (lanes 2-7, bottom arrow), which could be caused by secondary structure effects, or by hybridisation to shorter probe molecules arising from early termination of the SP6 polymerase. It is also seen in hybridisations using labelled total RNA preparations (see Section 4.2.4) and its origin was not investigated further. Analogous bands were observed in hybridisations using the hGH probe (Section 3.2.1).

The μ NP probe also protects a molecule which migrates in the expected position (Fig. 4.9, lanes 2-7, arrow). However, in these hybridisations much more smearing is evident, as are faint lower molecular weight bands at higher loadings. This could be due to heterogeneity in the probe, but the rRNA probe is heterogeneous to a similar degree and yet gives a much cleaner pattern. A more likely explanation is that the smearing is caused by nicking of the hybrid duplex molecules by the ribonuclease. This could be caused by the fact that the μ NP probe is not a perfect match for the WEHI231 μ mRNA, as they are of different allotypes; WEHI231 cells are derived from a NZB x BALB/c mouse (Gutman *et al*, 1981), and the μ NP probe is of C57bl/6 origin (Bothwell *et al*, 1981; Wood *et al*, 1984). In addition, there may be further mismatches in the J region. Nicking by the ribonuclease at these base pair mismatches could therefore produce a variety of smaller molecules which could account for the smearing effect. This creates a potential problem as it will lead to a reduction in intensity of the correct size protected band and thus may make quantitation difficult. To try and minimise the degree of nicking whilst still removing unhybridised molecules, an experiment was carried out to titrate the ribonuclease

FIGURE 4.9

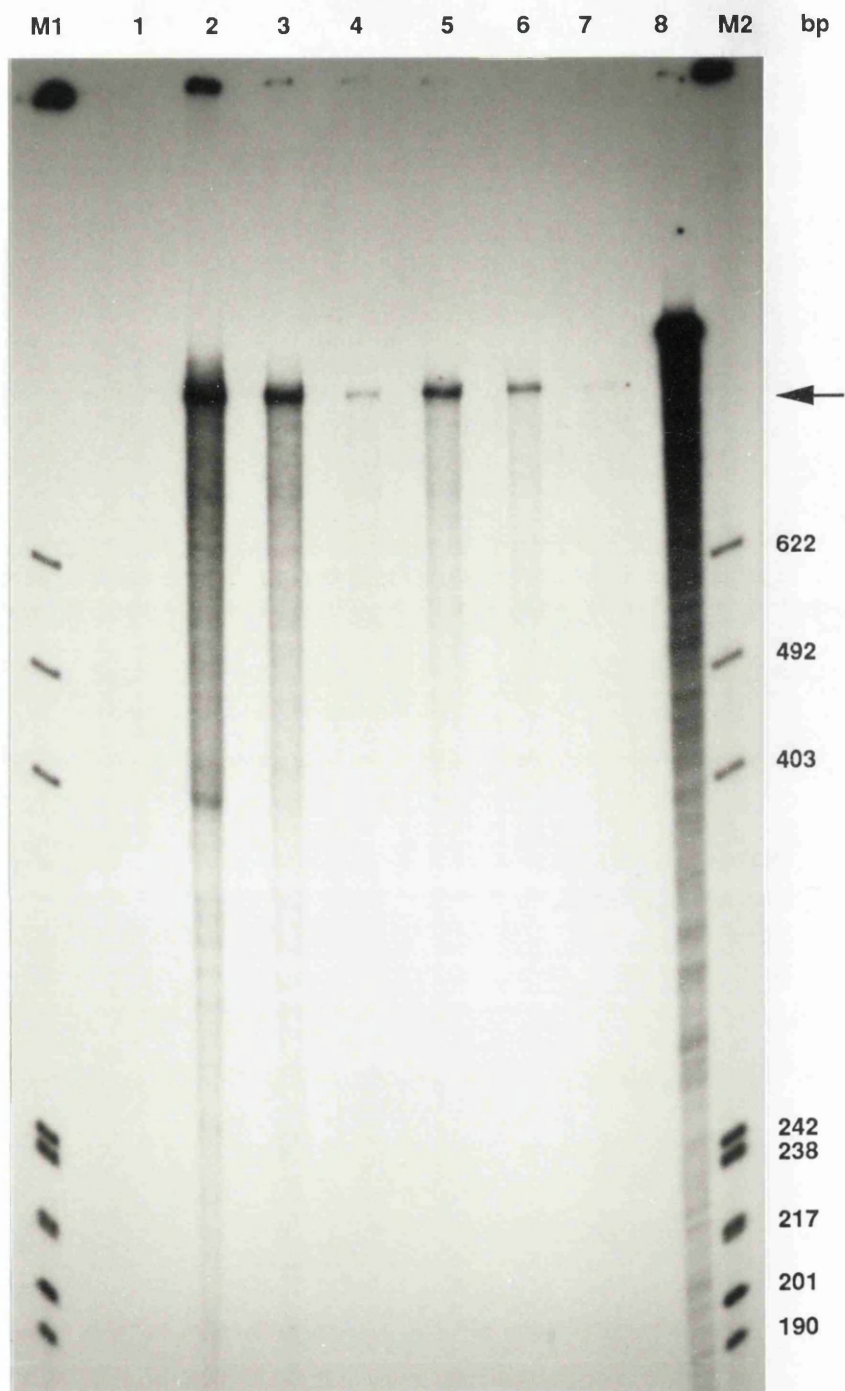


Figure 4.9: Ribonuclease protection using the μ NP probe.

A ribonuclease protection experiment was carried out in which total RNA from MXW231 and WEHI231 cells was hybridised to a labelled probe generated from pSP64 μ NP. 10 μ g/ml ribonuclease A and 0.5 μ g/ml ribonuclease T1 were used, at 18°C for 30 minutes. A range of amounts of total RNA were used, as follows; 100 μ g (lanes 2 and 5); 50 μ g (lanes 3 and 6); 10 μ g (lanes 4 and 7). Lane 1 contains a hybridisation to total RNA from CBMG cells and lane 8 contains a sample of undigested probe. Lanes M1 and M2 contain molecular weight markers generated as described in the legend to Figure 4.8.

digestion conditions.

MXW231 cells were labelled for 2 hours with ^3H uridine, and labelled RNA prepared as described in Section 2.3. A series of hybridisations containing a constant amount (100 μg) of labelled MXW231 cell RNA and unlabelled μNP probe were set up, and these were subjected to various conditions of ribonuclease concentration and digestion temperature. In Fig. 4.10 it can be seen that a band is detected in ^3H -labelled RNA (lanes 3-8) which co-migrates with the protected species produced by the ^{32}P -probe (lanes 9 and 10). This band is reduced in intensity relative to the background smear as the ribonuclease concentration and digestion temperature are increased (lanes 3-8). The band is not present in control hybridisations which do not contain probe (lanes 1 and 2), showing that it is produced by specific hybridisation with the probe. There are several bands and a degree of smearing which are independent of the presence of probe and are therefore presumably due to incomplete digestion of the labelled cellular RNA. These results show that it is not possible to completely remove the background and retain a reasonable signal from the protected species, because of the nicking effect described above. An experiment was therefore carried out to determine whether it was possible to obtain a quantitative signal response from the hybridisation, despite the background, using ribonuclease digestion conditions which gave maximum intensity of the 1.1kb band; 10 $\mu\text{g}/\text{ml}$ ribonuclease A and 0.5 $\mu\text{g}/\text{ml}$ ribonuclease T1 at 18°C for 30 minutes (Fig.4.10 lane 3).

Increasing amounts of labelled MXW231 RNA were hybridised to a constant amount of μNP probe, and the total amount of RNA in the hybridisation mix was made up to 200 μg with unlabelled RNA from J558L cells, so that the amount of RNA in each tube was equal. J558L cells do not produce any μ chain (Fig. 4.3; Oi *et al*, 1983). Similar hybridisations were also carried out without any probe in the hybridisation mix. After hybridisation and ribonuclease digestion, the protected 1.1 kb band was cut out from the gel, treated with solubiliser and subjected to liquid scintillation counting, as described for the hGH experiments in Chapter 3. The results shown in Fig. 4.11A indicate that a reasonable linear

FIGURE 4.10

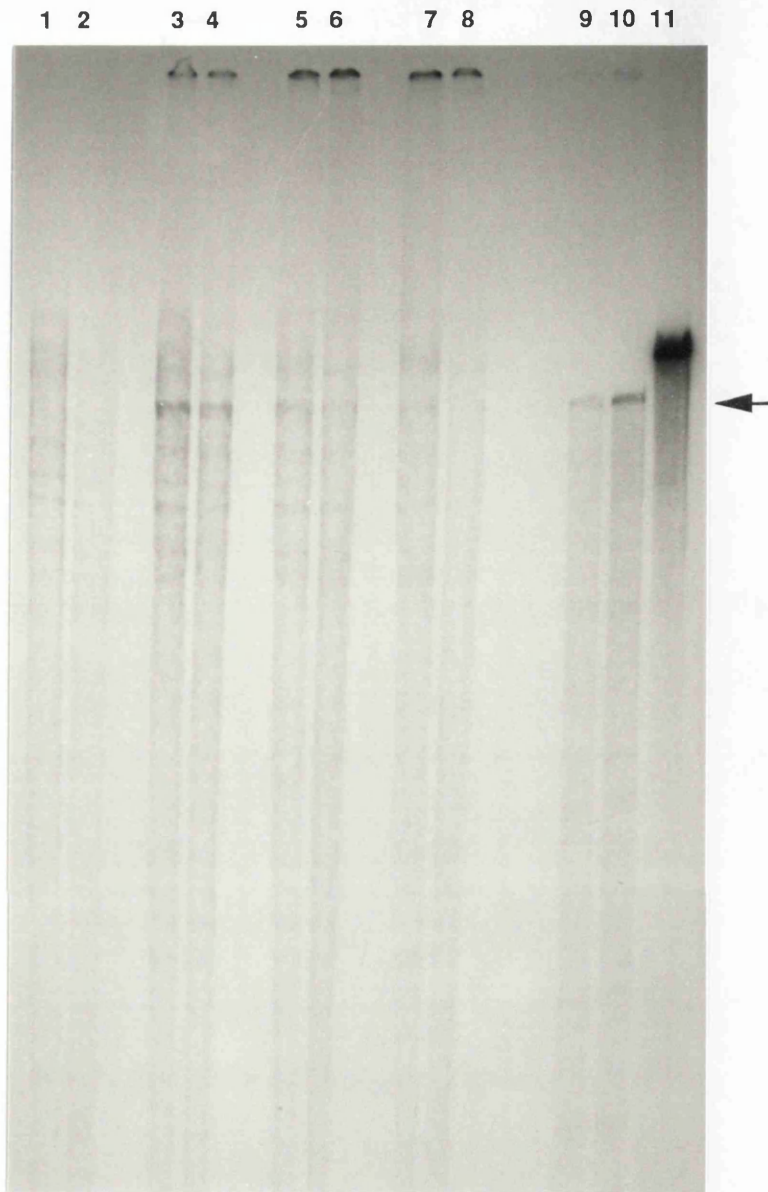
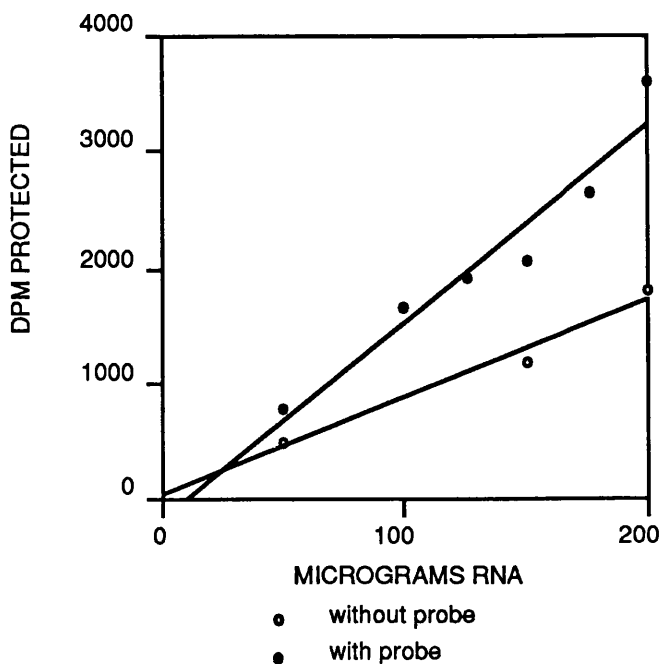


Figure 4.10: Ribonuclease protection experiment to titrate the ribonuclease digestion conditions.

A ribonuclease protection experiment was carried out in which 100 μg tritiated RNA from MXW231 cells was hybridised to unlabelled probe generated from pSP64 μNP , and a range of digestion conditions were employed. Lanes 1 and 2 show the results of hybridisations in the absence of probe. Digestion conditions were as follows; 20 $\mu\text{g}/\text{ml}$ ribonuclease A, 1 $\mu\text{g}/\text{ml}$ ribonuclease T1, 18 $^{\circ}\text{C}$ (lane 1); 20 $\mu\text{g}/\text{ml}$ A, 1 $\mu\text{g}/\text{ml}$ T1, 30 $^{\circ}\text{C}$ (lane 2); 10 $\mu\text{g}/\text{ml}$ A, 0.5 $\mu\text{g}/\text{ml}$ T1, 18 $^{\circ}\text{C}$ (lane 3); 10 $\mu\text{g}/\text{ml}$ A, 0.5 $\mu\text{g}/\text{ml}$ T1, 30 $^{\circ}\text{C}$ (lane 4); 20 $\mu\text{g}/\text{ml}$ A, 1 $\mu\text{g}/\text{ml}$ T1, 18 $^{\circ}\text{C}$ (lane 5); 20 $\mu\text{g}/\text{ml}$ A, 1 $\mu\text{g}/\text{ml}$ T1, 30 $^{\circ}\text{C}$ (lane 6); 40 $\mu\text{g}/\text{ml}$ A, 2 $\mu\text{g}/\text{ml}$ T1, 18 $^{\circ}\text{C}$ (lane 7); 40 $\mu\text{g}/\text{ml}$ A, 2 $\mu\text{g}/\text{ml}$ T1, 30 $^{\circ}\text{C}$ (lane 8); 10 $\mu\text{g}/\text{ml}$ A, 0.5 $\mu\text{g}/\text{ml}$ T1, 18 $^{\circ}\text{C}$ (lane 9); 10 $\mu\text{g}/\text{ml}$ A, 0.5 $\mu\text{g}/\text{ml}$ T1, 30 $^{\circ}\text{C}$ (lane 10). The hybridisations in lanes 9 and 10 were carried out with labelled probe as markers for the position of the protected band. Lane 11 contains a sample of undigested labelled probe. Molecular weight markers were loaded on this gel but are not visible in this exposure.

FIGURE 4.11

A



B

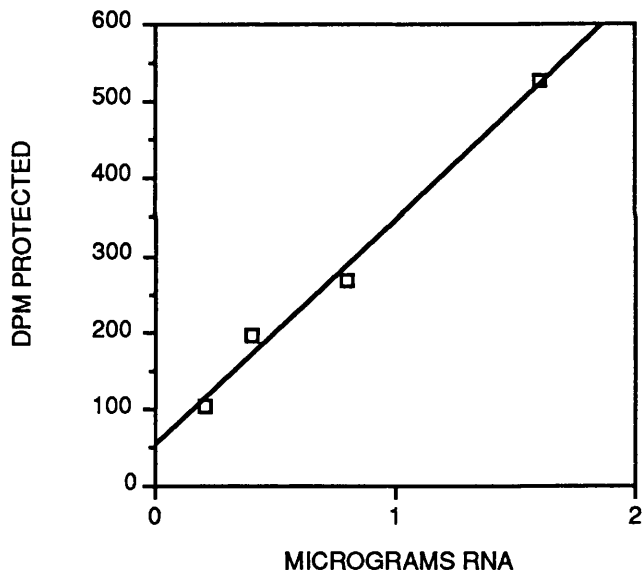


Figure 4.11: Relationship between hybridisation signal and amount of RNA hybridised.

(A) A ribonuclease protection experiment was carried out using the pSP64 μ NP probe with increasing amounts of tritiated total RNA from MXW231 cells. The 1.1kb μ -specific band was cut out from the gel, the radioactivity quantitated as described in Section 2.3.9, and plotted against RNA input (filled circles). Similar hybridisations were carried out in the absence of probe, and identical gel fragments were cut out at the 1.1kb position to determine the background (open circles). R-squared values for the lines of best fit were 0.932 (+probe) and 0.975 (-probe).

(B) Ribonuclease protection was carried out with the pSP65 rRNA probe using increasing amounts of tritiated total RNA from MXW231 cells. The 358 nucleotide 28S rRNA-specific band was quantitated as in (A) and plotted against RNA input. The R-squared value for the line of best fit was 0.990.

response is obtained over this range of hybridisable RNA (filled circles), and repeat experiments gave similar results. Identical gel slices were cut out at the 1.1kb position from the hybridisations without probe, and the background signal at this position is indicated by the open circles in Fig.4.11A. The slopes of the two lines (17.131 and 8.464 respectively) indicate that approximately half of the dpm obtained in the presence of probe are due to the background. This effect was minimised in subsequent experiments by taking as narrow a slice as possible when cutting out the bands from the gels, but the background smear did not seem to be a problem in pulse-chase experiments as its rate of decay paralleled that of the main band (see Section 4.2.4 below and 4.3 discussion).

Similar experiments were carried out using ^3H labelled RNA from MXW231 cells with the rRNA probe, and the results of one experiment are shown in Fig. 4.11B. In this case much lower amounts of total RNA are required, due to the high abundance of ribosomal RNA, but the signal is also linear over this range. No signal was obtained in the absence of probe in this case.

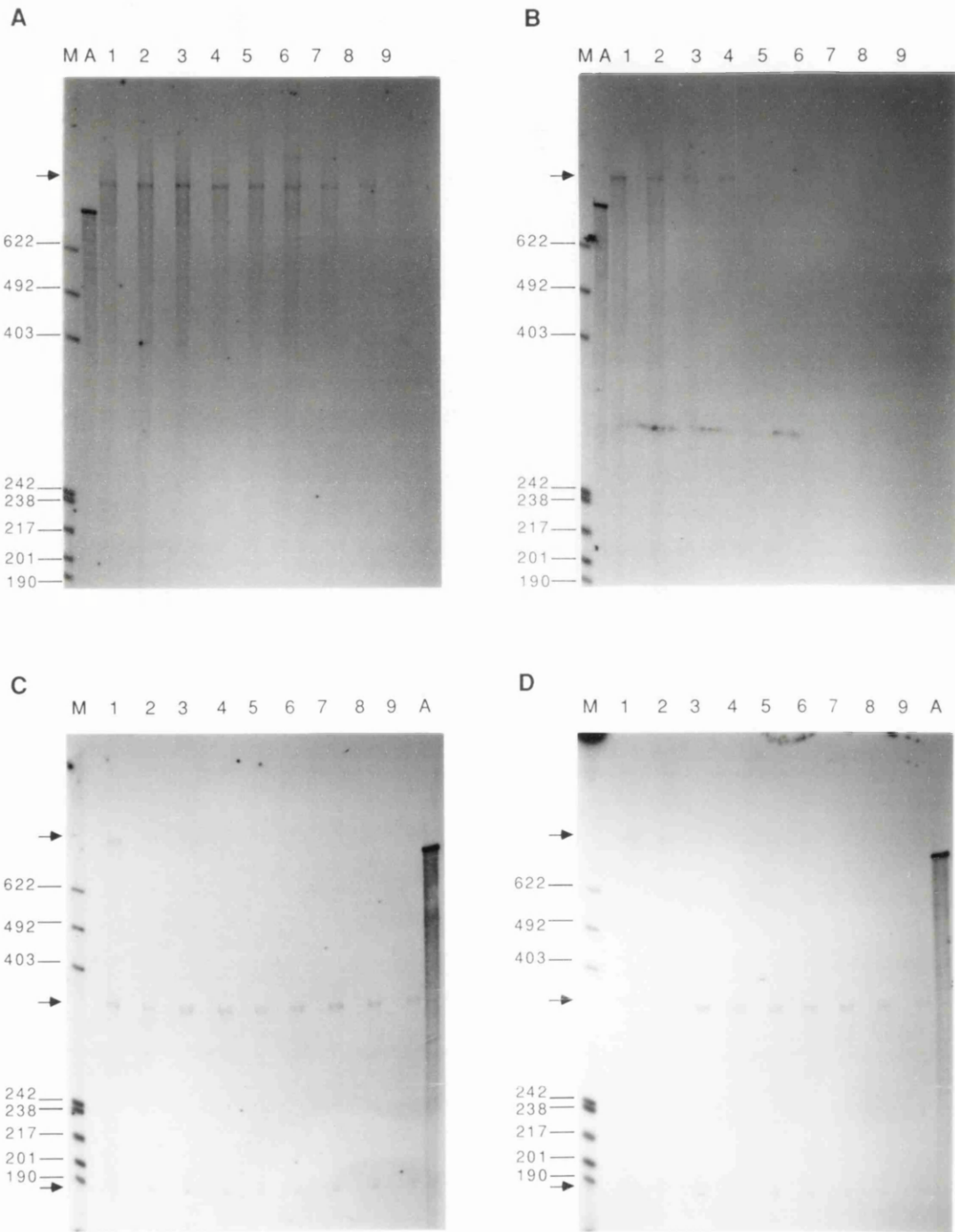
4.2.4: Pulse-chase analysis on WEHI231 and MXW231 cells

Pulse chase analysis was carried out on the two cell lines using cells in exponential growth, using the protocol described in Chapter 3, and the ribonuclease digestion conditions determined above. The results of one such experiment are shown in Fig. 4.12, where (A) and (B) panels show the results with the μNP probe, and (C) and (D) show the results with the rRNA probe. The 1.1kb band from the μNP probe decays faster in WEHI231 cells (Fig. 4.12B; arrow) than in MXW231 cells (Fig.4.12A; arrow). The 358 nucleotide rRNA band on the other hand decays at a similar rate in both cell types (Fig. 4.12C and D; middle arrow). As the half-life of rRNA in dividing cells is of the order of 100 hours (Emerson, 1971; Weber, 1972; Singer and Penman, 1973; Perry and Kelley, 1973; Abelson *et al*, 1974; Lowenhaupt and Lingrel, 1978; Guyette *et al*, 1979; Volloch and Housman, 1981;), little degradation would be expected to be observed over this time course, as is indeed the case. The small reduction in intensity of the band which is observed is presumably mainly

Figure 4.12: Pulse-chase analysis on WEHI231 and MXW231 cells.

A pulse-chase experiment was carried out as described in the text, and 150µg total RNA isolated at various time points during the chase was used in ribonuclease protection assays with unlabelled probe generated from pSP64µNP and pSP65 rRNA. (A), MXW231 RNA/ µNP probe; (B), WEHI231 RNA/ µNP probe; (C), MXW231 RNA/ rRNA probe; (D), WEHI231/ rRNA probe. Digestion conditions for the µNP probe were 10µg/ml ribonuclease A, 0.5µg/ml ribonuclease T1, at 18°C for 30 minutes (A and B), and for the rRNA probe, the same concentrations of ribonuclease at 30°C for 30 minutes (C and D). Lanes 1-9 correspond to 0, 2, 4.25, 10.5, 15.5, 20, 28.5, 38.25 and 44 hour time points respectively. Arrows indicate the positions of bands described in the text. Lane M contains molecular weight markers generated as described in the legend to Fig. 4.8. Lane A contains a sample of the 834 nucleotide tritiated hGH probe generated from pRHGH1 (described in Chapter 3) as a control for the fluorography procedure.

FIGURE 4.12



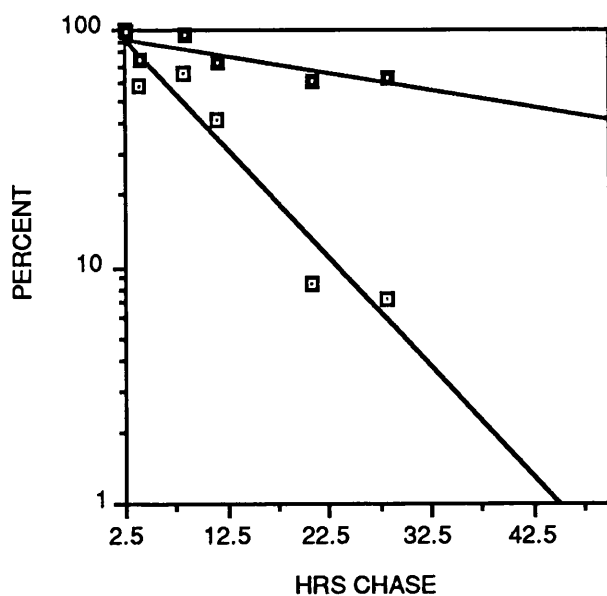
due to the dilution effect of cell division.

The 1.1kb μ NP band and the 358 nucleotide rRNA bands were cut out from the gels and quantified by liquid scintillation counting as before. The number of dpm in the μ NP bands were normalised to the number in the rRNA bands at each time point during the chase, to correct for cell division and any error in the measurement of the optical density of the RNA preparations. This normalisation assumes that rRNA has an infinite half-life, which is a reasonable approximation for the length of the time course of this experiment (see above references). The results of plotting the normalised μ NP signal against time for two pulse chase experiments are shown in Fig. 4.13. These graphs confirm that the μ mRNA in WEHI231 cells decays more rapidly than the μ mRNA in MXW231 cells. Linear regression analysis of the \log_e dpm values was used to calculate the slope of the line of best fit in each case, and the half-life was calculated using the relationship $T_{1/2} = \log_e 2 / \text{slope}$ (see Appendix). Table 4.2 shows the half-lives obtained in the two experiments, with the 95% confidence intervals given in brackets. The mean half-life of μ mRNA was found to be 5.5 hours in WEHI231 cells and 34.2 hours in MXW231 cells, indicating approximately a six-fold longer half-life in the more differentiated cell type. This difference in the rates of decay would make a significant contribution to the difference in steady state μ mRNA levels (see discussion, Section 4.3).

The high molecular weight rRNA band of 944 nucleotides mentioned in Section 4.2.3 is also observed in the pulse chase hybridisations (Fig. 4.12, C and D; top arrow), and it clearly decays rapidly. Fig. 4.14 shows the results of quantitation of this band for the experiment shown in Fig. 4.12. The graphs indicate that no significant label is detectable in this molecule in either cell type after 10 hours of chase. The half-life estimated from the time points up to 10 hours is 2.6 hours for both WEHI231 and MXW231 cells, but this is likely to be an over-estimation, as the specific activity of the UTP pool was found to be high at these early time points (see Section 4.2.5 below). The short half-life is consistent with the hypothesis that this band represents an rRNA precursor molecule. The 200 nucleotide band also described in Section 4.2.3. is faintly visible in

FIGURE 4.13

A



B

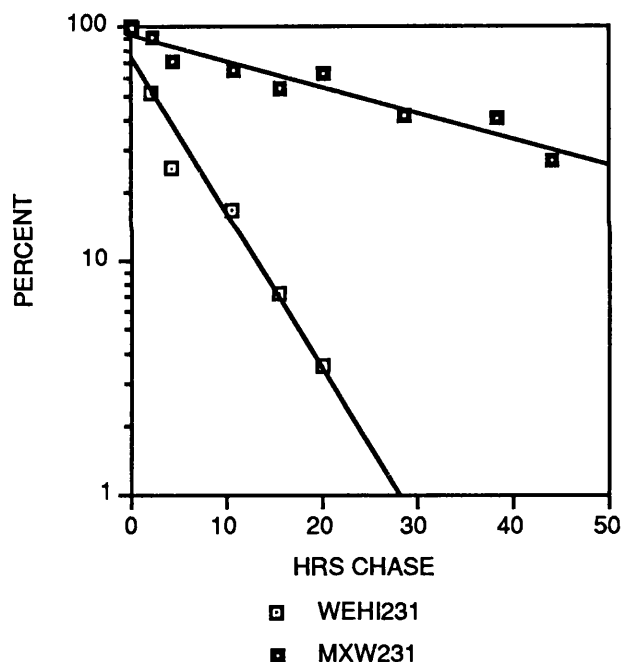


Figure 4.13: Quantitation of signal from two pulse-chase experiments

The 1.1kb μ -specific bands and 358 nucleotide rRNA-specific bands from two experiments such as the one shown in Fig. 4.12 were quantitated as described in Section 2.3.9. The μ -specific signal was normalised to the rRNA signal at each time point during the chase, expressed as a percentage of the initial time point and plotted on a log scale against time of chase. (B) represents the data from the experiment shown in Fig. 4.12. Initial dpm protected by the μ probe (after subtraction of background) were (A) WEHI231, 2051; MXW231, 1521 and (B) WEHI231, 3090; MXW231, 2591. Background dpm were (A) 144,163, (B) 229,205. R-squared for the lines of best fit were (A) WEHI231, 0.9306; MXW231, 0.6596; (B) WEHI231, 0.8919; MXW231, 0.9266.

FIGURE 4.14

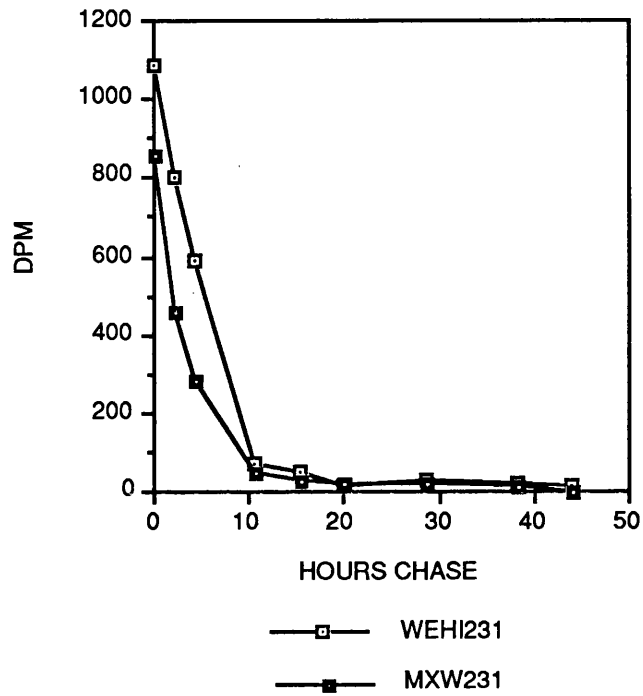


Figure 4.14: Decay of a putative rRNA precursor

The 944 nucleotide band was cut out from the gels illustrated in Fig. 4.12C and D, and the radioactivity quantitated as described in Section 2.3.9. The dpm were then plotted against time during the chase. Time points up to 10 hours were used to determine the half-life of this molecule by plotting the log dpm values against time and using linear regression analysis to determine the line of best fit (see text).

Table 4.2**HALF-LIFE (HRS)**

<u>Experiment</u>	<u>WEHI231</u>	<u>MXW231</u>	<u>RATIO</u>
1	6.5(4.7-10.5)	40.7(20.4- ∞)	6.2
2	4.6(3.6-6.3)	27.7(22.2-37.0)	6.1
mean	5.5	34.2	6.2

Table 4.2: The half-life of μ mRNA in MXW231 and WEHI231 cells

The half-life values for the experiments described in this Chapter are shown. The graphs for experiments 1 and 2 are shown in Fig. 4.13A and 4.13B respectively. The half-lives were calculated using linear regression analysis of the \log_e values of the ratios of dpm(μ NP)/dpm(rRNA) plotted against time to determine the slope of the regression line in each case. The significance values of the t statistic for experiment 1 were WEHI231, <1%; MXW231, <5%, and for experiment 2 both regression lines were significant to at least 0.1%. The half-lives were calculated from the relationship $t_{1/2} = \log_e 2 / \text{slope}$. The 95% confidence intervals, which are indicated in brackets, were calculated using the equation $\log_e(95\%CI) = \text{slope} \pm t_s$, where t is the value of the t statistic at n-2 degrees of freedom for 95% confidence, and s is the standard error of the slope.

these experiments (Fig.4.12, C and D bottom arrow) and it has a rate of decay similar to the main rRNA band.

4.2.5: Decay of UTP pool

As discussed previously, the large size of the ribonucleotide pool in mammalian cells can introduce errors into the measurement of half-life due to the poor chase conditions (Warner *et al*, 1966; Schimke, 1970; Scholtissek, 1971; Cowan and Milstein, 1974; Levis and Penman, 1977; Guyette *et al*, 1979). In the experiments to measure the rate of decay of hGH mRNA described in Chapter 3, continued incorporation of labelled nucleotide into hGH mRNA was clearly observed during the first four hours of the chase. To obtain an estimate of the lag time before the chase becomes effective, and to see whether there was any differential incorporation of label between the two cell types, the specific activity of the nucleotide pool was measured during the chase. Following the method of Brock and Shapiro (1983b), the amount of UTP in cell lysates was

quantitated by h.p.l.c. on an anion exchange column, measuring the absorption of the eluted material at 262nm. Details of the experimental procedure are described in Section 2.5.8. Initial experiments were carried out to determine the retention times and absorption spectra of nucleoside triphosphate standards in this system, and to verify that the amount of UTP eluted could be quantitated by this method.

In the preliminary experiments, the four nucleoside triphosphates and uridine diphosphate were applied to the column. The equipment used allowed the absorption spectra for specific peaks to be measured, as well as retention time on the column, and it was found that each of the four nucleoside triphosphates could be uniquely identified by its retention time and absorption spectrum, and uridine diphosphate could be distinguished from uridine triphosphate by retention time. The elution profile and spectrum for UTP are shown in Fig. 4.15. To determine whether a UTP peak could be identified in cell lysates, cell lysate from MXW231 cells was prepared as described in Materials and Methods and applied to the column. A peak was observed with retention time very close to that of the UTP standard, and when the absorption spectrum was obtained, it was found to be identical to that of UTP (Fig.4.16). A range of amounts of UTP was then loaded onto the column, to confirm that the area under the UTP absorption peak was proportional to the amount of UTP loaded. Fig. 4.17 shows that this was indeed the case, and therefore this method could be used to quantitate the amount of UTP in each cell lysate from pulse chase experiments.

A pulse-chase experiment was carried out and cell lysates prepared at suitable time points during the chase. These were applied to the h.p.l.c. column and three fractions were collected from each elution, at the following times: 20.0-20.49, 20.5-21.4, and 21.41-21.9 minutes. An aliquot of the second fraction was re-run on the column in a test experiment which confirmed that this fraction contained the UTP peak. This fraction from each sample was therefore added to liquid scintillation fluid for determination of the amount of radioactivity it contained, and the ratios dpm/area under absorption peak were calculated for each time point during

Figure 4.15: The h.p.l.c. elution profile and absorption spectrum of UTP.

A known amount of UTP was applied to the h.p.l.c. column and eluted as described in the text. The absorbance at 262nm was monitored during the elution. (A) shows the absorption spectrum of the UTP peak which was found to have a retention time of 20.829; the elution profile is shown in (B).

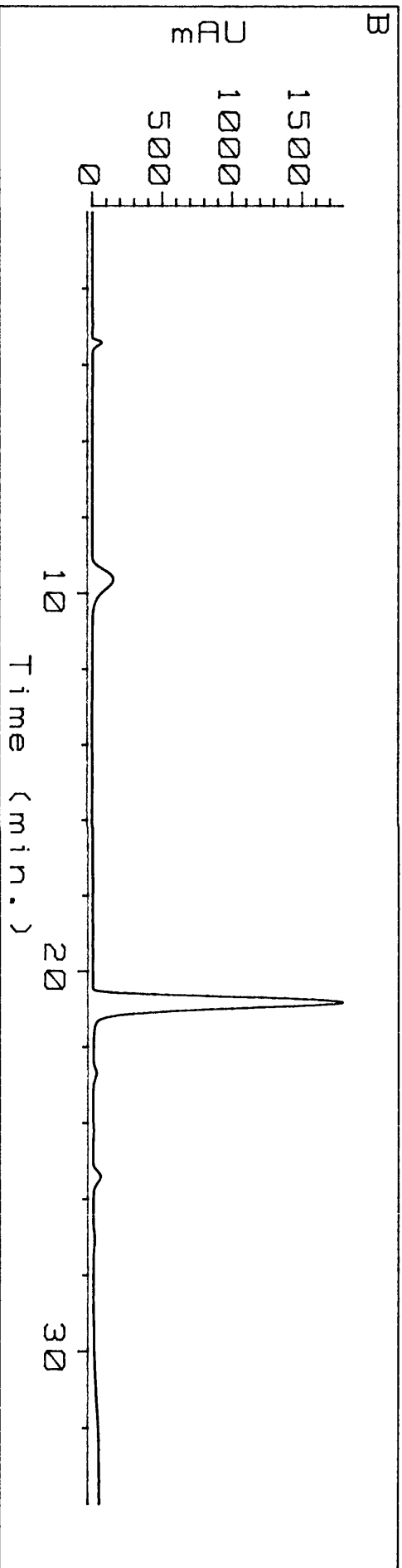
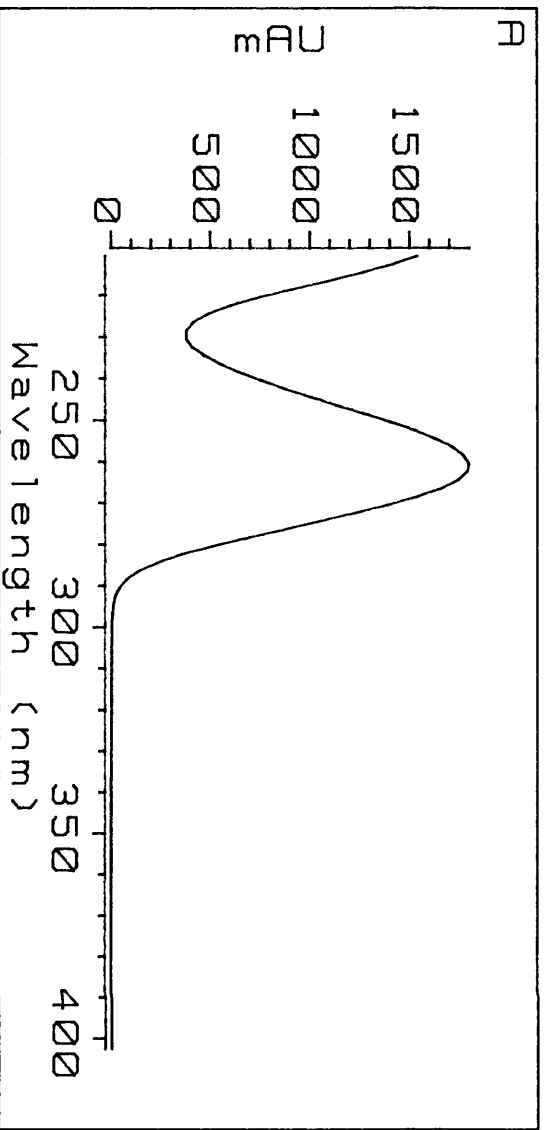


Figure 4.16: The h.p.l.c. elution profile of MXW231 cell lysate.

A sample of cell lysate prepared as described in the text was applied to the h.p.l.c. column, and the elution profile is shown in (B). The peak at 20.935 was judged to be the UTP peak by its retention time, compared to standards (eg Fig. 4.15), and its absorption spectrum, shown in (A). The other peaks in (B) represent the other nucleoside triphosphates, which were also identified by comparison to standards.

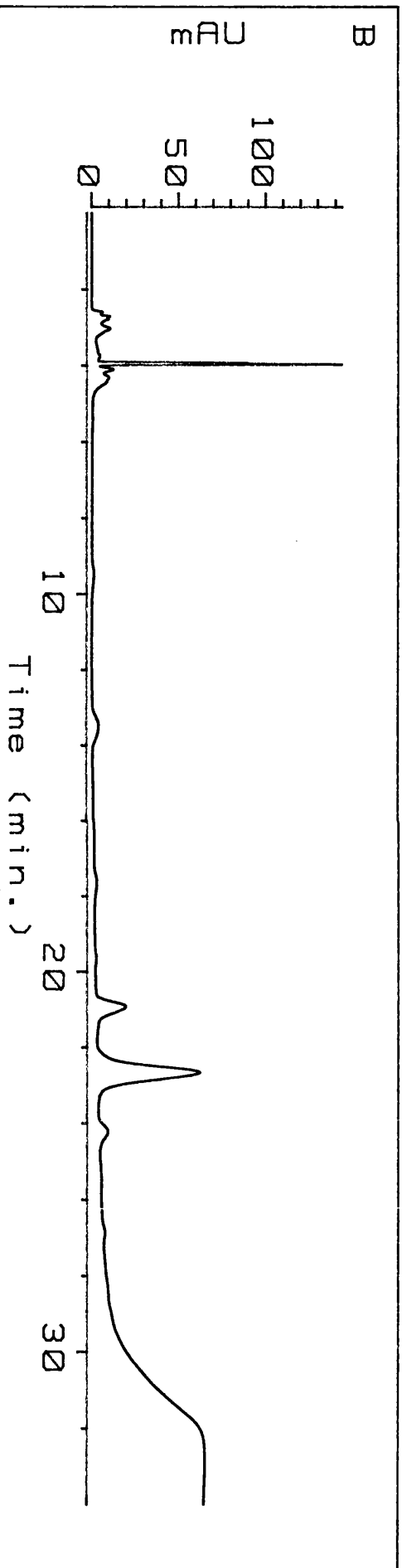
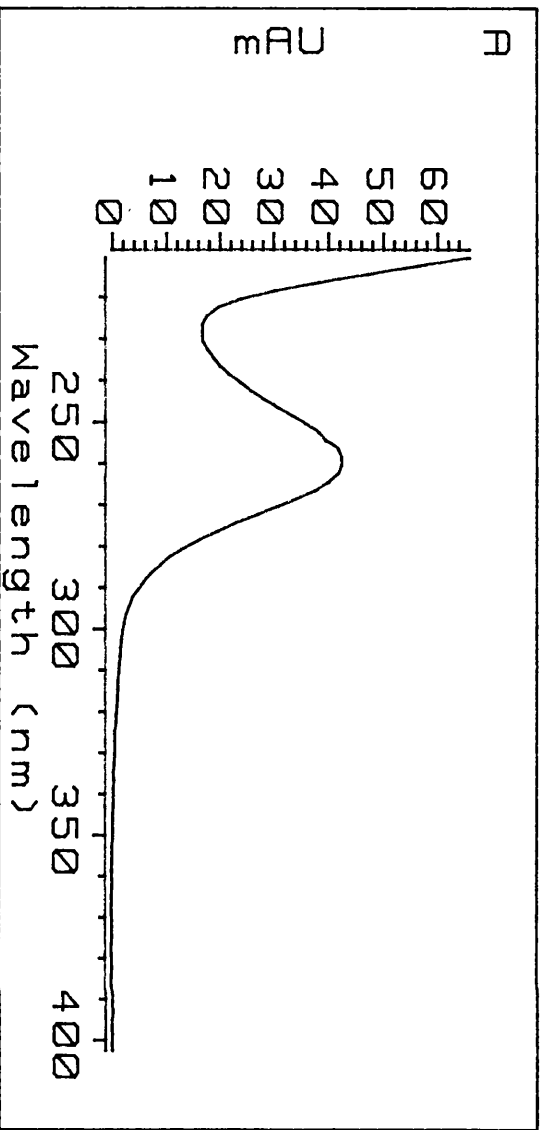


FIGURE 4.17

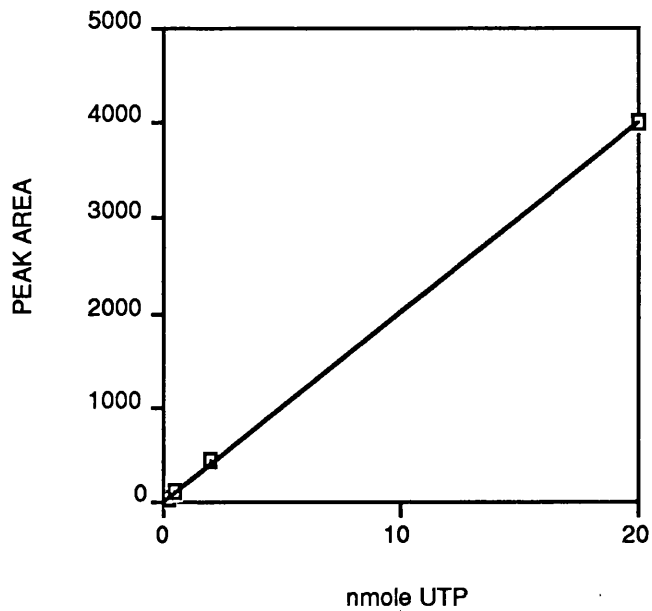


Figure 4.17: Calibration of UTP measurements

The UTP quantitation method was calibrated by applying UTP standards to the h.p.l.c. column and measuring the peak area of the eluted UTP peak. This was plotted against amount of UTP applied to the column. R-squared for the line of best fit=1.000.

the chase. When these values were expressed as percentages of the maximum value, the graphs shown in Fig. 4.18 were obtained for the two cell lines. They indicate that the decay of the UTP pool did not differ significantly between the two cell types and that therefore there is no significant difference in the chase conditions which might account for the difference in the rate of decay of μ mRNA. However, the specific activity of the UTP pool remains high, as expected, during the early part of the chase. The question of the effect of this on the half-lives is addressed in the discussion (Section 4.3).

4.2.6: Decay of total polyadenylated RNA

The observed difference in μ mRNA half-lives between WEHI231 and MXW231 cells could be accounted for by a general increase in the half-life of total messenger RNA in the MXW231 cells. To investigate this possibility, the decay of total polyadenylated RNA was analysed in a pulse chase experiment, by measuring its specific activity at various time points during the chase. Polyadenylated RNA was selected from total RNA by two rounds of oligo(dT) cellulose chromatography, as described in Section 2.3.3. The material which bound oligo(dT) cellulose was first analysed by Northern blotting to confirm that samples contained equivalent amounts of mRNA.

The concentration of the oligo(dT) purified material was measured by optical density determination at 260nm, and equal amounts (50ng) were loaded onto each track of a formaldehyde/agarose gel for Northern blotting. The results of one experiment after probing the Northern blot with the μ NP probe are shown in Fig. 4.19. The intensity of the signal is very similar in all tracks, with exceptions being lanes 5 and 8, where the RNA preparation is slightly degraded. This indicates that similar levels of mRNA are present in each sample. To estimate the variation in levels between the various time points, densitometric scanning of a series of timed exposures of the autoradiographs was carried out, and the slopes of the linear portions of the graphs of peak area against exposure time were obtained for each track. The average value for the MXW231 pulse chase was 1.51 and for WEHI231 0.17, with standard deviations of 0.23 and 0.03

FIGURE 4.18

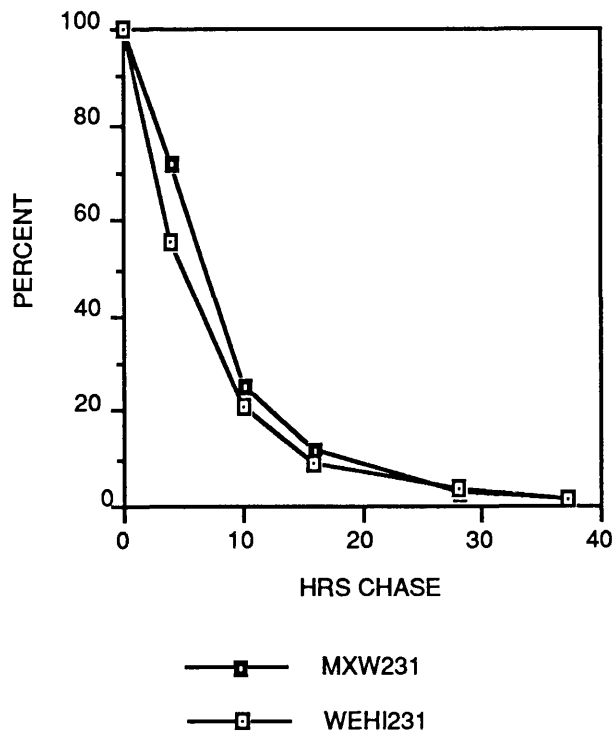


Figure 4.18: Decay of UTP pool

The specific activity of the UTP pool was measured at each time point during the chase by determination of the dpm in each eluted UTP peak and dividing by the area of the peak. This figure was expressed as a percentage of the maximum and plotted against time during the chase.

FIGURE 4.19

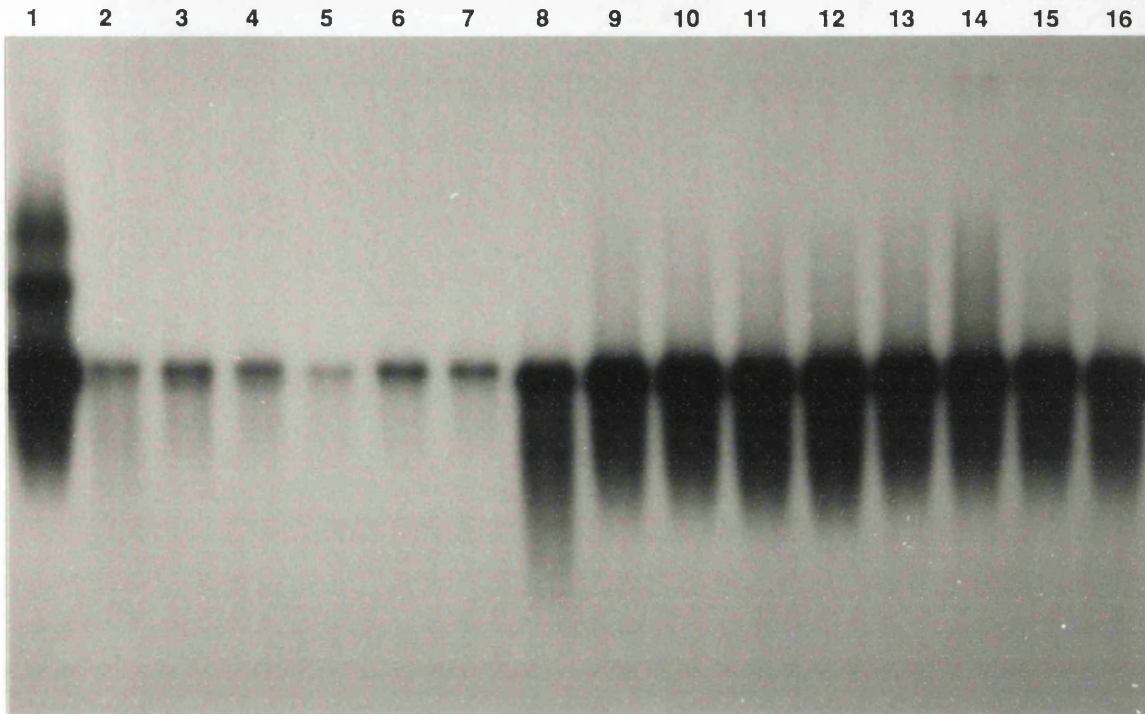


Figure 4.19: Northern blot analysis of polyadenylated RNA from a pulse-chase experiment.

A pulse-chase experiment was carried out on MXW231 and WEHI231 cells, and total polyadenylated RNA was isolated at various time points during the chase. 50ng of each RNA was loaded onto a formaldehyde/agarose gel and, after electrophoresis, transferred to "Hybond-N". The filter was probed with the pSP64 μ NP probe. 0.5 μ g total RNA from MXW231 cells was loaded as a positive control (lane 1). Lanes 2-7 correspond to 0, 4, 15, 27 and 43 hour time points, respectively, for WEHI231 RNA. Lanes 8-16 correspond to 0, 2, 4, 9, 15, 20, 27, 33 and 43 hour time points respectively for MXW231 RNA.

respectively. Some of this variability will inevitably arise from variations in gel loading and error in optical density measurement. Therefore it was concluded that a reasonably consistent level of recovery had been achieved from the oligo(dT) cellulose columns.

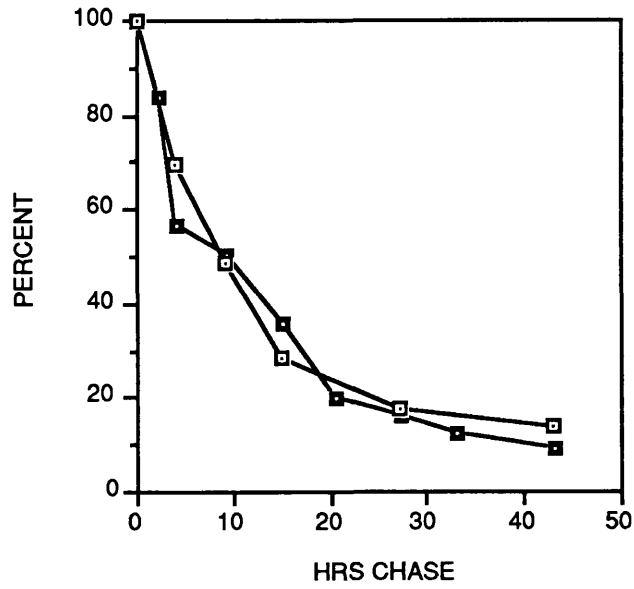
The above result indicates that there is unlikely to be significant contamination of the samples with ribosomal RNA, and to confirm this, the Northern blot was stripped and reprobed with the rRNA probe. No rRNA was detected, although it was not possible to determine the sensitivity of the experiment as there were not sufficient dilutions of the control total RNA on the gel. Having shown that the poly(+) RNA was relatively pure, the specific activities of the poly(A)+ RNA in dpm/ μ g were determined, and plotted as a function of time during the chase. Fig. 4.20 shows the results of this procedure for two duplicate experiments. In previous pulse chase experiments, little difference had been observed in the decay of rRNA between the two cell lines, and the chase conditions had been shown to be very similar (see above Section 4.2.4). If the specific activity of poly(A)+ RNA was normalised to the rRNA signal during the chase, as had been done for the μ mRNA signal, very similar graphs to those shown in Fig. 4.20 were obtained (not shown). Therefore the decay of poly(A)+ RNA is very similar in the two cell lines, indicating that there is no general increase in stability of mRNA when B cells differentiate. If anything, in Fig. 4.20B the decay of poly(A)+ RNA is slower in WEHI231 than in MXW231 cells. The pattern of decay of poly(A)+ RNA is complex, indicating several RNA populations; this is consistent with what has been observed in other cell types (Singer and Penman, 1973; Lowenhaupt and Lingrel, 1978; Guyette *et al*, 1979).

4.3: Discussion

This Chapter describes the characterisation of the WEHI231 and MXW231 cell lines with respect to their μ mRNA content, and the comparison of μ mRNA half-life between the cell lines using the pulse chase and ribonuclease protection methods described in Chapter 3. Northern blot analysis of total RNA from the two cell lines revealed approximately an

FIGURE 4.20

A



B

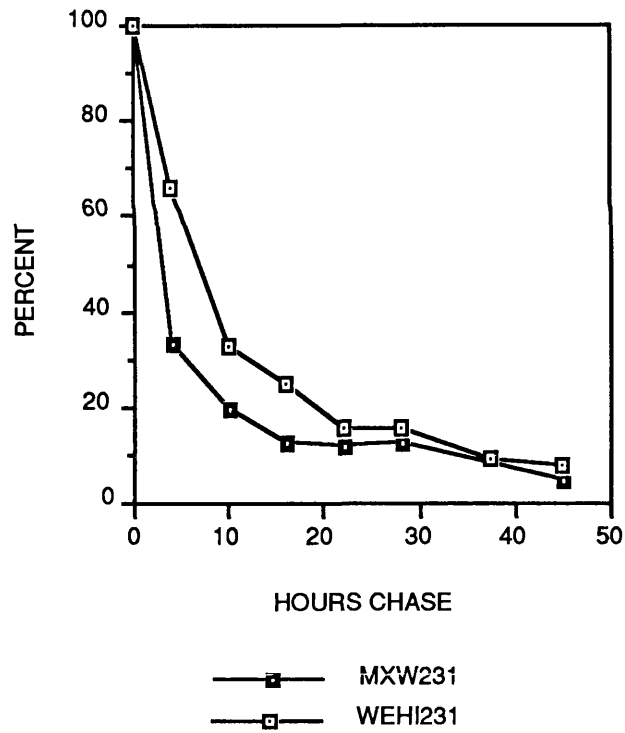


Figure 4.20: Decay of poly(A)+ RNA

Total polyadenylated RNA was isolated from MXW231 and WEHI231 cells during two pulse-chase experiments, and its specific activity was measured, expressed as a percentage of the maximum, and plotted against time during the chase.

order of magnitude difference in the steady-state μ mRNA levels, in good agreement with the result of Mason *et al* (1988), who observed a 15 fold difference with the same cell lines. Pulse-chase analysis showed that μ mRNA was approximately 6 fold more stable in MXW231 cells than in WEHI231 cells.

It was found that although the ribonuclease protection assay was straightforward when using perfectly matched probes such as the hGH and rRNA probes, problems were encountered with the μ NP probe which was not perfectly matched to the μ mRNA from the WEHI231 μ gene. The non-hybridised RNA molecules could not be completely removed by the ribonuclease without greatly diminishing the signal from the hybridisation to the μ NP probe. However, after careful titration of the ribonuclease concentration, conditions were found which allowed quantitative determination of the μ mRNA, which enabled pulse chase analysis to be carried out.

It must be born in mind that some non-specific component is still present in each hybridisation signal using this method, which obviously introduces unwanted error into the half-life determinations. However, the gels in Fig. 4.12 show that the background smear decays at approximately the same rate as the μ -specific band, so this error is not as great as it might at first appear. The background may largely be due to μ -related mRNA, as would be expected if the μ mRNA formed a large proportion of the total mRNA (Schibler *et al*, 1978). Alternatively, the majority of cellular mRNA could be decaying at the same rate as the μ mRNA in each cell type. The latter possibility seems unlikely as the total poly(A)+ RNA shows a similar decay pattern in both two cell types (Fig. 4.20). To eliminate the background completely from the half-life estimation, control hybridisations without probe would have to be carried out for each time point during the chase. This is rather impractical, however, owing to the large amounts of ^3H uridine that would be required and the overall increase in scale of the experiments. In conclusion, the ribonuclease protection assay offers the advantage of increased specificity when used with a perfectly matched probe, but with a mis-matched probe the signal:background ratio is reduced, and more care must be taken when isolating the specific band. To obtain a perfectly matched probe in

this case, it would be necessary to clone the μ cDNA from the WEHI231 cell line.

The problems of achieving perfect chase conditions in mammalian cells have been discussed in Chapter 3 in relation to hGH mRNA. In WEHI231 and MXW231 cells, there was no initial increase in the amount of radioactivity in μ mRNA at the start of the chase, as was observed for hGH mRNA in CBMG cells, but analysis of the specific activity of the UTP pool was carried out to determine when the chase became effective.

It was found that it takes 10-15 hours for the specific activity of the UTP pool to decay to an insignificant level (Fig. 4.18), so the value of the half-life of WEHI231 μ mRNA (5.5 hours) is likely to be over-estimated. Furthermore, any difference in the chase conditions between the two cell lines could contribute to the apparent difference in the rate of decay of μ mRNA. However, the data presented above (Fig. 4.18) indicate that the specific activity of the UTP pools decay at the same rate in both cell types, so there is no evidence for a difference in overall chase conditions. The rates of transcription of the gene in the two cell types have also been shown to be very similar, the MXW231 μ gene being transcribed 1.5 fold faster than the WEHI231 gene (Mason et al, 1988). This means that the rate of incorporation of labelled nucleotide into μ mRNA will be 1.5 fold faster in MXW231 cells, so this may affect the apparent relative rates of decay during the early part of the chase, when the specific activity of the UTP pool is still high. However this effect will be small (at most 1.5 fold), and will diminish rapidly as the specific activity of the UTP pool decreases. In addition, the rate of μ mRNA decay has been measured well beyond the time taken for the UTP pool to decay to 10% of its original value, thus minimising this factor. Therefore it can be concluded that differences in the chase conditions between the two cell types cannot account for the observed difference in the rate of decay of μ mRNA.

The putative rRNA precursor detected at the start of the chase also provides evidence that the chase conditions are very similar in the two cell lines, as it has an identical apparent half-life in both. The disappearance of this molecule by 10 hours into the chase gives a good indication that no further labelled nucleotide is available in the cells

after this time, in agreement with the UTP pool data. Further experiments could be carried out to confirm that this is a precursor molecule, perhaps using actinomycin D in conjunction with a shorter labelling time to demonstrate the precursor-product relationship.

Another reason for the increased stability of μ mRNA in MXW231 cells could be an overall increase in the stability of total polyadenylated RNA. This possibility ~~has~~ ^{seems to have} been ruled out by the finding that total poly(A)+ RNA from WEHI231 and MXW231 cells shows a very similar pattern of decay in both cell types, suggesting that some specific control is exerted over the decay of μ mRNA. It must be noted that this method of analysis is rather crude, and subtle differences in the degradation of poly(A)+ RNA would not be detected. However, there is clearly no gross difference in the decay of poly(A)+ RNA. Therefore the six-fold specific increase in μ mRNA half-life, together with the 1.5 fold difference in μ gene transcription observed by Mason et al (1988), almost fully accounts for the 9-13 fold difference in steady state μ RNA levels described in Section 4.2.2. The slight discrepancy could be due to inaccuracies in the measurements, or due to additional regulation at other levels of mRNA metabolism, for example mRNA processing or nuclear to cytoplasmic transport.

Other workers have also measured μ mRNA decay rates in immortalised cell lines representing different stages of B cell differentiation. Storb (1973) measured the rate of decay of total microsomal RNA in P3K MOPC21 myeloma cells and found the half-life to be 44 hours, with a half-life of total poly(A)+ RNA of 12 hours. Cowan and Milstein (1974) also used the P3K myeloma cell line and determined the half-life of sucrose gradient-fractionated RNA from polyribosome preparations. They obtained half-lives of 12-14 hours for the heavy and light chain fractions. Several reasons for the discrepancy in these results can be postulated. Different methods were used; Cowan and Milstein used a pulse chase protocol, whereas Storb used the continuous labelling to equilibrium method. Also, different fractionation methods were used, but both experimental procedures suffered from the major problem that the RNA fractions would not have been pure, and this is likely to introduce most inaccuracy into the results.

More recently Jäck and Wabl (1988) used transcriptional inhibitors actinomycin D and 5,6-dichloro-1- β -D-ribofuranosyl benzimidazole (DRB) to prevent mRNA synthesis, and showed that μ mRNA was more stable in plasmacytomas and hybridomas than in B cell lymphomas, consistent with the results shown in Section 4.2. These workers did not calculate half-lives, so it is not possible to quantitatively compare the results. Mason et al (1988) have used a heat shock promoter to induce a pulse of μ mRNA synthesis, and measured the rate of decay of this mRNA using a ribonuclease protection assay. With this system they demonstrated a half-life of 14 hours in MXW231 cells and 3 hours in WEHI231, a 4.5 fold difference. Considering the difficulties involved in measuring half-lives of messenger RNAs, the slight quantitative difference between these results and those presented here is probably not significant, and the ratios obtained between the two cell lines are very similar. The heat shock system is more convenient for analysis of several samples, and was adopted for further experiments (see Chapters 5 and 6).

Yuan and Dang (1989) studied μ_s and μ_m RNA decay in the cell line BCL₁, that can be induced to differentiate. They found, using α -amanitin to prevent mRNA synthesis, that there was approximately a two-fold increase in stability of μ_s and μ_m after differentiation, and that μ_m was more stable than μ_s both before and after differentiation. This two fold increase is sufficient to account for the increase in μ mRNA levels observed in these cells (Yuan and Dang, 1989), and reflects the fact that the cells do not fully differentiate upon induction. The half-lives which they obtained were also quite different from the results described above; the half-life of μ_s increased from 8 hours to 18 hours, and that of μ_m went from 16 to 40 hours. The reason for this difference is not clear, but may be due to the use of a different cell line, and/or differences in methodology. Despite the quantitative differences, the results are consistent with the principle of an increase in half-life of total μ mRNA upon differentiation.

More recently, Genovese and Milcarek (1990) have also studied μ mRNA stability in tumour cell lines, including WEHI231. Their results, again obtained using DRB as a transcriptional inhibitor, are in good agreement with those

already described; the half-life of μ mRNA in WEHI231 was found to be 3.5-4 hours, with a 4-9.4 fold ratio in half-lives between early and late differentiation stages.

It is clear, then, that the data obtained from various laboratories using transformed cell lines shows that μ mRNA in cells of the plasma cell phenotype is more stable than that in cells representing earlier stages of differentiation. However, it must be remembered that although transformed cells provide a convenient means of analysing large numbers of cells of a uniform phenotype, they do not necessarily provide perfect models of B cell differentiation. Quantitative differences are apparent between the phenotypes of normal and transformed B cells. For example, the transcription rate of the μ gene increases 8-10 fold when normal lymphocytes are activated with LPS, and the steady state μ mRNA level increases 25-80 fold (Lamson and Koshland, 1984; Yuan and Tucker, 1984a, 1984b). When the corresponding cell lines are analysed, the transcription rate of the μ gene differs at most by 2-5 fold, and the steady state μ mRNA levels differ by 10-100 fold (Perry and Kelley, 1979; Mather et al, 1984; Kelley and Perry, 1986; Gerster et al, 1986; Jäck and Wabl, 1988; Mason et al, 1988; Genovese and Milcarek, 1990). Therefore although it is clear that post-transcriptional processes are important for regulation of μ gene expression in both normal and transformed B cells, transcriptional control may make a larger contribution to the final steady state levels in normal B cells. This is supported by the work of Tsuda et al, (1978) who studied κ -chain induction after LPS treatment of mouse spleen cells. They were able to fully account for the increase in κ mRNA levels by the increase in the rate of transcription, However, this result is not fully reliable as it was determined only in the presence of actinomycin D, which is very likely to introduce other spurious effects (Singer and Penman, 1972; Kostura and Craig, 1986). There may also be differences in the mode of regulation of κ chain and μ chain which would account for this observation.

Studies on μ mRNA stability also have some bearing on the control of the production of mRNA coding for μ_s and μ_m polypeptides. Yuan and Dang (1989) found μ_m mRNA always to be more stable than μ_s mRNA, therefore it is unlikely that a

selective increase in μ_s mRNA stability can be responsible for the switch from μ_m to μ_s production when B cells differentiate. Also, when RNA synthesis was inhibited, they did not see the usual switch from μ_m to μ_s mRNA production, indicating that this event is dependent on RNA synthesis. Therefore it seems from these results that changes in μ mRNA stability are not related to the switch from μ_s to μ_m mRNA production. However, a more recent paper describes results which are in contradiction to those of Yuan and Dang (1989). Berberich and Schimpl (1990) used normal resting B cells from 2-4 month old mice to analyse μ mRNA decay before and after stimulation with LPS. Using actinomycin D and DRB, they found that the half-life of μ_s increased from 8 to 16 hours upon stimulation, similar to the results with the BCL₁ cells. However, the half-life of μ_m remained at less than 4 hours before and after stimulation, a result very different from that obtained with the BCL₁ cells. Berberich and Schimpl also measured the rate of decay of μ_s and μ_m mRNA in WEHI231 cells and found that both had a half-life of 3 hours, consistent with the results shown in this Chapter and those of Mason *et al* (1988). They argue that immortalised B cell lines regulate the stability of μ mRNA differently to normal B cells, and that the switch from μ_s to μ_m RNA production can be accounted for by changes in mRNA stability, in contrast to Yuan and Dang's results.

At present it is difficult to conclusively prove whether these differences arise from genuine differences between transformed and normal B cells. It must be born in mind, however, that in dealing with normal B cells the cell populations are likely to be impure, and for example, an over-representation of earlier stage B cells, which contain predominantly μ_m RNA with a shorter half-life, could bias the results. Examination of the decay graphs of Berberich and Schimpl (1990) reveals that the decay is non-exponential, particularly in the resting B cell population, which provides evidence that the cell population is mixed. In addition, other circumstantial evidence argues against selective stabilisation of μ_s mRNA when B cells differentiate. When constructs which only allow the expression of μ_m mRNA are transfected into myeloma cells, the resulting messages are expressed at levels comparable to those of μ_s (Danner and

Leder, 1985; Peterson and Perry, 1986; Sitia *et al*, 1987; Tsurushita *et al*, 1987; Peterson and Perry, 1989).

From the above discussion it is unclear whether the 3' terminus of the μ mRNA plays a role in controlling its stability, but other evidence indicates that sequences that determine μ mRNA stability reside at the 5' end of the molecule. Kelley and Perry (1986) showed that "sterile" transcripts, which initiate downstream of the V region promoter and do not contain the 5' leader, are not regulated in the same way as productive transcripts when B cells differentiate to plasma cells. In addition, Mason *et al* (1988) found that a mutant μ mRNA which was not localised on membrane-bound polysomes was unstable even in myeloma cells. Further work was therefore carried out to investigate the role of 5' sequences in the control of μ mRNA stability in myeloma cells, and this work is described in Chapters 5 and 6.

CHAPTER 5

TRANSFECTED CELL LINES CONTAINING THE μ GENE5.1: Introduction

The data described in the previous Chapter, and that of other laboratories (Jäck and Wabl, 1988; Mason et al, 1988; Yuan and Dang, 1989; Genovese and Milcarek, 1990), indicate that μ mRNA is more stable in B cell lines with a fully differentiated phenotype. Control of the rate of degradation of a particular message must occur via interactions between the mRNA and other cellular components; in order to understand the control mechanism it is necessary to determine the site of these interactions on the mRNA, and the cellular factors involved.

Two lines of evidence indicate that sequences at the 5' end of the μ mRNA molecule may be important in the regulation of its stability. Firstly, transcripts which initiate at cryptic promoters downstream of the normal promoter are not regulated in the same way as the full length transcripts (Kelley and Perry, 1986). These "sterile" transcripts (Kemp et al, 1980; Alt et al, 1982) initiate at two sites; in the D region of alleles which have undergone DJ rearrangement, and in the J-C intron (Nelson et al, 1983; Reth and Alt, 1984; Lennon and Perry, 1985). In pre-B cells, the "sterile" transcripts represent 20-30% of the total μ mRNA, but in plasma cells they constitute only a negligible fraction of the total, and there is no significant difference in their rate of transcription or accumulated levels between the two cell types (Kelley and Perry, 1986). This means that the *trans*-acting factors which act to increase the stability of productive μ mRNA are not able to recognise the "sterile" transcripts, indicating that the important region of the mRNA in this regard may be at the 5' end.

The second piece of evidence that the 5' end of the mRNA is involved in control of mRNA stability comes from the work of Mason et al, (1988). They noted that when B cells differentiate to plasma cells, there was a large increase in the amount of endoplasmic reticulum in the cells. This is significant when considering the translation of immunoglobulins, which, like that of other secreted and

membrane-bound proteins, occurs on polysomes which are associated with the endoplasmic reticulum (Harrison *et al*, 1974; Mechler and Rabbitts, 1981; Mechler, 1981a). The association of the polysomes with the endoplasmic reticulum relies on the presence of the signal peptide at the amino-terminus of the protein (Blobel and Dobberstein, 1975; Milstein *et al*, 1972; von Heijne, 1983; Perlman and Halvorson, 1983). When the nascent polypeptide chain emerges from the ribosome, the signal sequence is recognised by the signal recognition particle, which causes translational arrest and transfer of the polysome to the endoplasmic reticulum. Here the signal recognition particle interacts with its receptor, which allows translation to continue and the signal recognition particle to be released (Gilmore and Blobel, 1983). The polysome binds to the endoplasmic reticulum, and the protein is co-translationally inserted through the membrane, whilst the signal peptide is cleaved from the protein in the endoplasmic reticulum by signal peptidase (reviewed in Wickner and Lodish, 1985).

In myeloma cells, around 90% of immunoglobulin mRNA is found in membrane bound polysomes (Mechler and Rabbitts, 1981), and it has been shown that initiation of immunoglobulin translation occurs on free ribosomes (Mechler, 1981b). Resting B cells contain a much lower content of endoplasmic reticulum; this led Mason *et al* to hypothesise that the increase in the amount of endoplasmic reticulum when a B cell differentiates would sequester a greater proportion of the μ mRNA in the cell for translation by membrane-bound polyribosomes. This they found to be the case, and they further hypothesised that immunoglobulin mRNA translated on membrane bound polyribosomes would be more stable than that translated on free polysomes. In order to test this, they constructed a μ gene with a mutated leader sequence, which encoded hydrophilic amino acids instead of the normal hydrophobic signal sequence. The mutated leader sequence would not be expected to target the polysomes to the endoplasmic reticulum for translation, as it would not be recognised by the signal recognition particle (Perlman and Halvorson, 1983; von Heijne, 1983). When this mutant was transfected into a myeloma host cell, the protein produced was not glycosylated, did not associate with light chain and

was not secreted into the culture medium, consistent with it being translated by cytoplasmic ribosomes (Sitia *et al*, 1987; Mason *et al*, 1988). The rate of decay of the mutated μ mRNA was determined using the heat shock promoter system (see below). It was found that the mutated mRNA had a short half-life in plasmacytomas, equivalent to that of unmutated mRNA in B cell lymphomas. This indicates that targetting of the μ mRNA to membrane bound polyribosomes may be important in the regulation of its stability.

Cellular localisation has also been shown to be crucial to the regulation of histone mRNA stability, discussed in Section 1.6.2. In this case, the correct degradation of histone mRNA at the end of DNA synthesis is prevented if the mRNA is localised on membrane bound instead of the normal cytoplasmic polyribosomes (Zambetti *et al*, 1987). Therefore cellular localisation of the mRNA may turn out to be a more general important feature in control of mRNA stability.

It was decided to continue this line of investigation, using different mutants, to further characterise μ mRNA sequences that might be involved in the control of μ mRNA stability. To analyse the effects of mutations on mRNA stability, the mutant genes must first be generated *in vitro*, and introduced into the appropriate cell type by transfection. It was initially hoped that the pulse-chase technique already described might then be used to determine the half-lives of mRNAs from transfected genes. However, preliminary experiments indicated that the transcription rates of transfected genes were too low for pulse-labelling techniques to be used, as insufficient label was incorporated into the μ mRNA during the pulse, making detection difficult (data not shown). Therefore the system used by Neuberger and colleagues (Mason *et al*, 1988) was employed. This will now be described in more detail.

Genes of interest are inserted in a plasmid vector downstream of a promoter which can be induced by subjecting the cells containing it to a short heat shock. The heat shock thus produces a burst of transcription which ceases after the cells are returned to the normal growth temperature, and the rate of decay of the mRNA thus produced can be determined by Northern blot analysis at various time points after the heat shock. The hsp70 promoter from

Drosophila melanogaster is highly inducible in murine cells, and has a very low background level of transcription, so is well suited to this purpose (Pelham, 1982; Mason et al, 1988). Plasmid constructs containing the μ gene are transfected into the myeloma cell line J558L, which does not produce any heavy chain mRNA, but constitutively produces λ 1 light chain (Fig 4.2; Oi et al, 1983). This cell line is highly transfectable (Oi et al, 1983; Neuberger and Williams, 1986), and allows the easy analysis of heavy chain protein and mRNA from transfected genes in the absence of endogenous heavy chain. Transfection of a plasmid encoding the μ heavy chain with specificity for the hapten NP (4-hydroxy-3-nitrophenacetyl) into these cells produces reconstituted antibody with hapten specificity (Neuberger et al, 1984). This provides a convenient means for detecting heavy chain expression using an anti-NP ELISA (Engvall and Perlman, 1971, 1972). This system is therefore relatively straightforward for the comparison of different mutant mRNAs with wild-type. The basis for the choice of mutations that were analysed with this system will now be described in more detail.

From the data presented above it seems that the 5' signal peptide sequence of μ mRNA is involved in the mechanism for stabilising μ mRNA in myeloma cells. Its sole function in this regard may be to localise the message, via translation of the signal peptide, onto membrane-bound polyribosomes, where other factors act to stabilise the mRNA. These factors could interact with the 5' end or other regions of the message. Alternatively, the localisation of the mRNA may be irrelevant, and the mRNA sequence which encodes the signal peptide may itself form a stabilising determinant. To investigate this problem further and to try and distinguish between these possibilities, a mutant was constructed which retained as much of the signal sequence as possible but would still be expected to be located on free polysomes.

Mutagenic oligonucleotides, for use in site-directed mutagenesis (Kramer et al, 1984), were designed such that the initiating AUG codon would be destroyed, and a new AUG codon would be inserted at the start of the mature polypeptide. Translation of eukaryotic mRNAs normally begins at the first available AUG codon (Kozak, 1983). Therefore this mutant mRNA would be expected to have the property that the leader

sequence, including the signal peptide, would not be translated, but rather translation would start at the AUG at the beginning of the mature protein. As the signal peptide would not be translated, the mRNA would not be expected to localise to membrane-bound polysomes. If the signal peptide coding sequence itself stabilises the μ mRNA, independently of the mRNA location in the cell, this mutant mRNA should remain stable, unless the point mutations at either end by chance disrupt the critical sequence or secondary structure. On the other hand, if the localisation of the mRNA to membrane-bound polysomes is important for stabilisation, the mutant mRNA will be unstable.

It is also of interest to know whether alterations in the 3' untranslated region affect mRNA stability, as has been shown for the *c-fos* gene and others (Treisman, 1985; Shaw and Kamen, 1986; Wilson and Treisman, 1988). In particular, the polyadenylation signal from SV40 is commonly used in mammalian cell expression vectors (Mulligan and Berg, 1981; Southern and Berg, 1982), and therefore its effect on mRNA stability is of interest. To this end, plasmid constructs containing the μ gene followed by the SV40 late polyadenylation signal were made to allow comparison with the μ_s 3' untranslated region and polyadenylation signal.

This Chapter describes the site directed mutagenesis to alter the position of the μ initiation codon, and the plasmid constructions for the control μ gene and the μ gene with the SV40 polyadenylation signal. Also this Chapter covers the transfections into J558L cells, and analysis and choice of transfectants for half-life analysis.

5.2 Results

5.2.1 Construction of control plasmids

A series of plasmid constructions were made based on two plasmids, p516 and p372, kindly provided by M. Neuberger. The plasmid p516 (Fig. 5.1A) is based on pSV2neo (Southern and Berg, 1982) and contains the *Drosophila melanogaster* hsp70 promoter (Pelham, 1982) which directs heat-shock inducible transcription of a μ minigene which has had all introns removed except the leader intron (Neuberger, personal communication). This μ gene contains the anti-NP variable

FIGURE 5.1

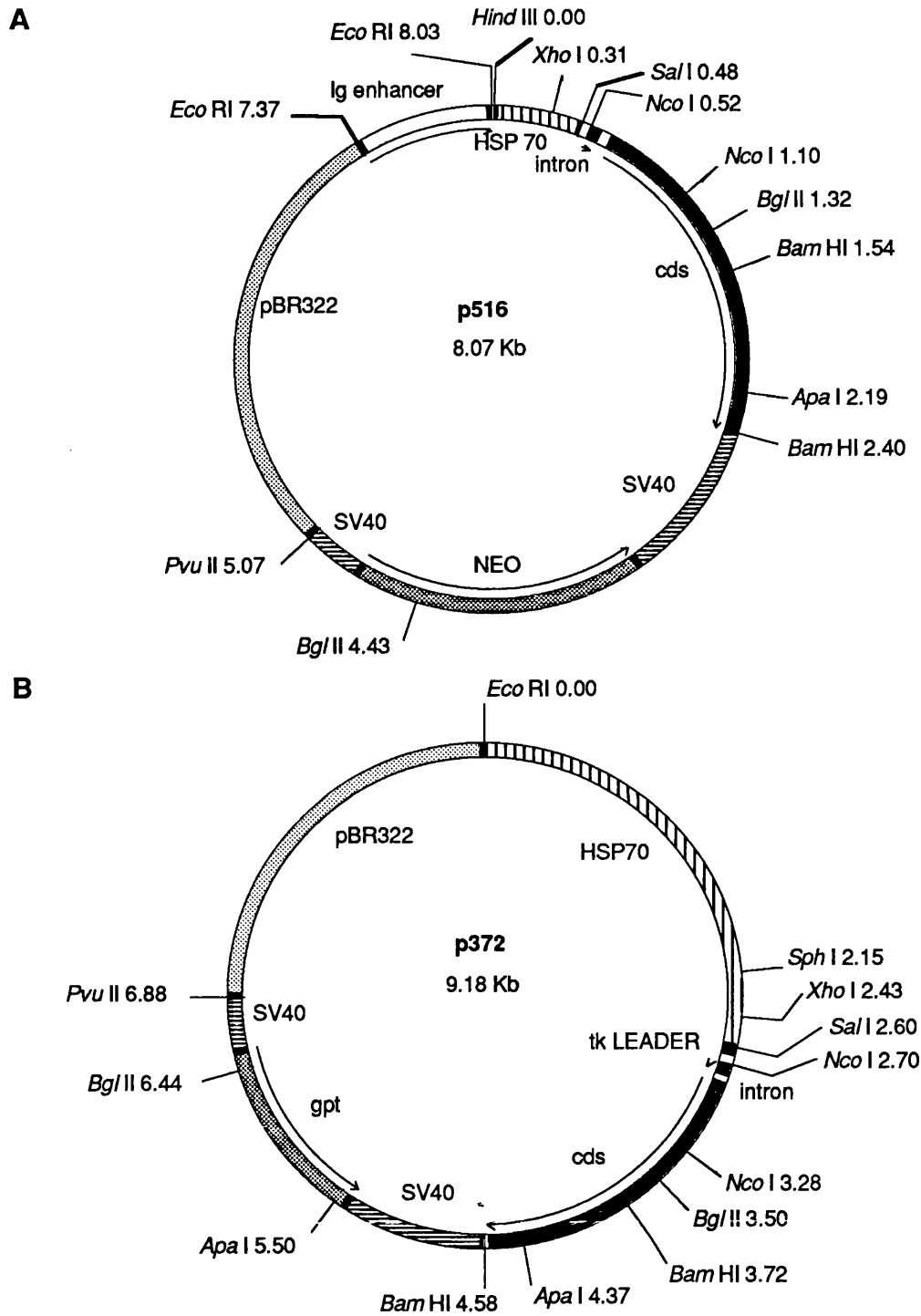


Figure 5.1: Starting plasmids for transfection constructs.

(A) p516, (B) p372

See text for details; cds indicates μ coding sequence.

region from the B1-8 hybridoma, and is identical in coding sequence to the gene in pNP1, used to construct pSP64 μ NP (Section 4.2.1; Bothwell *et al*, 1981). The immunoglobulin heavy chain enhancer is inserted at the *Eco* RI site in this plasmid. The plasmid also contains the neomycin resistance gene which encodes a phosphotransferase that confers resistance to the antibiotics neomycin and kanamycin in bacteria. Mammalian cells are not affected by these antibiotics, but they are affected by the analogue G418, which is also inactivated by the phosphotransferase (Southern and Berg, 1982). Therefore, under the control of a eukaryotic promoter (in this case the early region promoter of SV40) the neomycin resistance gene allows selection of cells which have taken up the plasmid DNA by virtue of their resistance to the antibiotic G418. Between the *neo* gene and the μ gene is an 850bp fragment containing the splicing and polyadenylation signals from SV40. The orientation of this fragment is such that the immunoglobulin transcript contains the SV40 late region polyadenylation sequence and the *neo* transcript contains the SV40 early region splice donor and acceptor sites (from the small t antigen intron) and early region polyadenylation sequence.

The plasmid p372 (Fig. 5.1B) is very similar to p516, except it contains a larger fragment of the heat shock promoter, and 62 nucleotides of the HSV thymidine kinase leader sequence before the normal μ leader. Also, p372 is based on pSV2gpt (Mulligan and Berg, 1981) and so has the *gpt* gene in place of the *neo*^R gene. The *gpt* gene encodes the bacterial enzyme hypoxanthine-guanine phosphoribosyl transferase, which allows selection of cells in mycophenolic acid. Mycophenolic acid inhibits the enzyme IMP dehydrogenase, which is necessary for the formation of guanosine monophosphate from inosine monophosphate. This inhibition can be overcome if the *gpt* gene is expressed, if xanthine is supplied in the growth medium. Normally, mammalian cells are not able to utilise xanthine, but the *gpt* enzyme is able to convert xanthine to GMP, thus bypassing the requirement for IMP.

A control construct was made in which the μ _S 3' untranslated region and polyadenylation signal were inserted

at the 3' end of the μ gene in p516, so that the μ mRNA generated from the heat shock promoter would be identical to wild type μ mRNA. The 3' region of the μ gene was obtained from pSP65C μ 343 (Fig. 5.2A). This plasmid contains a genomic μ gene *Hind* III fragment inserted at the *Hind* III site of pSP65 (A. Cox unpublished). The *Hind* III fragment contains the 3rd and 4th exons of the μ gene, including the μ_s tail and 3' untranslated region. After appropriate restriction enzyme digestion of p516 and pSP65C μ 343, and separation of the resulting fragments by agarose gel electrophoresis, three fragments were purified as follows. The 520 base pair *Apa* I to *Bam* HI fragment from pSP65C μ 343, the 1.89 kb *Xho* I to *Apa* I fragment of p516 containing the hsp70 promoter and most of the μ gene, and the 5.98 kb *Xho* I to *Bam* HI fragment from p516 containing the 5' portion of the hsp70 promoter, heavy chain enhancer, and the pSV2neo sequences. These three fragments were ligated together as indicated in Fig. 5.2B, and the ligation mix was transformed into *E. coli* LM1035. Mini-plasmid preparations from the resulting colonies were digested with appropriate restriction enzymes to identify a transformant containing the desired plasmid, called p516 μ_s neo (Fig. 5.3A).

The above approach, using the *Xho* I, *Apa* I and *Bam* HI restriction enzyme sites in 3-way ligations, was useful in creating a variety of related plasmids. The plasmid p372 μ_s gpt (Fig. 5.3B) was also created in a similar manner, using the equivalent *Xho* I to *Apa* I and *Xho* I to *Bam* HI fragments from p372. It had been reported that the presence of the HSVtk leader sequence gave better induction from the heat shock promoter (M. Neuberger, personal communication), so p372 μ_s gpt was analysed in parallel with the main experiments. In order that all the constructs used for transfection would be comparable, the heavy chain enhancer was deleted from p516 and p516 μ_s neo, to yield p516neo Δ E and p516 μ_s neo Δ E (Figs. 5.4A and B respectively). This was done by digesting the plasmids with *Eco* RI, purifying the large fragment after agarose gel electrophoresis, and religating. After transformation into *E. coli* LM1035, a transformant was selected which did not contain the enhancer, as determined by restriction enzyme analysis. In addition, the 3-way ligation was repeated using the *Xho* I to *Apa* I fragment from p516, the

FIGURE 5.2

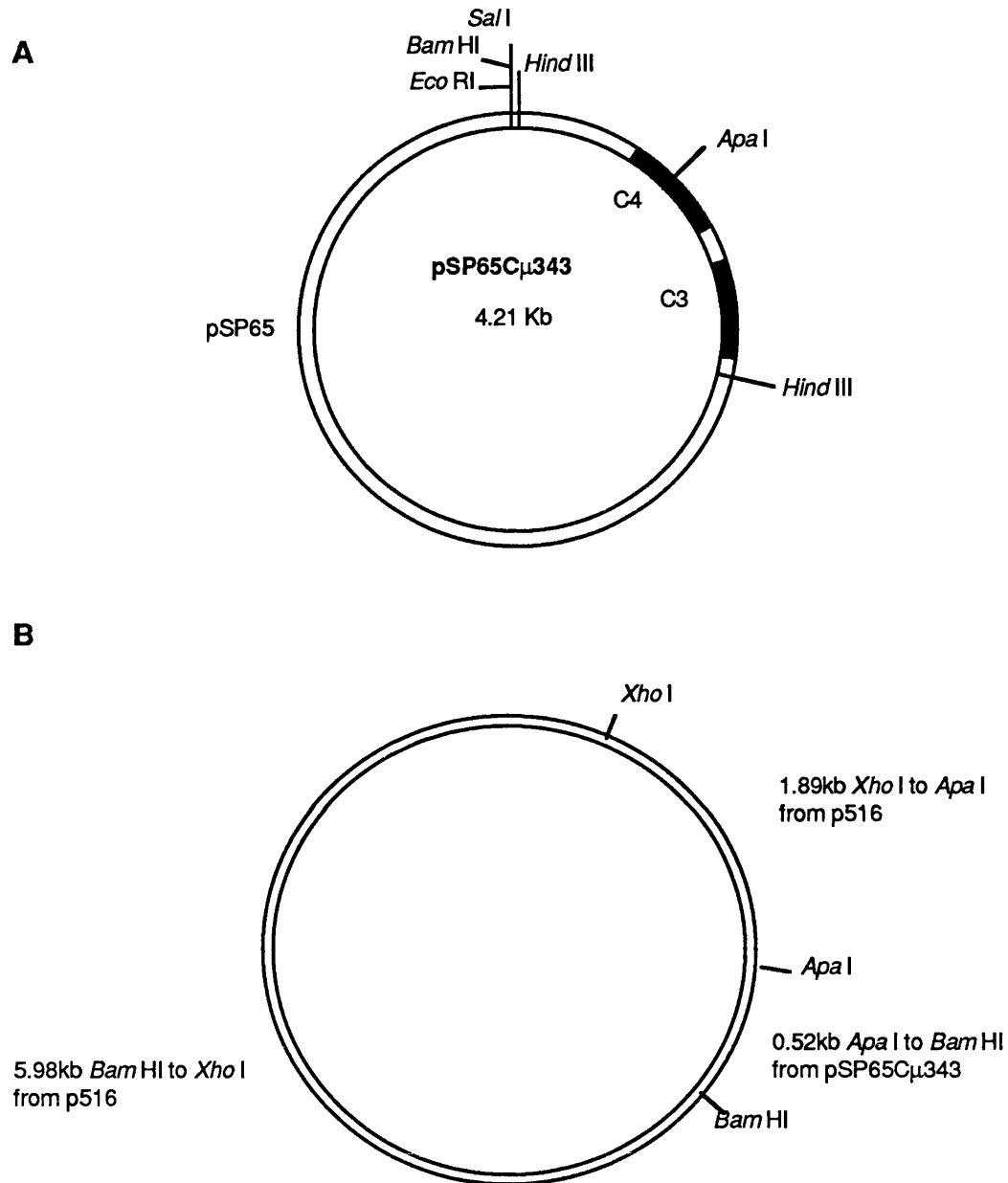


Figure 5.2: 3-way ligation.

(A) pSP65C μ 343 is shown with relevant restriction enzyme sites marked. The 0.52kb *Apa* I to *Bam* HI fragment was used to insert the μ secreted form polyadenylation site into the plasmids p516 and p372, by 3-way ligation as indicated in (B).
(B) Typical 3-way ligation to generate p516 μ sneo (Fig.5.3A).

FIGURE 5.3

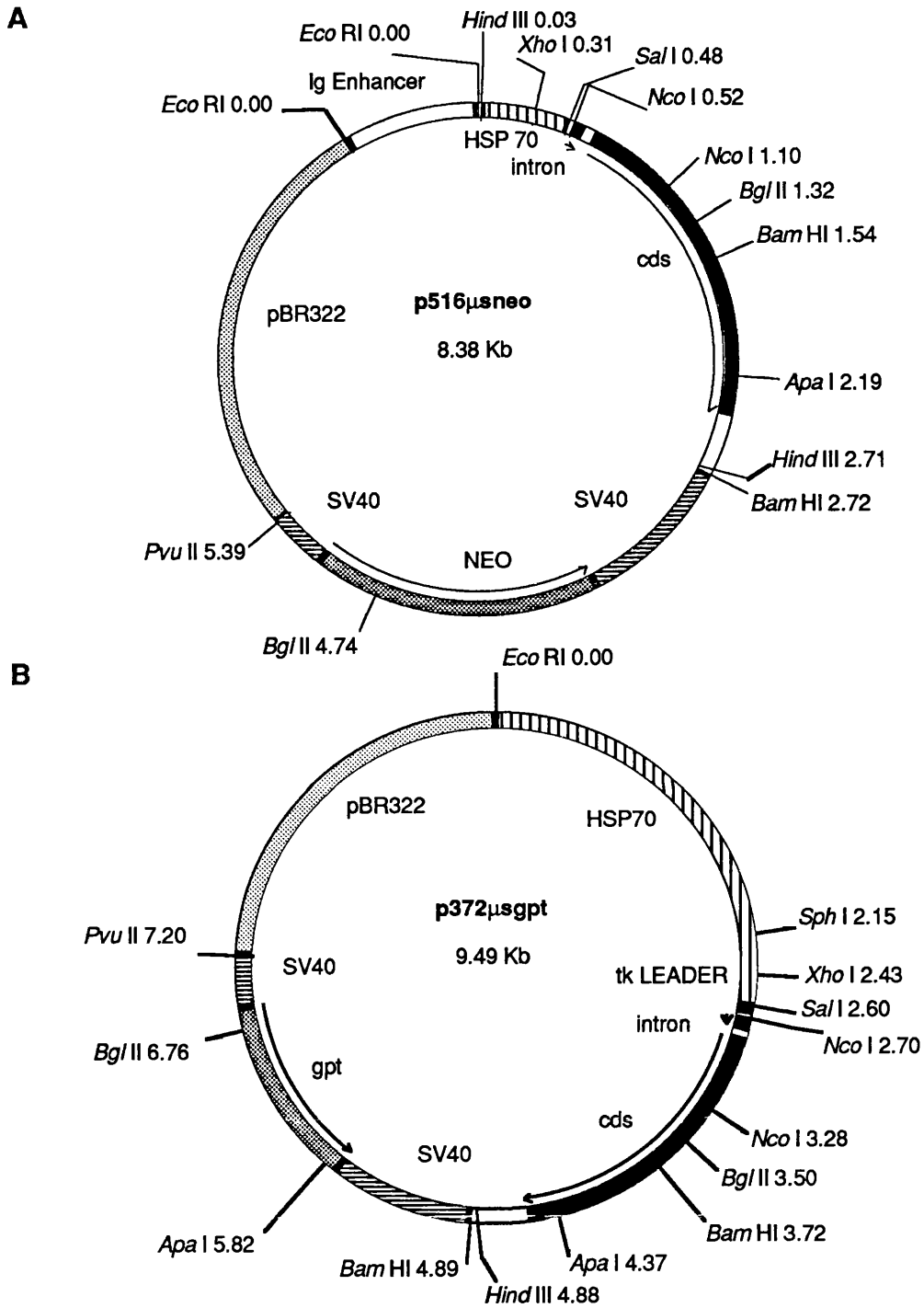


Figure 5.3: Results of 3-way ligations

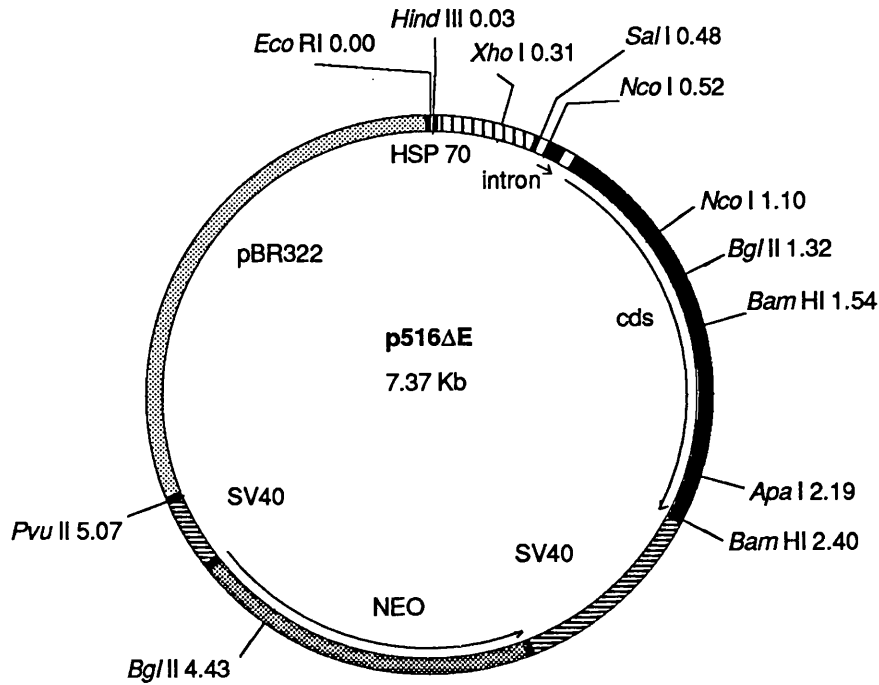
(A) p516 μ sneo

(B) p372 μ sgpt

For details see text; cds indicates μ coding sequence

FIGURE 5.4

A



B

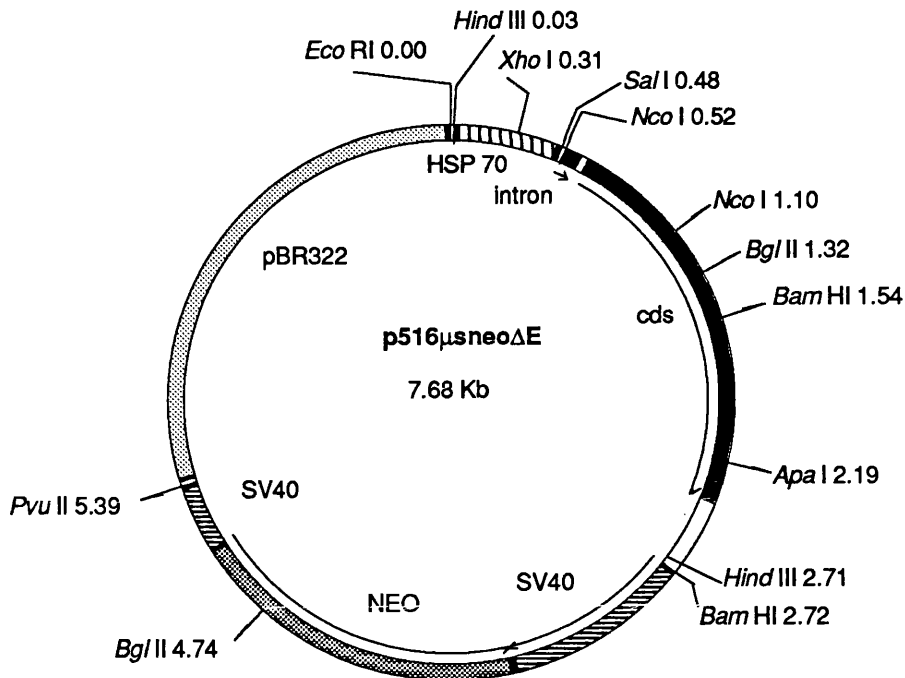


Figure 5.4: Deletion of immunoglobulin heavy chain enhancer.

(A) p516neoΔE, derived from p516.

(B) p516μsneoΔE, derived from p516μsneo.

For details see text; cds indicates μ coding sequence.

Xho I to *Bam* HI from p372 and the *Apa* I to *Bam* HI from pSP65C μ 343, to yield p516 μ_s gpt (Fig. 5.5), so the control μ gene was available with both *neo* and *gpt* selectable markers (p516 μ_s neo Δ E and p516 μ_s gpt respectively). The constructs p516neo Δ E and either p516 μ_s neo Δ E or p516 μ_s gpt can be used to compare the half-lives of μ mRNA with either the SV40 late region polyadenylation signal or the μ_s polyadenylation signal respectively. A summary of the μ genes contained in these plasmids is shown in Fig. 5.6(A-C).

5.2.2 Mutagenesis of the μ leader sequence.

As mentioned in the introduction, a μ gene with a mutated leader sequence was constructed by *in vitro* site-directed mutagenesis in bacteriophage M13 using the gapped duplex method (Kramer *et al*, 1984; Carter *et al*, 1985). The principles of this procedure are outlined in Section 2.2.8 and illustrated further in Fig. 5.7. The mutagenesis is carried out on the target sequence in the vector M13mp8, which, like other M13 vectors, contains a multicloning site in the β -galactosidase gene. This allows detection of phage which are recombinant at this site with the chromogenic substrate X-gal. (In the presence of the inducer IPTG, non-recombinant phage produce blue plaques and recombinant phage produce white plaques (Messing *et al*, 1977)). M13mp8 also contains an *amber* mutation that is the basis for the selection of the mutants (see below). After the mutagenesis and selection of the correct products, the mutated DNA fragment is subcloned into the desired vector.

The mutagenic oligonucleotides used, R1216 and R1217, are shown in Fig.5.8, which also indicates the nucleotide changes. R1216 is designed to mutate the initiating ATG codon to ATT. In addition, as there is a second ATG codon 2 bp downstream, this is also mutated, to CTG, to prevent any spurious initiation. R1217 is designed to insert an ATG codon in front of the first codon of the mature μ protein, so that translation will initiate here and the signal peptide will not be translated, although its coding sequence will be present in the mRNA. To optimise translation initiation at this site, a Kozak consensus sequence for translational initiation was also created (Kozak, 1987; Kozak, 1984). Fortunately, the first four bases of this consensus, CCAC

FIGURE 5.5

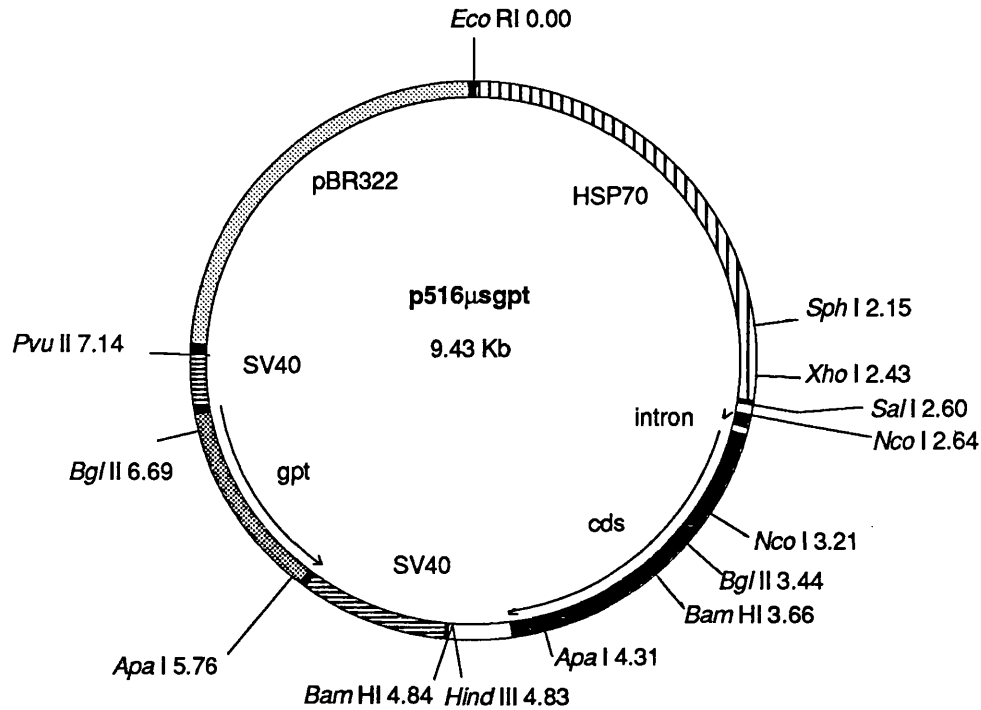


Figure 5.5: Alternative selectable marker for control construct.

p516μsgpt, generated by 3-way ligation of the *Xho*I to *Bam*HI fragment from p372, the *Xho*I to *Apa*I fragment from p516 and the *Apa*I to *Bam*HI fragment from pSP65Cμ343. For details see text; cds indicates μ coding sequence.

FIGURE 5.6

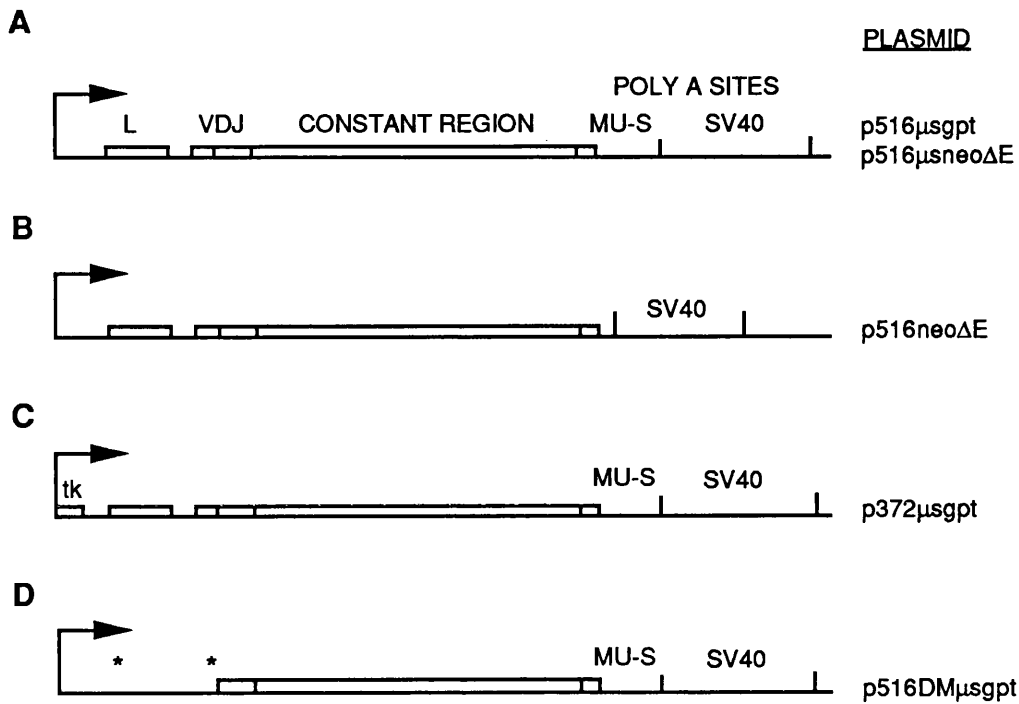


Figure 5.6: Summary of μ genes contained in various plasmids.

Open boxes represent coding regions.

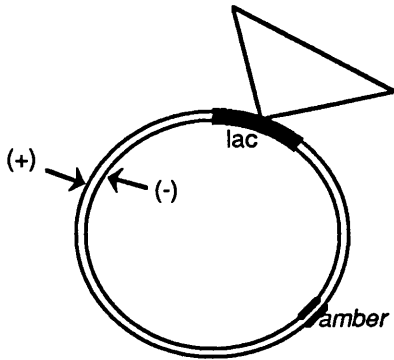
(A) The control μ gene with the μ s polyadenylation site, contained in plasmids p516 μ sgpt and p516 μ sneo Δ E.

(B) The μ gene with the SV40 polyadenylation site, contained in p516neo Δ E.

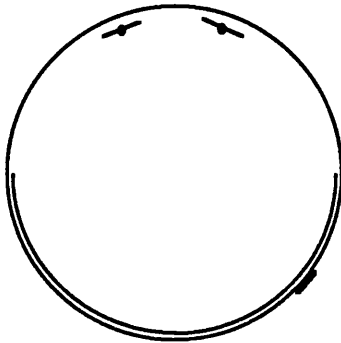
(C) The μ gene containing 62bp of tk leader sequence, contained in p372 μ sgpt.

(D) The μ gene with the two mutations (marked with asterisks) such that translation begins at the start of the mature protein.

FIGURE 5.7

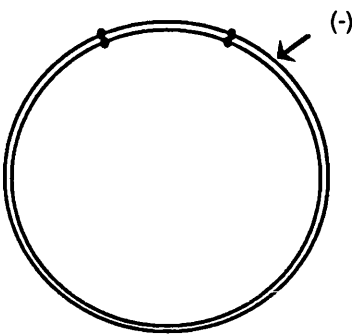


Subclone fragment into M13mp8 (which contains *amber* mutation), select white plaques and screen for correct orientation of insert.

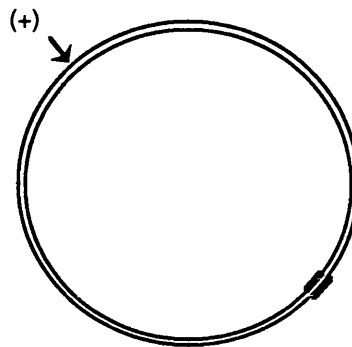


Prepare single-stranded phage DNA (+ strand). Anneal wild-type M13 to generate gapped duplex. Anneal mutagenic oligonucleotides

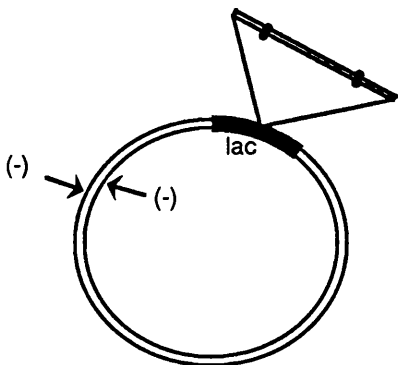
Fill in with Klenow polymerase and transform into repair deficient strain. Plate out into *sup 0* strain to select for (-) strand.



(-) strand does not contain *amber* mutation and will grow on *sup 0* strain



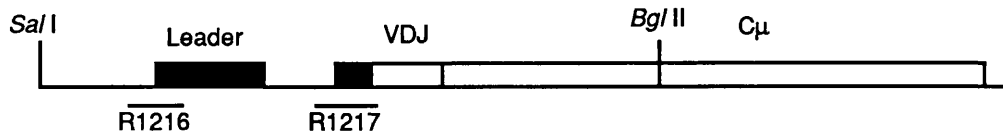
(+) strand contains *amber* mutation and will not grow on *sup 0* strain.



Sequence insert and subclone out of M13 into desired vector.

FIGURE 5.8

A



B

AATGTCGCGAAAAGAGCGCCGGAG TATAAA TAGAGGCGCTTCGTC

GACCAGATCACAGTTCTCTCTACAGTTACTGAGCACACAGGACCT
CCTGGA

T C
↑ ↑
 CACCATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGC
 GTGGTAACCGACCTCGACATAG

M G W S C I I L F L V A T A

TACAGGTAAGGGGCTCACAGTAGCAGGCTTGAGGTCTGGACATAT
T G

ATATGGGTGACAATGACATCCACTTTGCCTTTCTCTCCACAGGTG
GTGTCCAC
V

CATG
↑
 TCCACTCCAGGTCCAAGTGCAGCAGCCTGGGGCTGAGCTTGTGA
 AGGTG GTCCAGGTTGACG
 GTAC
 H S Q V Q L
M +1 → MATURE PROTEIN

Figure 5.8: Mutagenesis oligonucleotides

(A) shows a schematic diagram of the μ primary transcript from the hsp70 promoter, the coding sequences are shown as open boxes and the untranslated regions as a single line. The position of the signal sequence is shown as a filled box. The restriction sites used to subclone the region to be mutated into M13mp8 are indicated.

(B) shows the relevant portion of the sequence to be mutated. The top line is the message sense sequence and the mutagenic oligonucleotides are shown underneath in the positions at which they hybridise. The "TATA" box is boxed, and the mRNA start is marked with an arrow. The positions of the mutations are indicated by arrows and the new sequence is shown above. The leader intron is underlined and the amino acid sequence is shown underneath the nucleotide sequence.

are already present in the correct position, requiring only the insertion of an extra C in front of the ATG. The mutagenesis procedure will now be described in more detail.

(i) Subcloning of the target fragment for mutagenesis.

Firstly, a *Sal* I to *Bgl* II fragment from the starting vector p516, containing the target sequence for the mutagenesis, was subcloned into the vector M13mp8. Use was made of the fact that the restriction enzyme sites *Xho* I and *Sal* I have compatible cohesive ends. An oligonucleotide adaptor, of the sequence GATCTCGA, was used to create a *Xho* I site at the *Bgl* II site in the C μ constant region (see Fig. 5.7A for the position of this site). The plasmid p516 (Fig. 5.1) was digested with restriction enzyme *Bgl* II, and the adaptor oligonucleotide was treated with T4 polynucleotide kinase to phosphorylate the 5' OH groups. The adaptor and linear DNA were then ligated together using a 30-50 fold molar excess of adaptor. After inactivation of the ligase, the DNA was digested with *Xho* I and *Sal* I to yield a 900 basepair fragment with *Sal* I compatible ends, which was purified after preparative agarose gel electrophoresis. The M13mp8 vector was linearised in the multicloning site by restriction with *Sal* I, and then treated with calf intestinal phosphatase to remove 5' terminal phosphates. After ligation of the 900 basepair fragment and linearised M13 vector, 20% of each ligation mix was transformed into *E. coli* JM101 and plated in the presence of IPTG and X-gal. Control ligation mix containing only vector DNA yielded 8 blue plaques, whereas the test ligation yielded 6 blue and 13 white plaques. 7 white plaques were grown up in liquid culture and the replicative form DNA was isolated from bacterial pellets using the mini plasmid preparation method. These were test digested with various enzymes to determine the orientation of the insert, and a transformant was chosen which had the orientation such that the (+) strand found in the phage particles corresponded to the message sense of the insert. Single stranded DNA from the phage particles was prepared from culture supernatant of this transformant as described in Section 2.2.8, to provide the template for the mutagenesis reactions.

(ii) Mutagenesis

First, gapped duplex DNA was prepared as described in Section 2.2.8, by annealing the single stranded recombinant M13mp8 DNA, and linear double stranded M13mp18 DNA (which is wild-type for the *amber* mutation). The mutagenic oligonucleotides (Fig. 5.8) were then annealed to the gapped duplex. A control template using the L42 oligonucleotide and non-recombinant M13mp8 was also prepared (see Section 2.2.8). This oligonucleotide introduces a mutation into the *lac* region, which leads to the production of white plaques and thus allows an estimate to be made of the efficiency of the mutagenesis reaction and selection. After the primer extension reactions had been carried out using Klenow polymerase and DNA ligase, 10% and 20% of the reaction mixes were transformed into the repair deficient *mut* L strain HB2154 (which allows replication of both strands), and plated out on a lawn of the *sup 0* strain HB2151. As this strain is a non-suppressor, there is selective pressure for the growth of phage derived from the (-) strand of the primer extension reaction, which does not contain the amber mutation. The control reaction yielded 80% white plaques, indicating that the mutagenesis procedure was efficient and the selection system was working well. In addition, the parental RF was plated on both HB2151 and JM101 (a suppressor strain), and plaques were only detected on JM101, confirming the efficiency of the selection procedure.

(iii) Plaque screening

Plaques were screened by in situ hybridisation to the labelled oligonucleotide probes according to the method of Grünstein and Hogness (1975). These procedures are described in detail in Section 2.2.8. Duplicate lifts were taken from each plate and these were probed with the probes specific for each mutation. The R1217 oligonucleotide (see Fig. 5.8) was used to screen for the insertion of the CATG nucleotides, and the oligonucleotide R1302 (GCTCCAGCCAATGGT) was used to screen for the removal of the two ATG codons. Plates of parental transformants in JM101 were used as negative controls. The T_m of the hybridisation probe R1302 was estimated as described in Section 2.2.8(v), and the filters

were washed at $(T_m - 5)^\circ\text{C}$. With the longer probe, it was not possible to estimate the T_m accurately, so filters were washed at increasingly higher temperature until the background was removed from the control plates. The majority of plaques from the mutagenesis reactions were positive with at least one probe, and many of these were positive with both. The latter group would be expected to contain both mutations, and 6 of these were picked and grown up in liquid culture. Single stranded DNA from the culture supernatant was prepared for DNA sequencing, and RF DNA was prepared from the bacteria for restriction enzyme analysis.

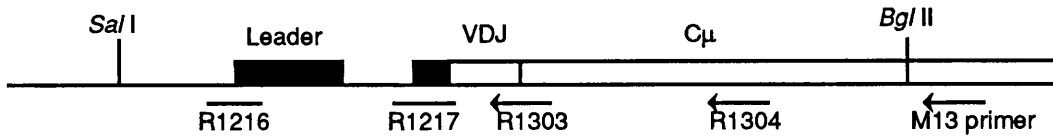
(iv) Sequencing and restriction analysis of the putative mutants

To confirm the presence of the mutations, the single stranded DNA was sequenced as described in Section 2.2.8, using the R1303 oligonucleotide shown in Fig 5.9. The sequence was compared to that of the parental DNA. All 6 of the transformants picked contained the desired mutations, and one, called p516DM6, was further sequenced using the oligonucleotides R1304 and the M13 reverse primer (Fig. 5.9), to confirm that no additional mutations had been introduced into the target DNA. The sequence of the mutant DNA was indeed found to be identical to that of the control DNA except at the positions of the desired mutations. RF DNA was also prepared from the same cultures and used for restriction enzyme analysis. Removal of the initiating ATG destroys a site for the restriction enzyme *Nco* I, so this site should be present in the unmutated M13mp8 recombinant, but not in the mutant. This was found to be the case, confirming the sequence data.

(v) Subcloning of mutated fragment

To reinsert the *Sal* I -*Bgl* II fragment containing the mutations back into the parent vector, p516DM6 was digested with *Sal* I and *Bgl* II and the 900 basepair fragment isolated by preparative gel electrophoresis. The vector p372 was digested partially with *Bgl* II and completely with *Sal* I, and the 8.3kb fragment was purified in the same way. The two fragments were ligated together and the ligation mix transformed into LM1035. Mini plasmid preparations from the

FIGURE 5.9



M13 primer: AACAGCTATGACCATG
R1303: CAGCTTCACTGAAGCCCC
R1304: GGAGACTGTGAGAGTGGTG

Figure 5.9: Oligonucleotides used for sequencing.

The positions of the oligonucleotides used to sequence the mutated fragment of DNA are shown, relative to the mutagenesis oligonucleotides, and their sequences are given below in a 5' to 3' direction. Open boxes represent coding regions, filled box indicates the signal sequence. Sequencing oligonucleotides are indicated with arrows, mutagenic oligonucleotides are shown as plain lines. Not to scale.

resulting colonies were test-digested with various restriction enzymes to identify a plasmid with the correct insert. The resulting plasmid is called p516DMgpt (Fig. 5.10A). This plasmid contains only two sites for the enzyme *Nco* I whereas the parental contains three sites, confirming the presence of the mutated segment of DNA. This plasmid then had the μ_s polyadenylation site inserted by 3-way ligation in a similar manner to that described above for the control plasmids, using the *Xho* I/*Apa* I fragment from 516DMgpt, the *Xho* I-*Bam* HI from p372, and the *Apa* I-*Bam* HI from pSP65C μ 343. This yielded the plasmid 516DM μ_s gpt (Fig. 5.10B), which was then used in transfection experiments. It differs from the control plasmid p516 μ_s gpt only at the sites of the mutations (see Fig. 5.6D).

5.2.3: Transfection Experiments

(i): Linearisation at the *Eco* RI site.

The first round of transfections was carried out with the plasmids p516 μ_s neo Δ E, p516neo Δ E and p372 μ_s gpt. 40 μ g of each plasmid was linearised at the *Eco* RI site 5' to the heat shock promoter by restriction enzyme digestion, and transfected into the myeloma cell line J558L (Oi et al, 1983) by electroporation (Neumann et al, 1982; Chu et al, 1987), as described in Section 2.5.5. Linear DNA has been shown to give higher stable transfection efficiencies than covalently closed circular DNA (Neumann et al, 1982; Chu et al, 1987). The transfected cells were plated out at 10^4 and 10^3 cells per well in 96-well microtitre plates and the frequencies of colonies obtained after the appropriate selection procedures are shown in Table 5.1. The reason for the difference in frequency between the *gpt* and *neo* selection systems is not known, but similar results were also obtained in later experiments. Cells from wells containing single colonies were grown up and stored frozen in liquid nitrogen until they could be analysed.

The first transfectants to be tested for their level of induction upon heat shock were those containing the p372 μ_s gpt plasmid; this was done using Northern blotting to analyse the μ mRNA produced by the cells. The heat shocks were carried out as described in Section 2.5.6; 10^6 to 10^7 cells of each

FIGURE 5.10

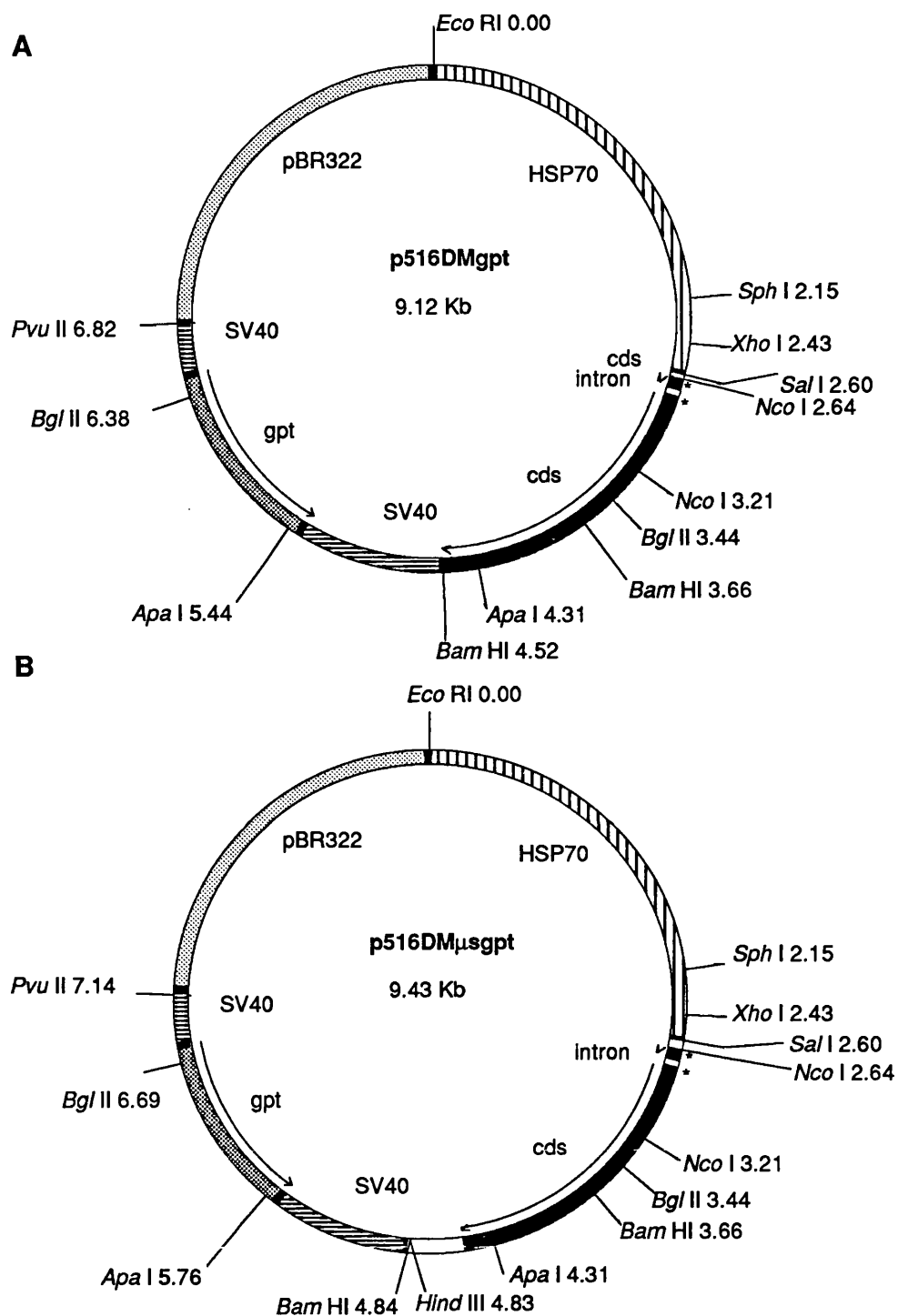


Figure 5.10: Plasmids containing the mutated μ gene.

(A) p516DMgpt, derived by *Sal* I and partial *Bgl* II digestion of p372, and ligation of the 8.3kb fragment thus obtained to the 900bp *Sal* I to *Bgl* II fragment containing the mutations in the μ gene.

(B) p516DMμsgpt, derived by 3-way ligation using the *Xho* I-*Apa* I fragment from p516DMgpt, the *Xho* I-*Bam* HI fragment from p372, and the *Apa* I-*Bam* HI fragment from pSP65Cμ343.

For details see text; cds indicates μ coding sequence, and mutations are marked as asterisks.

Table 5.1

p516 μ_s neo Δ E	10 ³ cells/well	42/96
p516neo Δ E	10 ³ cells/well	36/96
mock neo	10 ⁴ cells/well	0/96
p372 μ_s gpt	10 ⁴ cells/well	7/96
p372 μ_s gpt	10 ³ cells/well	1/96
mock gpt	10 ⁴ cells/well	0/96

Table 5.1: Transfection efficiencies.

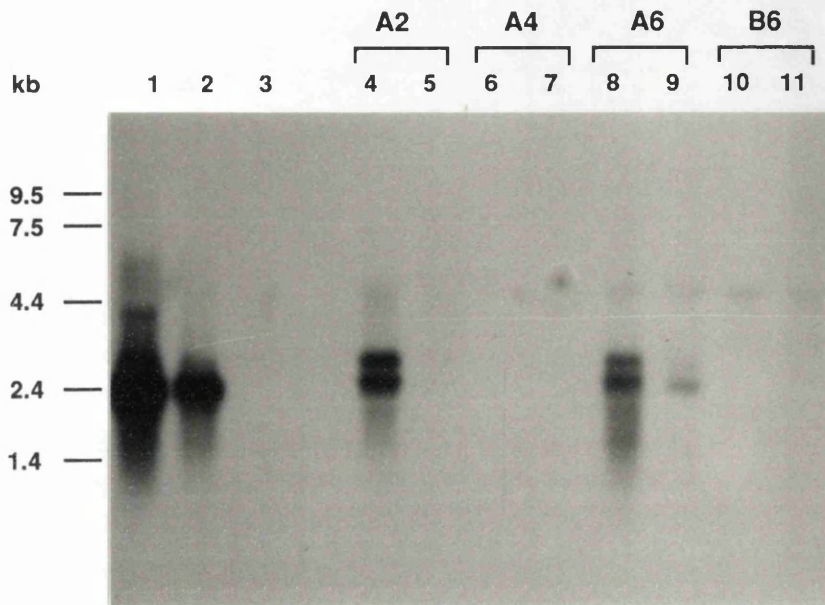
The number of colonies in 96 well plates at each cell dilution is shown. For the p516neo Δ E and p516 μ_s neo Δ E transfections, colony densities were too high to count when plated at the 10⁴ cells/well.

line were heat shocked at 42.5°C for 1 hour and allowed to recover at 37°C for 1 hour before total RNA was prepared from them, as described in Section 2.3.2. Total RNA was also prepared from control cells from each line, which had not been heat-shocked. The RNA samples were then subjected to gel electrophoresis through formaldehyde/agarose gels, transferred to "Hybond-N", and probed with the pSP64 μ NP probe described in Chapter 4 (Fig. 4.1). A positive control cell line, JW122, kindly provided by M. Neuberger, was also loaded onto the gel. This cell line is a stable transfectant of J558L containing the plasmid pSV-V μ 1 which also encodes the anti-NP μ heavy chain, but under the control of the natural VNP promoter (Neuberger, 1983).

The results shown in Fig. 5.11 indicate that good induction of the μ mRNA was obtained for several of the transfectants. All inductions gave rise to two bands upon heat shock; one which migrates close to the band in the control JW122 at around 2.4 kb, and a higher molecular weight band 300 or so bases larger. One possible explanation for the origin of the latter band is that the μ_s poly(A) site is not 100% efficient, resulting in a proportion of the transcripts being polyadenylated at the downstream SV40 late region polyadenylation site. The size of such an mRNA molecule would be consistent with that observed here.

FIGURE 5.11

A



B

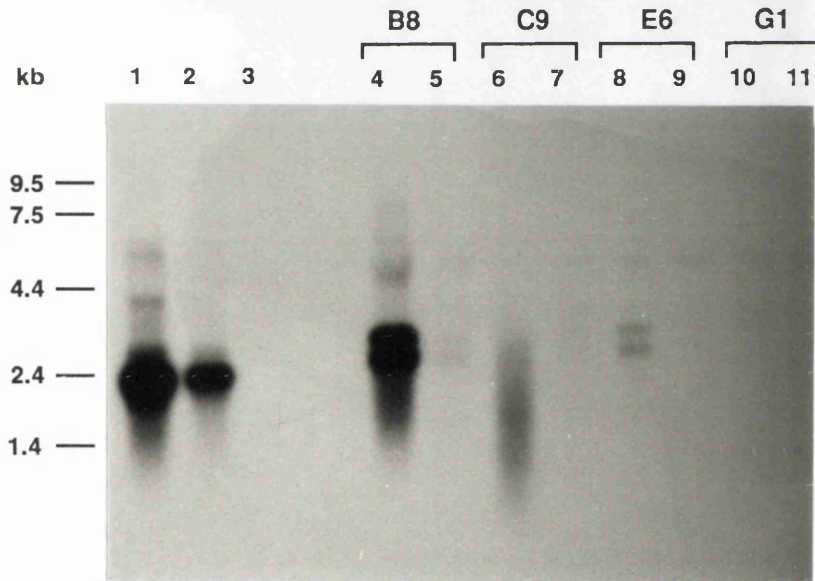


Figure 5.11: Heat shock induction from p372 μ sgpt in J558L cells.

(A) and (B) show the results of two Northern blots of total RNA from various cell lines derived from transfection of J558L cells with p372 μ sgpt. Lanes 1 and 2 contain 2.5 μ g and 1 μ g total RNA from JW122 cells, respectively (see text). Lane 3 contains 2.5 μ g total RNA from untransfected J558L cells. Lanes 4,6,8 and 10 contain samples of RNA from heat shocked cells, and lanes 5,7,9 and 11 contain RNA prepared from non-heat shocked cells. The names of the cell lines are indicated above the appropriate lanes.

Further experiments to confirm this hypothesis are described below. Despite the slightly unexpected result of obtaining two bands, it is clear that 4/8 of these lines give good induction, B8 producing the highest level of induction, followed by A2 and E6. A6 has a high background level of transcription, and C9 also seems to induce although that RNA preparation is degraded, so it is not possible to determine the level of induction in this case. Cell line B8 was used as a positive control in the analysis of other transfectants, and was also used for half-life experiments, described below and in Section 6.2 respectively.

Having established heat shock conditions which gave good induction of the promoter, cell lines from the p516 μ_s neo Δ E and p516neo Δ E transfections were heat shocked in the same way. The RNA samples from heat shocked cells were initially analysed in the absence of the samples from non-heat shocked cells, using a slot-blot apparatus as described in Section 2.3.6. The use of the slot-blot apparatus facilitated the handling of many samples. The heat-shocks were carried out in several batches, using the B8 cell line as a positive control for each batch. After transfer of the RNA to nitrocellulose, the filter was hybridised to the pSP64 μ NP probe exactly as described previously for Northern blots (Section 2.3.5), and the result of this experiment is shown in Fig. 5.12. The B8 samples give reasonably consistent signals for each heat shock (indicated by the letter C in Fig. 5.12), indicating that the heat shock procedure was reproducible. RNA preparations from several lines which gave a strong signal in the slot-blot experiment were selected for Northern blot analysis. These were p516neo Δ E:- A1, B3, D6; p516 μ_s neo Δ E:- A2, B11, D3, and D5.

RNA samples from heat shocked and non-heat shocked cells were analysed by Northern blotting, probing with the pSP64 μ NP probe as described above, and the result is shown in Fig. 5.13. The samples from heat shocked cells of all the cell lines derived from p516 μ_s neo Δ E show two bands which migrate close to those from the control B8 cell line. This is consistent with the hypothesis that the two bands arise from alternative polyadenylation sites, as p516 μ_s neo Δ E also contains the μ_s and the SV40 polyadenylation sites downstream of the μ gene. The two species of μ mRNA from the p372 μ_s gpt

FIGURE 5.12

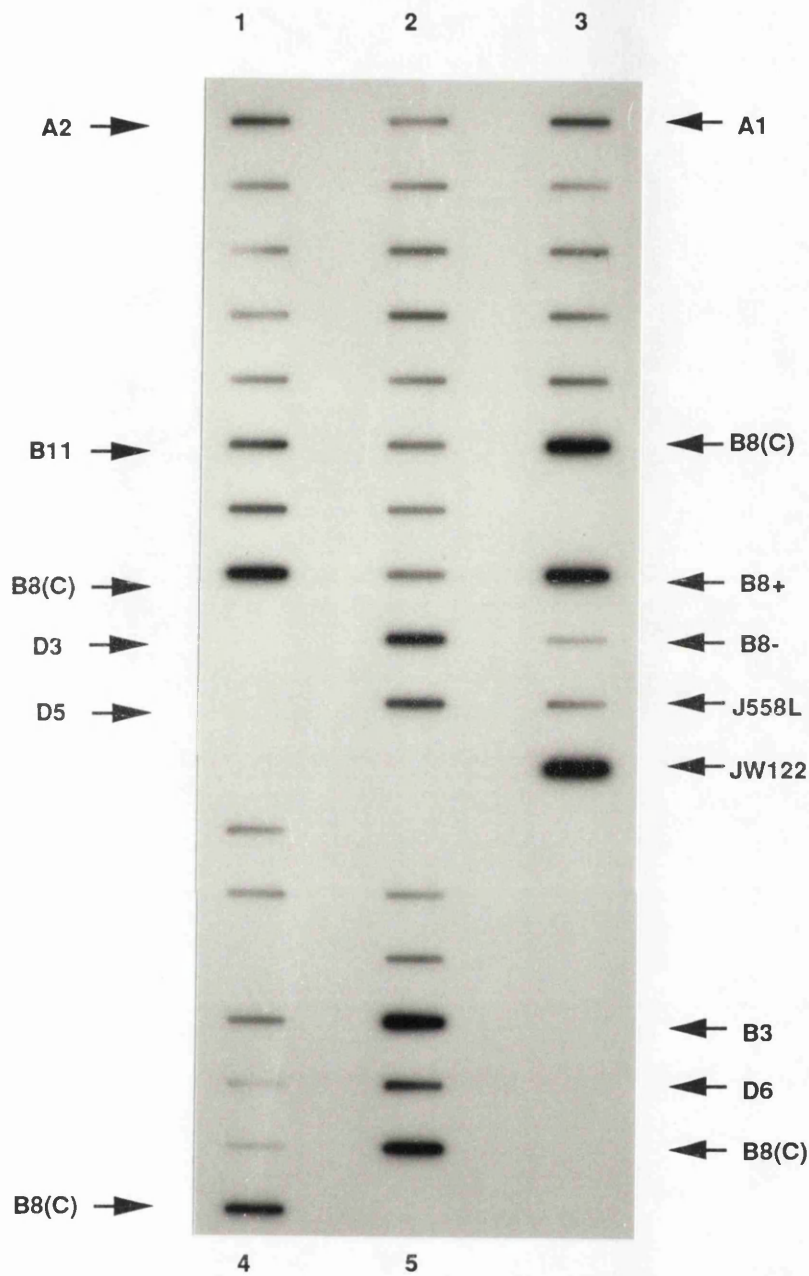


Figure 5.12: Heat shock induction from p516neo Δ E and p516 μ sneo Δ E.

Various cell lines derived by transfection of J558L cells with the plasmids p516neo Δ E and p516 μ sneo Δ E were heat shocked as described in the text. 2.5 μ g of total RNA from the heat shocked cells was analysed using a slot blot apparatus. The heat shocks were done in batches, and a positive control, B8 (see Fig. 5.11) was included with each batch. Batches 1 and 2 are cell lines derived from p516 μ sneo Δ E and batches 3, 4 and 5 are derived from p516neo Δ E. The strongest positives are labelled with their cell line name.

FIGURE 5.13

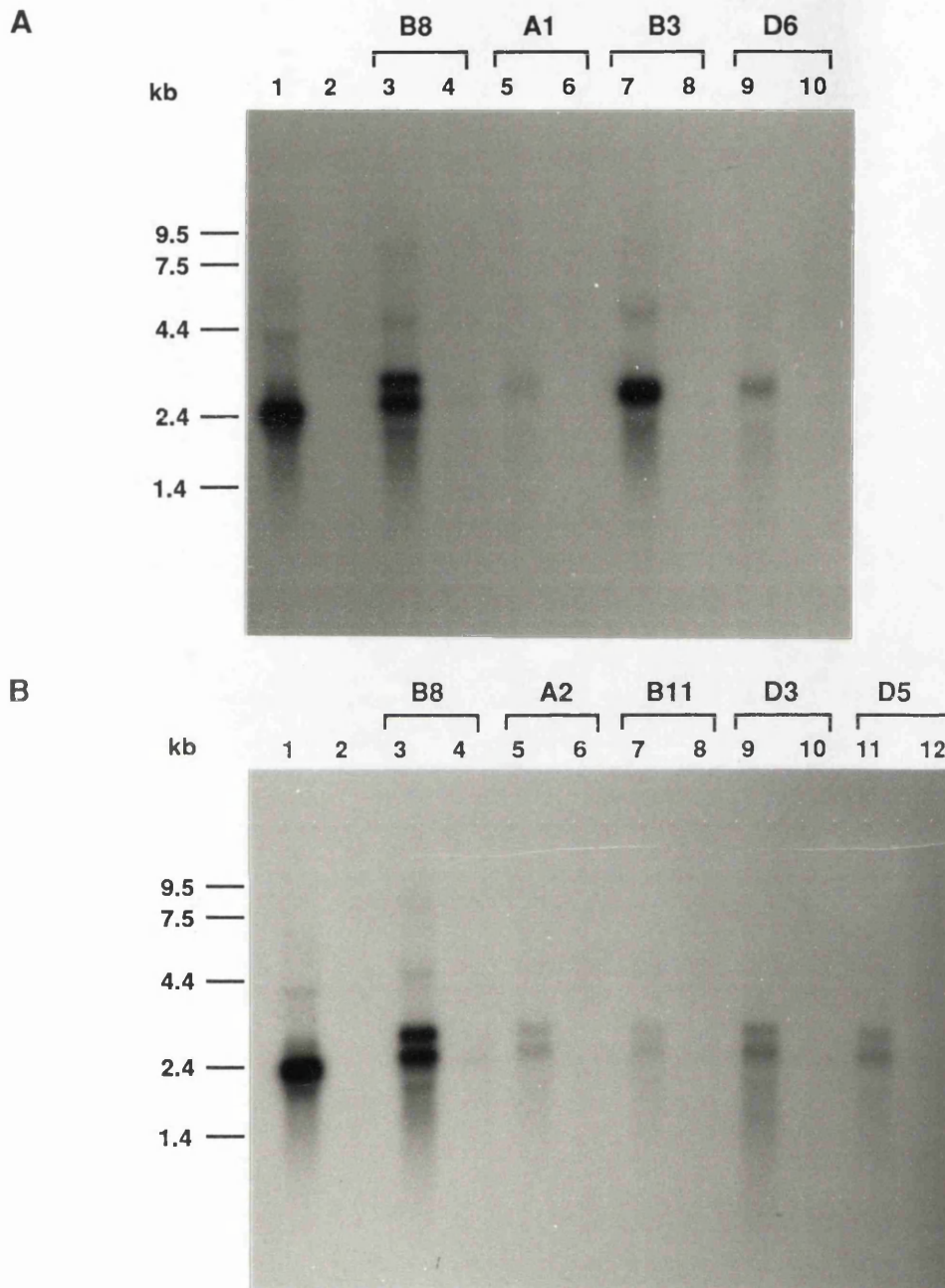


Figure 5.13: Heat shock induction from p516neo Δ E and p516 μ sneo Δ E: Northern blot.

The results of two Northern blots of total RNA from cell lines which gave a positive signal in the slot blot assay (Fig. 5.12). Lanes 1 and 2 contain 2.5 μ g total RNA from JW122 cells and J558L cells, respectively. Lanes 3, 5, 7 and 9 contain samples of RNA from heat shocked cells, and lanes 4, 6, 8 and 10 contain RNA prepared from non-heat shocked cells. The line B8 (lanes 3 and 4) was included as a positive control.

(A) Cell lines from p516neo Δ E.

(B) Cell lines from p516 μ sneo Δ E.

construct would both be expected to be 62 nucleotides longer than the corresponding species from p516 μ_s neo Δ E, but this difference is not detectable on these gels. The 516neo Δ E derived samples contain a single band, consistent with the presence of only the SV40 polyadenylation signal on this plasmid. All the cell lines tested in this experiment have low background levels of transcription in the non-heat shocked controls, so the cell line from each transfection with the highest level of induction was chosen for half-life experiments; p516 μ_s neo Δ E/D3 and p516neo Δ E/B3. The experiments to measure μ mRNA half-life in these cell lines are described in Chapter 6. In addition, further transfection experiments were carried out, to investigate the origin of the higher molecular weight band, and to transfect the plasmid containing the mutant leader sequence into J558L cells.

Transfection experiments (ii):- linearisation at the *Hind* III site.

If the two μ mRNA species do arise from alternative polyadenylation sites, it should be possible to prevent the production of the larger species by linearising the plasmid between the two sites prior to transfection, as transfection of linear DNA normally leads to integration at the ends of the molecule (Folger et al, 1982; Roth et al, 1985; Thomas et al, 1986). The plasmids p516 μ_s gpt and 516DM μ_s gpt were therefore linearised at the *Hind*III site (see Figs. 5.5 and 5.10) and transfected into J558L cells. Transfections, selection, grow up of single colonies and heat shock of the resulting cell lines was carried out as before, and Fig. 5.14 shows a Northern blot of total RNA from heat-shocked and non-heatshocked cells of some of these lines. It is clear that the pattern of bands produced is more complex than was anticipated. Two lines, F8 and A12, from the 516DM μ_s gpt transfection (Fig 5.14A), seem to induce for a band of the expected size after heat shock, but contain two higher molecular weight bands before heat shock which decrease in intensity after heat shock. B3 and B1, from the p516 μ_s gpt transfection (Fig. 5.14B), contain bands of similar sizes, but in this case all three bands decrease in intensity after heat shock. Other cell lines, like H9, F6, G2, E3, and A7 do

FIGURE 5.14

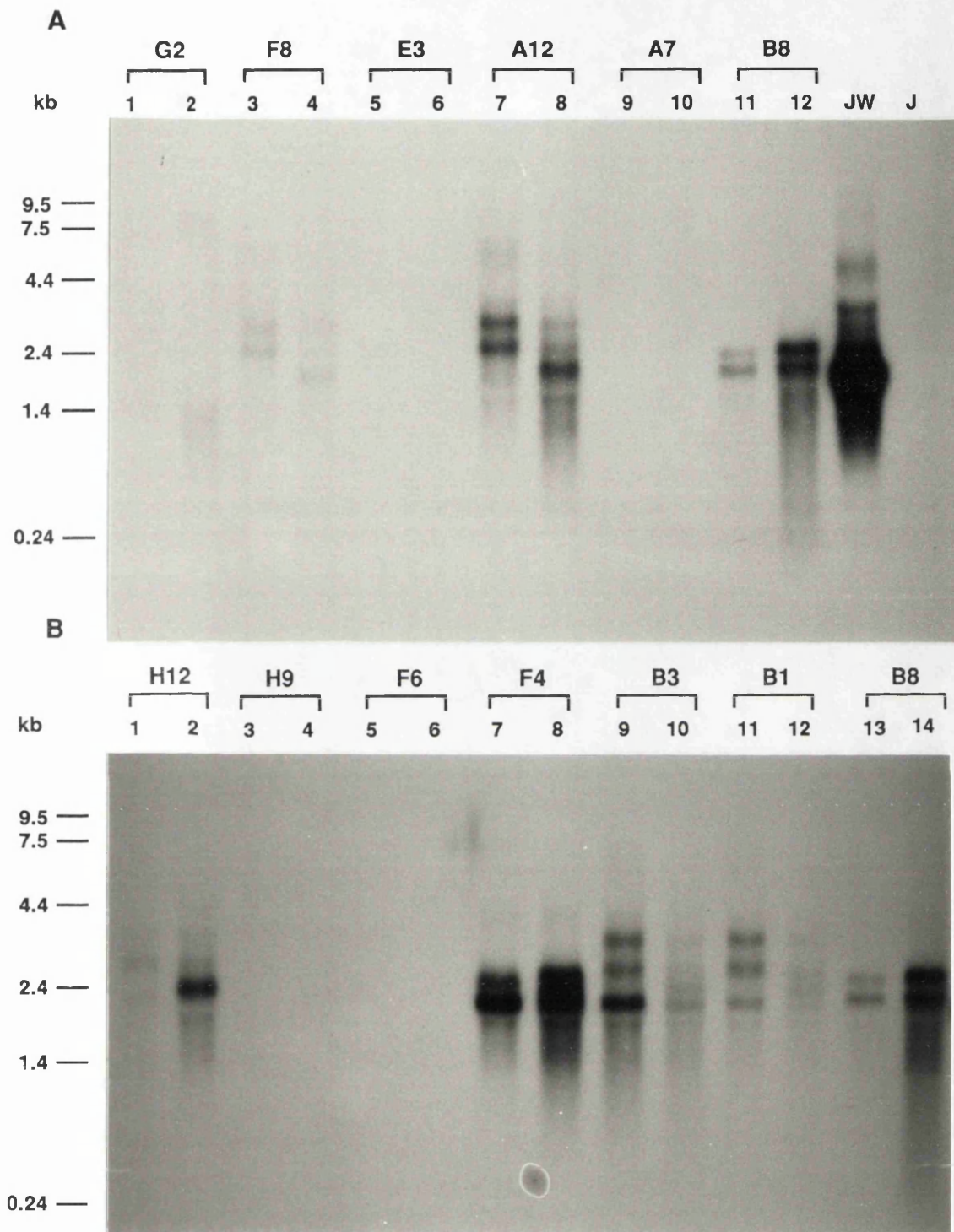


Figure 5.14: Transfectants derived from plasmids linearised at the *Hind* III site.

Plasmids p516DM μ sgpt and p516 μ sgpt were linearised at the *Hind* III site prior to transfection, rather than the *Eco* RI site used previously (Figs. 5.11-13). Lanes 1,3,5,7,9,11 and 13 contain total RNA from non-heat shocked cells and lanes 2,4,6,8,10,12 and 14 contain RNA from heat shocked cells. The two right-hand lanes contain 2.5 μ g total RNA from JW122 and J558L cells respectively.

(A) Cell lines derived from p516DM μ sgpt.

(B) Cell lines derived from p516 μ sgpt.

not contain detectable μ mRNA before or after heat shock. Only one line, p516 μ _sgpt/H12, produces a high level of the expected sized band after heat shock, but it still contains a low level of the two higher molecular weight bands.

It is clear then, that rather than producing a single μ mRNA species as expected, linearisation at the Hind III site has lead to the production of further higher molecular weight bands. It is most likely that these aberrant transcripts have arisen from read-through transcription from the SV40 late region promoter (see Figs. 5.4B and 5.10), but they could also have arisen from promoters in the J558L genome, which are adjacent to the integration sites of the plasmids. However, the uniformity of the sizes of the bands from different transfected lines argues against the latter hypothesis. These cell lines were not thought to be suitable for use in half-life experiments, due to the presence of multiple transcripts through the same region, so the spurious bands were not investigated further. Instead, another approach was taken to confirm the origin of the higher molecular weight band in the *Eco* RI-linearised transfectants, that of probing a Northern blot of these lines with an SV40 late region probe.

A plasmid which could be used to generate an RNA probe to detect SV40 late region transcripts was prepared as follows. The plasmid EE8 (Fig. 5.15A) was prepared from *E. coli* gm242, a *dam*⁻ strain, to allow restriction with *Bcl* I. (*Bcl* I is sensitive to *dam* methylase.) This plasmid was then digested with the restriction enzymes *Bcl* I and *Bam* HI, and the 235 base pair fragment containing the polyadenylation region of SV40 was isolated by preparative gel electrophoresis. As restriction with *Bcl* I produces cohesive ends that are compatible with *Bam* HI, this fragment could be inserted into the *Bam* HI site of pSP65. pSP65 was restricted with *Bam* HI, treated with calf intestinal phosphatase, and the resulting fragment isolated from an agarose gel. The two fragments were ligated together and transformants were obtained containing the insert in both orientations, as determined by restriction enzyme analysis (Fig. 5.15B). pSP65SV40pA9 was used to generate ³²P-labelled RNA for probing Northern blots, as described in Section 2.3.7. This plasmid

FIGURE 5.15

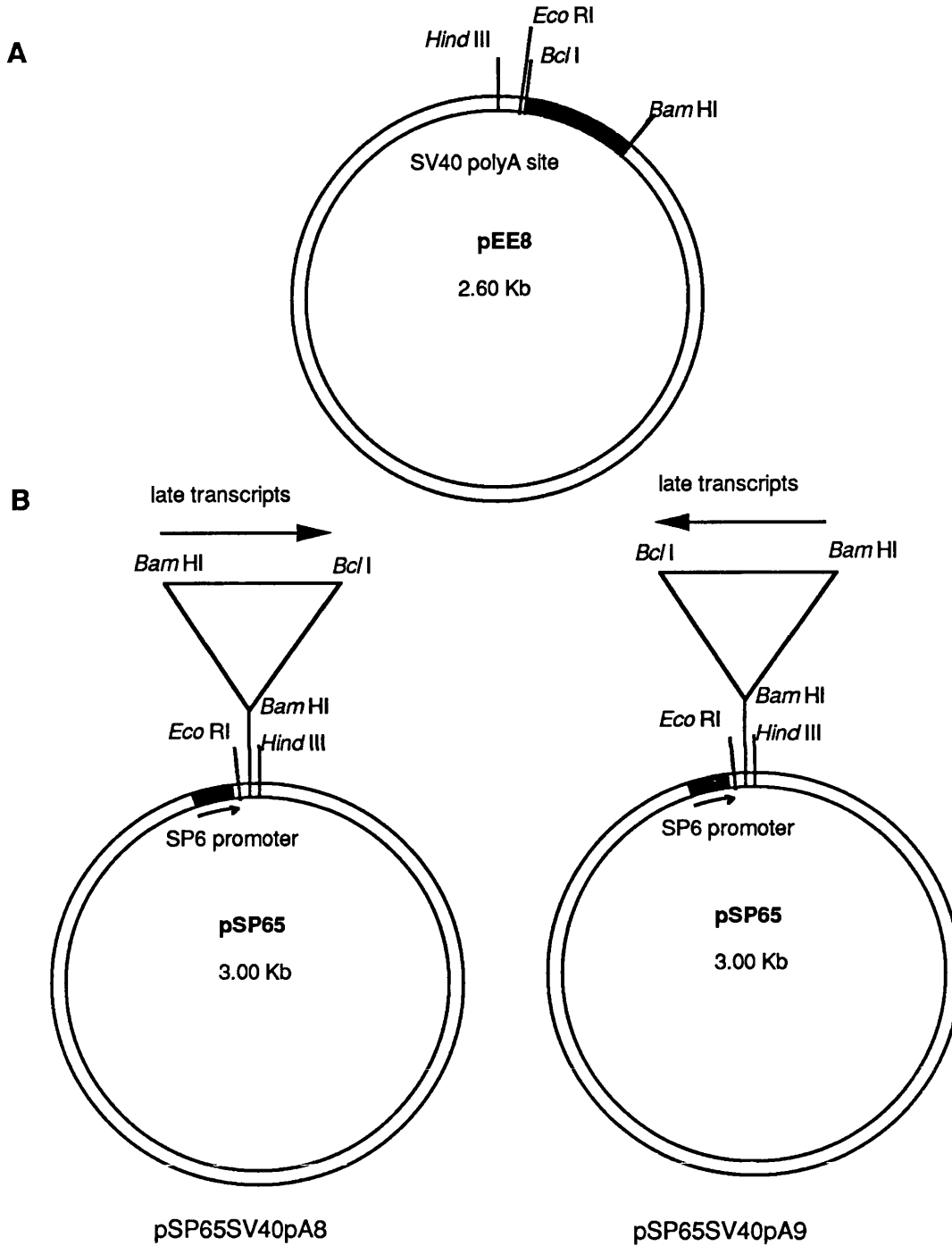


Figure 5.15: Construction of plasmids containing the SV40 late region polyadenylation site.

(A) pEE8, used to obtain the *Bcl* I-*Bam* HI fragment containing the SV40 late region polyadenylation site.

(B) The resulting plasmids containing the SV40 late region polyadenylation site in both orientations in pSP65.

contains the insert in the orientation such that SP6 transcripts from it are complementary to SV40 late region RNA.

A Northern Blot was then prepared using RNA from several of the cell lines described above. Two of these, p516 μ _Sgpt/H12 and 516DM μ _Sgpt/A12, are derived from transfections of the *Hind* III-linearised plasmids, and three others, p372 μ _Sgpt/B8, p516 μ _Sneo Δ E/D3 and 516neo Δ E/B3, are derived from *Eco* RI-linearised plasmids. This blot was probed with the SV40 probe and the result is shown in Fig. 5.16A. The probe detects a single band in RNA from the three cell lines containing the *Eco* RI-linearised plasmids, and there is no signal in the tracks containing the RNA derived from *Hind* III-linearised plasmids, as would be expected. The sizes of the bands in B8, D3 and B3 correspond to those detected with the pSP64 μ NP probe which are predicted to contain SV40 sequences; namely the single B3 band and the upper of the two bands in B8 and D3. To confirm that this was the case, the blot was stripped and reprobed with the pSP64 μ NP probe, and the single band in B3, and the higher molecular weight bands in B8 and D3, were found to exactly correspond to the bands hybridising to the SV40 probe (Fig. 5.16B). This indicates that these mRNA species do contain SV40 late region sequence, confirming the hypothesis that they arise from polyadenylation at the SV40 polyadenylation site. The H12 and A12 μ mRNA species give a much weaker signal, but are clearly visible on a longer exposure of the autoradiograph (not shown). The lower level of expression may be a result of transcriptional interference between the two promoters (see Discussion).

No further experiments were carried out with the cell lines derived from plasmids linearised at the *Hind* III site, owing to the complexity of the mRNA species produced (Fig. 5.14) and the low level of induction compared to the other cell lines (Fig. 5.16B). The two μ mRNA species produced from the *Eco* RI-linearised plasmids on the other hand are well defined and highly inducible on heat shock. Further transfections were therefore carried out with p516 μ _Sgpt and 516DM μ _Sgpt plasmids linearised at the *Eco* RI site.

FIGURE 5.16

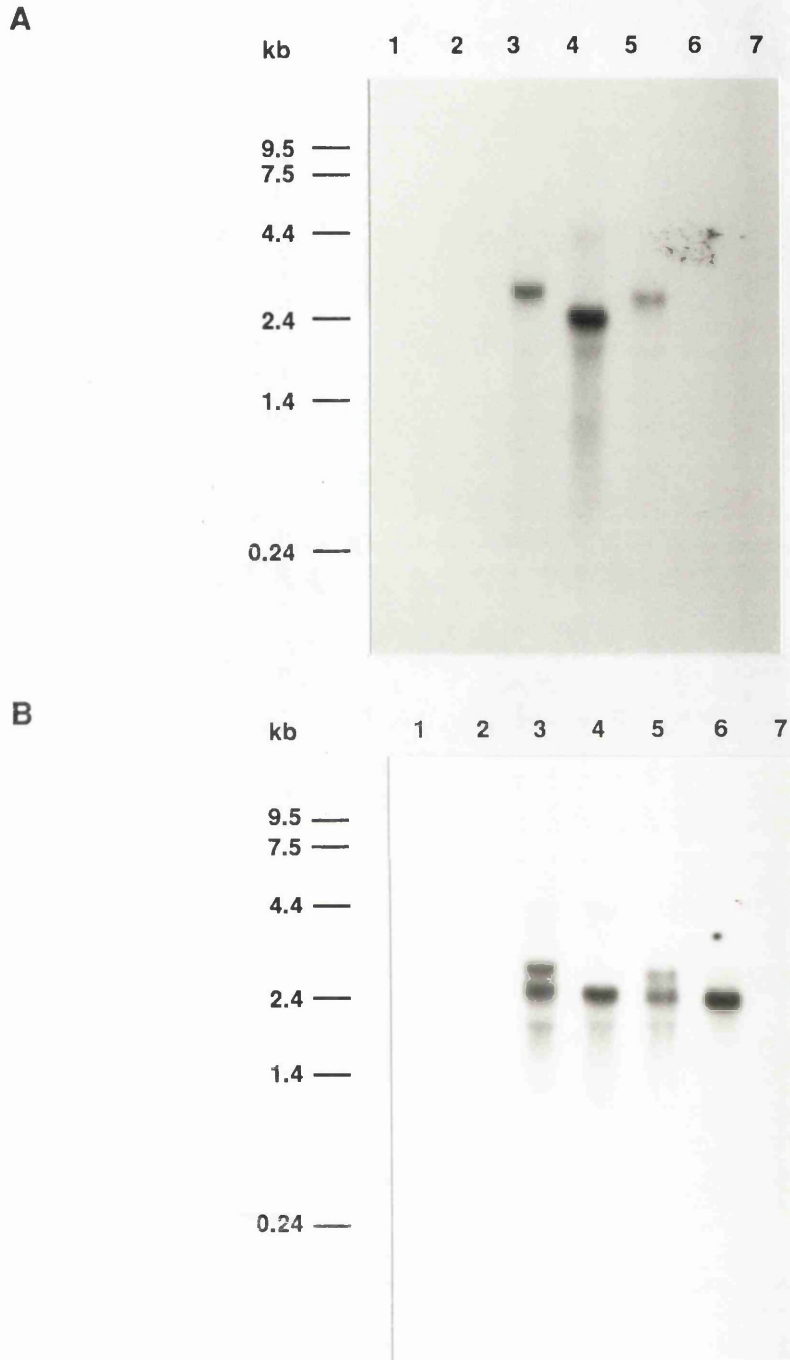


Figure 5.16: Experiment to identify mRNA species containing SV40 sequences.

A Northern blot was carried out with total RNA from heat shocked cells from various transfected cell lines, and probed with (A) pSP65SV40pA9 and (B) pSP64 μ NP. Lane 1; p516 μ sgpt/H12, lane 2; p516DM μ sgpt/A12, lane 3; p372 μ sgpt/B8, lane 4; p516neo Δ E/B3, lane 5; p516 μ sneo Δ E/D3, lanes 6 and 7; 2.5 μ g total RNA from JW122 and J558L cells respectively. Lanes 1 and 2 are examples derived from plasmids linearised at the *Hind* III site and lanes 3-5 are derived from plasmids linearised at the *Eco* RI site.

Transfection experiments (iii):- the Eco RI site revisited

After transfection into J558L of p516 μ _Sgpt and 516DM μ _Sgpt plasmids linearised at the Eco RI site, cell lines were isolated as before and initially screened by slot-blotting (not shown). Those lines which gave the highest signals were then analysed by Northern blot as before, ^{with the μ NP probe} and the result is shown in Fig. 5.17. Once again two bands were produced on induction in all the cell lines investigated, at the same positions as seen previously. In addition, two lines, 516DM μ _Sgpt/D3 and D6, produce a low molecular weight band below 1kb. This band was not investigated further at this stage, but it may be a stable degradation intermediate produced in the cells, or a result of the RNA preparation procedure. These two preparations look more degraded than the others, indicating the presence of ribonuclease during the preparation. All the lines shown give good induction, and p516 μ _Sgpt/Z5 and 516DM μ _Sgpt/D6 and D1 were chosen for half-life analysis. These experiments are described in Chapter 6.

5.3 Discussion

This chapter describes the construction of plasmids containing various μ genes under the control of the *Drosophila* hsp70 heat shock promoter, and the transfection of these plasmids into J558L cells. One or two cell lines from each transfection were selected to be used in experiments to determine the half-life of μ mRNA, and the structure of the μ gene contained in each of these lines is summarised in Fig. 5.6. During the course of the generation of these lines, several interesting observations were made.

Firstly, two μ mRNA species of different sizes were found to be generated from the same transcription unit. An SV40 late region probe was used to demonstrate that the longer of these mRNA molecules was derived by polyadenylation of the transcript at the SV40 late region polyadenylation site. The two species are produced in approximately equal proportion, indicating that the μ _S site is approximately 50% efficient in the presence of the SV40 polyadenylation site. This result is interesting when compared to the results obtained by Peterson and Perry (1989), who were investigating

FIGURE 5.17

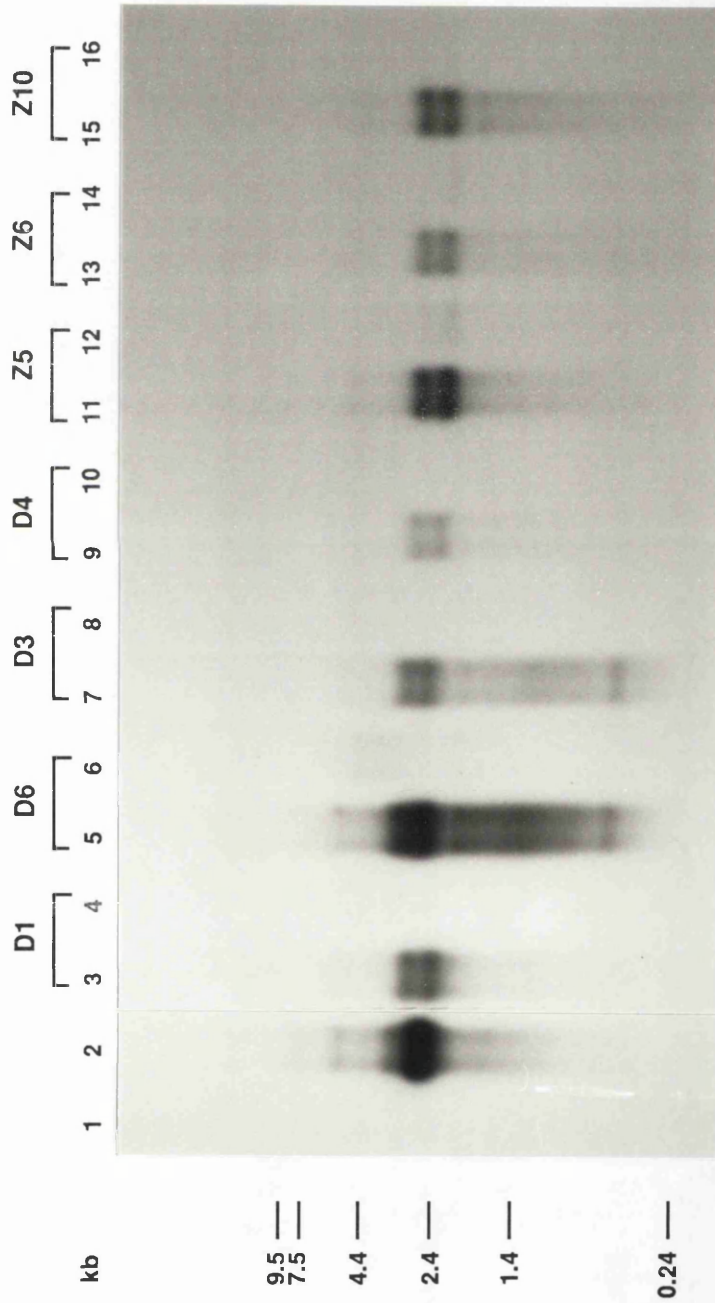


Figure 5.17: Heat shock induction from mutant and control plasmids linearised at the *Eco* RI site.

Lanes 3,5,7,9,11,13, and 15 contain total RNA from heat shocked cells, and lanes 4,6,8,10,12,14, and 16 contain total RNA from non-heat shocked cells. Lanes 1 and 2 contain 2.5µg of total RNA from J558L and JW122 cells respectively. Z10, Z6 and Z5 are derived from p516µsgpt, and D4, D3, D6 and D1 are derived from p516DMµsgpt.

the regulation of μ_s and μ_m mRNA production using a transient transfection system. When they substituted the SV40 late polyadenylation site for the μ_m polyadenylation site, and assayed the mRNA produced in plasmacytoma cells S194, they detected 11 fold more mRNA polyadenylated at the μ_s site. However, when they carried out the same experiment with part of the intron between the secreted and membrane exons deleted, (i.e. with a reduced distance between the polyadenylation sites), the ratio was reduced to 1.2, a value much closer to that observed here. These results are consistent with the hypothesis that when either μ_s and μ_m or μ_s and SV40 polyadenylation sites are available, the distance between the two sites is one of the factors which affects their relative usage. The greater the distance between the sites, the longer the time interval during which the upstream site is available on the nascent transcript, and hence the greater proportion of the total mRNA is polyadenylated at the upstream site (Guise et al, 1989). As discussed in Chapter 1, this is an important factor in the control of the relative levels of μ_s and μ_m mRNA during B cell development (Peterson and Perry, 1989; 1986; Galli et al, 1987, 1988).

The second interesting observation was that when the transfecting plasmids were linearised 3' to the heat shock promoter, at the *Hind* III site, new mRNA species appeared which were down-regulated after the heat shock. Such transcripts must be being spliced, to yield bands of the observed sizes, perhaps using cryptic splice donor sites and the acceptor site at the 3' end of the heavy chain leader intron. As the sizes of the aberrant transcripts appear very similar in several transfectants, it seems reasonable to suggest that they arise from the SV40 late region promoter present in the plasmid in the same orientation as the heat shock promoter. The transcripts are indeed of the same sense as those from the heat shock promoter, as they are detected with the single-stranded μ NP probe. Such transcripts would be likely to disappear when the heat shock promoter was activated, due to transcriptional interference (Kadesch and Berg, 1986; Proudfoot, 1986), as is observed. The insertion of sequences known to be polymerase II termination regions into plasmids with multiple transcription units is an obvious

means of avoiding this problem in the future, and one such termination region has been identified downstream of the μ_m polyadenylation site (Law et al, 1987).

The presence of two transcripts from the *Eco* RI-linearised plasmids was not seen to be a particular problem, as the two mRNAs are easily distinguishable on a Northern blot, and both are tightly regulated by the heat shock promoter. Therefore these transfectants were chosen for half-life analysis in preference to those derived from the *Hind* III-linearised plasmids. Another reason for studying these transfectants is that they allow comparison of the rate of decay of the longer mRNA, (containing both the μ_s and SV40 polyadenylation signals), with the control mRNA, in the same cells. The longer mRNA may or may not have the same half-life as the mRNA with only the SV40 polyadenylation signal produced from p516neo Δ E.

CHAPTER 6

MEASUREMENT OF μ mRNA HALF-LIFE USING HEAT SHOCK
INDUCTION**6.1 Introduction**

Chapter 5 described the construction of a series of cell lines derived by the transfection of the myeloma cell line J558L with plasmids containing various μ genes under the control of the *D. melanogaster* heat shock promoter. The structures of the mutant μ mRNAs produced in these cell lines are summarised in Fig. 5.6. This Chapter describes experiments to compare the rates of decay of the μ mRNAs after heat shock induction, using Northern blot analysis, and also to characterise the μ protein which they produce. Preliminary experiments were carried out with three of the cell lines described in Chapter 5, those containing the μ mRNA species shown in Fig. 5.6A-C, and a more detailed analysis was carried out on cell lines containing the mutant μ mRNA indicated in Fig. 5.6D.

6.2 Results**6.2.1: Probes used for Northern blot analysis.**

Three SP6-based plasmids were used to generate the ^{32}P -labelled probes used in the experiments described below. These were the pSP64 μ NP probe described in Section 4.2.1 (Fig.4.1A), pSP65rRNA, also described in Section 4.2.1 (Fig. 4.1B), and in addition, a probe to detect actin mRNA. The latter was used as an internal control for the amount of mRNA loaded in each track of formaldehyde/agarose gels. As before, the probe sequence was inserted into a vector containing the SP6 promoter, in order to generate RNA probes *in vitro*. This was done as follows. The plasmid pAM91 (Minty *et al*, 1981) containing the coding region of the mouse α -actin gene, was digested with restriction enzymes *Eco* RI and *Bam* HI. A 1.4kb fragment containing 730 bp of actin sequence was then isolated by preparative agarose gel electrophoresis, and ligated to pSP64 DNA which had been digested with the same enzymes and purified in the same manner. After transformation of the ligation mix into *E.*

coli LM1035, mini-plasmid preparations from the resulting transformants were test-digested with appropriate restriction enzymes to identify a clone containing the correct insert. The resulting plasmid, pSP64actin, was linearised at the *Eco*RI site for use in SP6 transcription reactions. Fig. 6.1 shows the region of the probe transcript that will hybridise to actin mRNA.

6.2.2: Quantitative Northern Blot Analysis.

As was illustrated in Chapter 4, it is important that the amount of total RNA loaded onto Northern blots is such that the hybridisation conditions are quantitative. As the signals from the heat shock transfectants are relatively low compared to equivalent loadings of MXW231 RNA (data not shown), it was expected that the Northern blot hybridisations would be in the linear range. This was confirmed using the control JW122 cell line (described in Chapter 6), which yields a more intense μ NP signal for equivalent loadings than any of the cell lines transfected with the heat shock promoter constructs. 1 μ g and 2.5 μ g of JW122 RNA were loaded onto a formaldehyde/agarose gel, which was blotted and probed using the pSP64 μ NP probe as described previously. A series of timed exposures of the autoradiograph were obtained and densitometric scanning was carried out on each track for each exposure, as described in Section 2.3.5. The optical density signal of the μ NP band was then plotted against the exposure time, and the results are shown in Fig. 6.2. The relative amounts of μ NP mRNA in the two tracks can be determined from the ratio of the slopes of these lines, and this was found to be 2.6, in good agreement with the amount of total RNA loaded. As the hybridisation conditions are quantitative for JW122 RNA, it seems reasonable to assume that they will also be quantitative for the cell lines transfected with the heat shock promoter constructs, provided the loadings are kept such that the signal is below that obtained from 2.5 μ g JW122 RNA. Therefore a known amount of JW122 total RNA was loaded on each gel used to determine half-life, to confirm that the signals from the test samples were within this range.

FIGURE 6.1

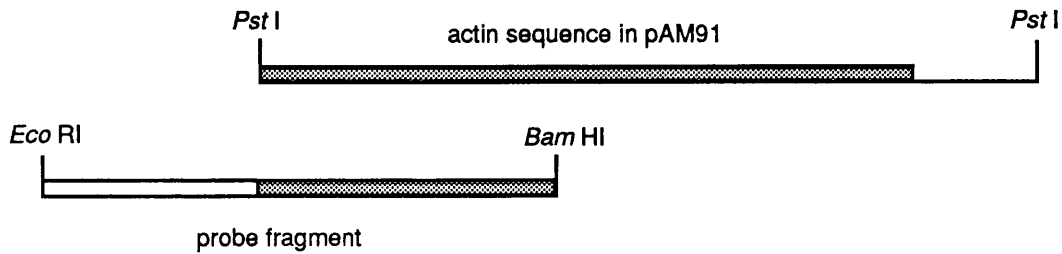


Figure 6.1: Actin probe.

The actin cDNA contained in the plasmid pAM91 (Minty *et al*, 1981) is shown at the top, with the coding sequence shaded. The *Eco* RI to *Bam* HI fragment from pAM91 which was subcloned into pSP64 is shown underneath, with the actin sequence shaded. The remainder of this fragment contains pBR322 sequence.

FIGURE 6.2

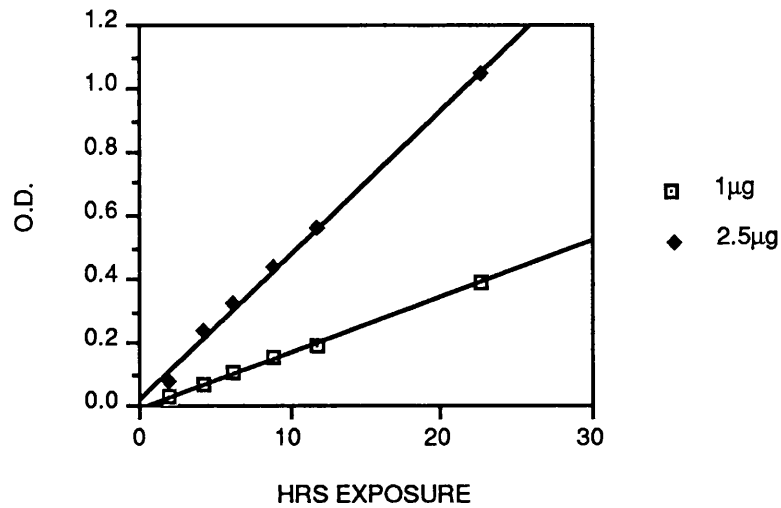


Figure 6.2: Quantitation of Northern blots.

2.5µg and 1µg of total RNA from JW122 cells were loaded on a formaldehyde/agarose gel for Northern blotting as described in the text. The blot was probed with the µNP probe and a series of timed exposures of the resulting autoradiographs were obtained. These were scanned with a densitometer, and the optical density from the µ mRNA peak was plotted as a function of exposure time. The values of R-squared for the lines of best fit were 1µg:0.999, 2.5µg:0.997.

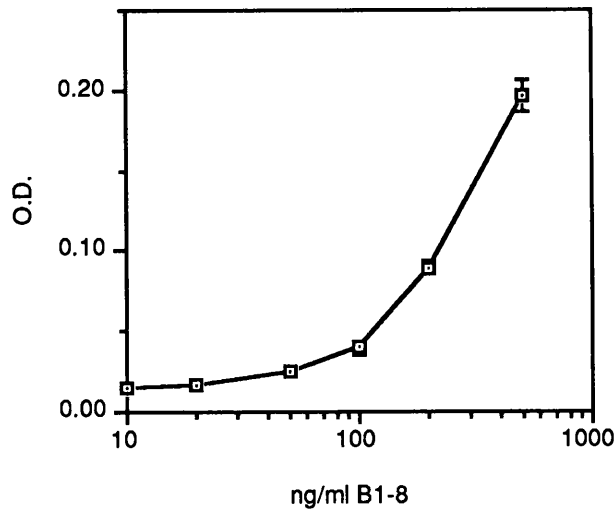
6.2.3: Induction of μ mRNAs containing leader sequence from the *tk* gene, and SV40 late region polyadenylation sequence.

The first transfectants to become available were those containing the constructs shown in Fig. 5.6A-C. These were 516 μ _sneo Δ E/D3 (wild-type), 516neo Δ E/B3 (SV40 polyadenylation sequence), and 372 μ _sgpt/B8 (*tk* leader), referred to as D3, B3, and B8, respectively. Derivation of these cell lines is described in Section 5.2.3. For the half-life experiments, the heat shock promoter was induced as before by taking cells in exponential growth, subjecting them to a heat shock at 42.5°C for one hour, then diluting them into growth medium at 37°C. This procedure is described in detail in Section 2.5.8. At suitable time points after the heat shock, equal volumes of cells were harvested for the preparation of total RNA as described in Section 2.3.2. Viable cell counts were also determined at each time point after the heat shock, to allow an estimate of the cell doubling time to be made.

To check that the heat shock inductions were successful, anti-NP ELISA assays were carried out on supernatant samples obtained at various times after the heat shock. The ELISA, described in Section 2.4.2, makes use of the fact that VNP heavy chain produced after heat shock induction can combine with the endogenous λ 1 light chain to produce active anti-NP antibody (Neuberger et al, 1984), and thus can be used as a rapid method for checking that μ polypeptide has been produced. To carry out the ELISA, 96-well microtitre plates were coated with NP/BSA (1:1) conjugate, and 100 μ l samples of neat culture supernatant were assayed in duplicate, together with a series of dilutions of standard. The standard used was anti-NP IgM, purified from supernatants produced by the B1-8 hybridoma, from which the μ NP cDNA used for the transfection experiments was obtained (see Chapter 5). Therefore this antibody contains the same heavy chain variable region and NP binding specificity as that produced from the transfectants. The material bound to the plates was detected using a goat anti-mouse IgM antibody conjugated to horse radish peroxidase, which was revealed with chromogenic substrate. A typical set of results from such an assay is shown in Fig. 6.3, where Fig. 6.3A indicates the standard curve and Fig. 6.3B shows the results from supernatants from D3, B3 and B8 cells after heat shock. While it is not

FIGURE 6.3

A



B

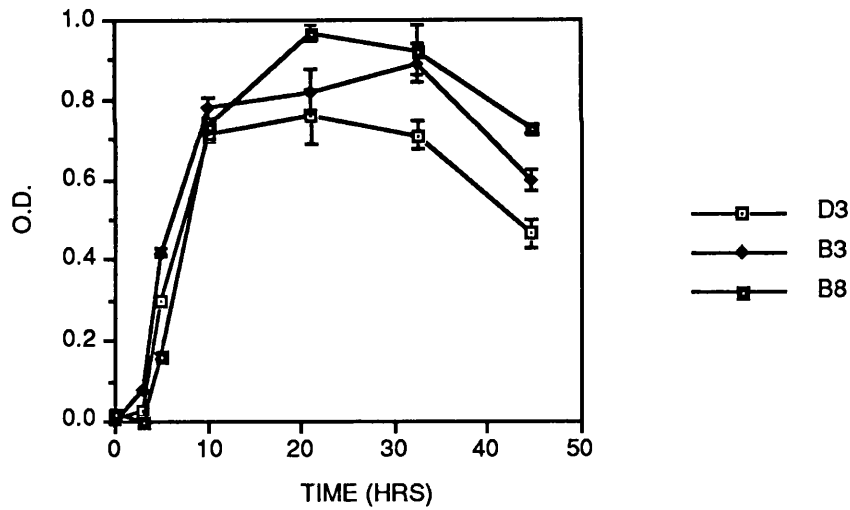


Figure 6.3: Analysis of culture supernatants by ELISA assay.

Anti-NP ELISA assays were carried out as described in the text. (A) shows the result of titration of the standard, B1-8 μ , in duplicate. (B) shows the result of assay of neat supernatant samples taken at various time points during a heat shock induction experiment, where time zero indicates the start of the heat shock.

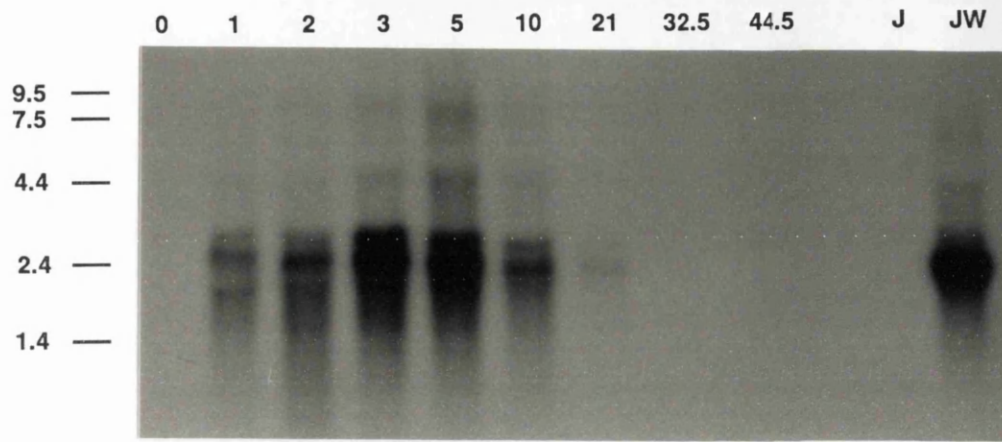
possible to quantitatively determine the amount of antibody produced by the cells without doing a series of dilution curves for each sample, it is clear by comparison of the neat samples with the B1-8 standard that the cells produce greater than 1 μ g/ml of antibody, indicating that the induction has worked efficiently.

Total RNA samples obtained in the above experiment were then analysed by Northern blotting, using 4 μ g of each RNA sample, and the Northern blots were carried out as described previously. 2 μ g of RNA from J558L cells (described in Chapter 4) was loaded as a negative control, and 2 μ g of JW122 cell RNA was loaded as the hybridisation control, as described in Section 6.2.2. The blots were initially probed with the μ NP probe and the results are shown in Fig. 6.4. The pattern of bands observed is the same in each case as was found in the experiments described in Chapter 5 (see Figs. 5.11 and 5.13), and, as predicted from the ELISA result shown in Fig. 6.3, good induction was obtained in all three cell lines. In order to determine whether the loadings in each track were equivalent, the μ NP probe was removed from the filters as described in Section 2.3.5, and the blots were reprobed using the actin probe. Fig. 6.5 shows that a single actin band, of the expected size of 2.1kb (Minty *et al*, 1981) was observed. Whilst there is some variation in the actin signal intensity, particularly in the B3 experiment, it is clear that the diminution in the μ NP signal with time is not solely due to loading variation.

In order to determine the rate of decay of the μ NP mRNA, the hybridisation signal in each track was quantitated by densitometric scanning of a series of timed exposures of the autoradiographs, for both μ NP and actin, as described in Section 2.3.5. It can be seen from Figs. 6.4 and 6.5 that some of the RNA preparations are partly degraded; it is assumed that the μ NP and actin mRNAs are degraded to the same extent so that normalisation to the actin signal takes account of this. In all the results described in this Chapter, optical densities of the peaks have been used, rather than peak areas, although it was found that use of peak areas gave very similar results (not shown). The peak heights were used in preference because of the difficulty of separating the peak areas of the two μ NP bands in the partly

FIGURE 6.4

A



B

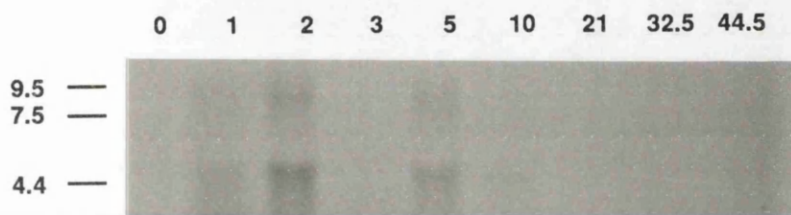


FIGURE 6.5

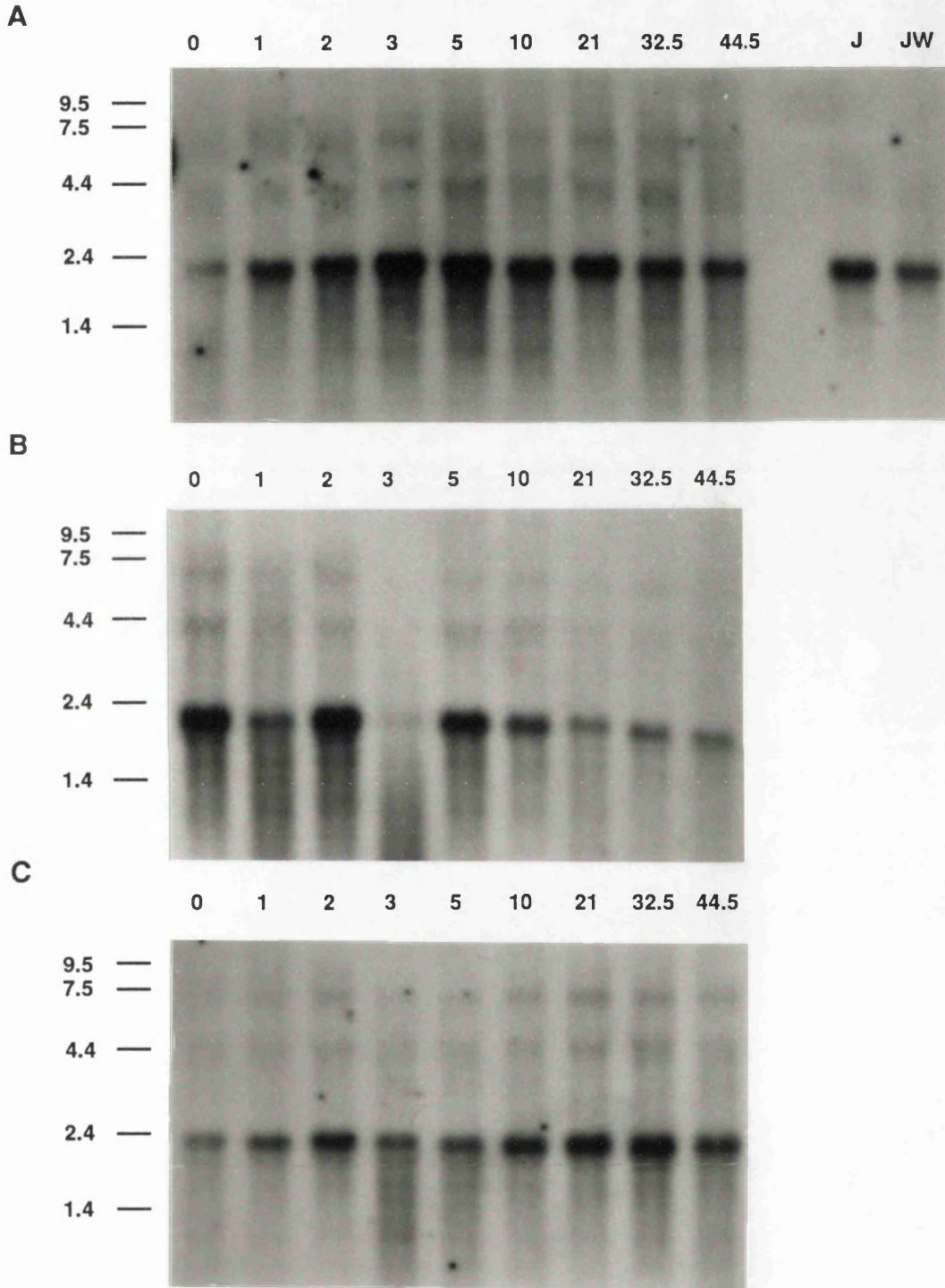


Figure 6.5: Northern blot analysis of total RNA isolated after heat shock induction; actin probe.

The Northern blots shown in Fig. 6.4 were stripped and reprobed with the actin probe. For other details, see the legend to Fig. 6.4.

(A) RNA from D3 cells, (B) RNA from B3 cells, and (C) RNA from B8 cells.

degraded samples. Grossly degraded samples, of which there is only one example, (B3 3 hour time point; Fig. 6.4B), were ignored in the half-life calculations.

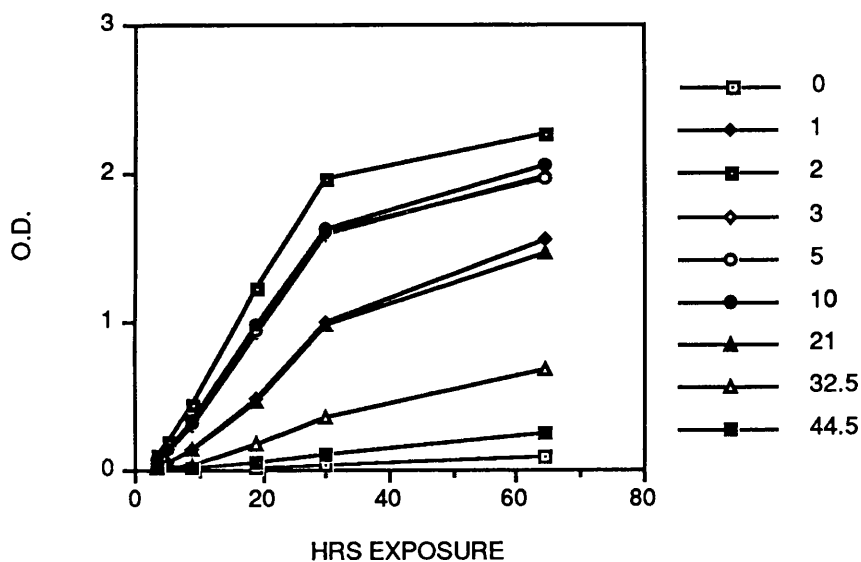
Typical results of the scanning procedure, in this case for the B8 cell line, are shown in Fig. 6.6, where the optical density of the relevant peaks have been plotted against exposure time. The slopes of the linear regions of these graphs were calculated, and, for each time point during the chase, the slope derived from the μ NP probe was divided by that from the actin probe to control for the loading in each track. The μ NP/actin ratio was then plotted on a logarithmic scale against the time after heat shock, (where the zero time point marks the beginning of the heat shock, and the 1 hour point is at the end of the heat shock), to obtain the graphs shown in Figs. 6.7A, 6.8A, and 6.9A. For the D3 cell line (Fig. 6.9A) it was not always possible to resolve the bands derived from polyadenylation at the μ_s and SV40 sites clearly, so the optical density for the most intense band, (i.e. the band derived from polyadenylation at the μ_s site), has been shown, normalised to the optical density of the actin band. The viable cell counts plotted against time are shown in Figs. 6.7B, 6.8B, and 6.9B. For the B3 and D3 cell lines, the rate of cell division is such that the μ NP mRNA is diluted out very quickly, and therefore insufficient time points were obtained to be able to calculate the half-life of μ NP mRNA in these cell lines. However, examination of the autoradiographs (Fig. 6.4) and the graphs (Fig. 6.7-6.9) indicates that the half-lives are likely to be similar. For further work, an alternative cell line Z5, containing the p516 μ_s gpt construct, was used as the control, as this cell line has a slower rate of cell division (see Section 6.2.4 below).

Due to the longer doubling time of the cell line B8, it was possible to calculate the half-life of the B8 μ mRNA, by plotting the values of $\log_e(\mu\text{NP}/\text{actin})$ against time, and calculating the slope of the linear region of the resulting graphs (called S1). To correct for the diluting effect of cell division, the values of $\log_e(\text{cell no.})$ were also plotted against time, and the slope of this line calculated (called S2). The half-life was then calculated using the equation:-

$$T_{1/2} = (\log_e 2) / (S1+S2)$$

FIGURE 6.6

A



B

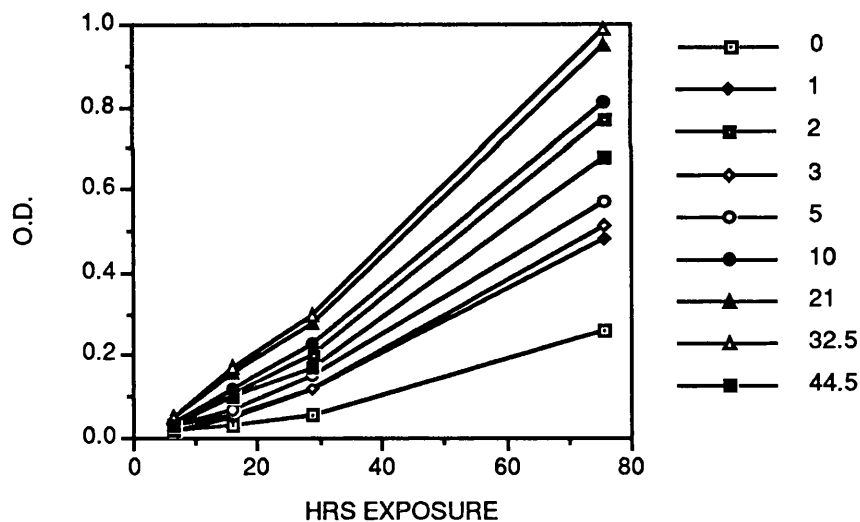


Figure 6.6: Typical scanning results from heat shock induction.

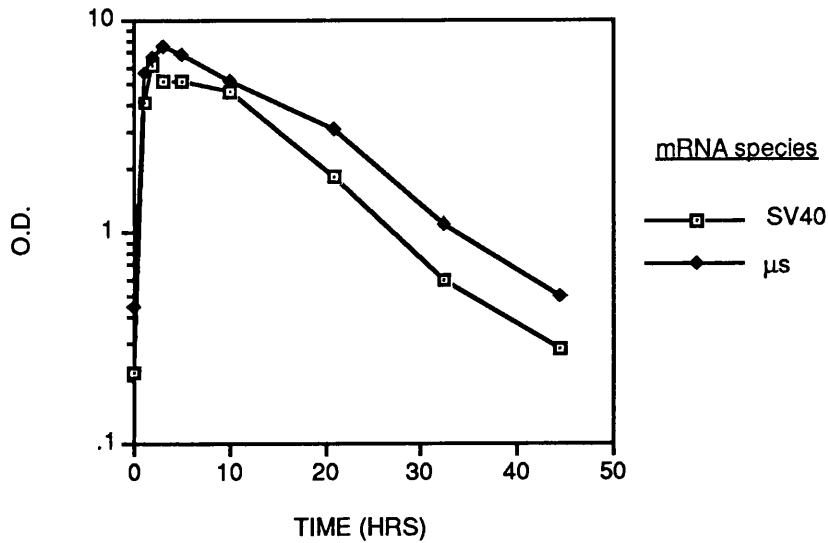
Several exposures of the autoradiographs shown in Figs. 6.4C and 6.5C were scanned with a densitometer, and the optical densities of the peaks were plotted against exposure time for each time point. The time points during the induction are indicated in hours to the right of the Figure.

(A) Data from Fig. 6.4C: μ NP probe, B8 cell line

(B) Data from Fig. 6.5C; actin probe, B8 cell line.

FIGURE 6.7

A



B

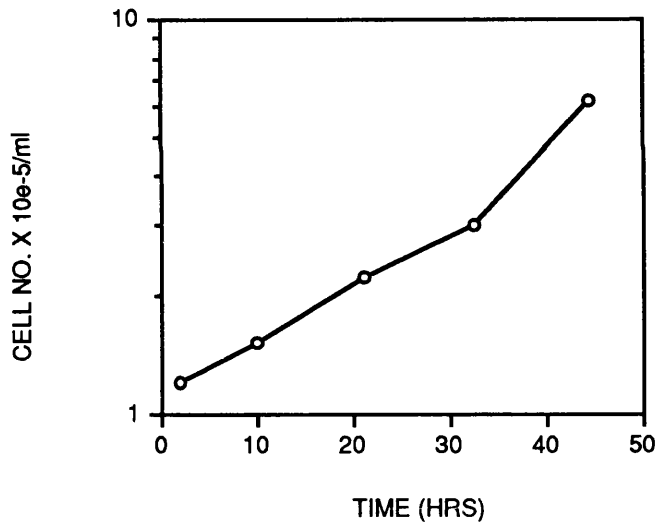


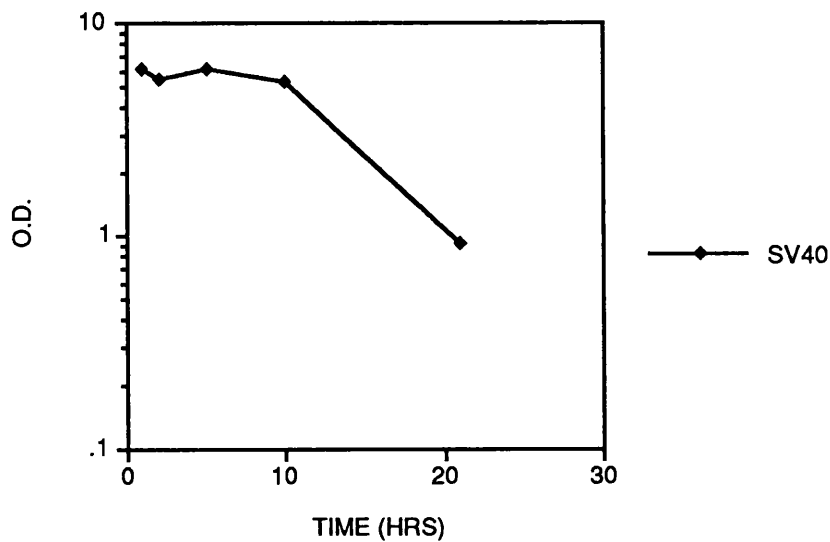
Figure 6.7: Decay of μ mRNA in B8 cells.

(A) The slopes of the linear regions of the graphs shown in Fig. 6.6 were calculated, and the ratio μ NP/actin plotted for each time point. The mRNA species are indicated on the right; μ s indicates the mRNA with the μ s polyadenylation sequence and SV40 indicates the mRNA polyadenylated at the SV40 site. These graphs were used to calculate the half-lives shown in Table 6.1. The slopes of the lines were calculated from the lines of best fit, using time points from 5 hours onwards. The R-squared values were μ s: 0.990, SV40: 0.989.

(B) shows the graph of B8 cell number against time for the experiment shown in (A). The slope of this line was calculated from the line of best fit, and is shown in Table 6.1. R-squared was 0.973. Doubling time was 18.6 hours.

FIGURE 6.8

A



B

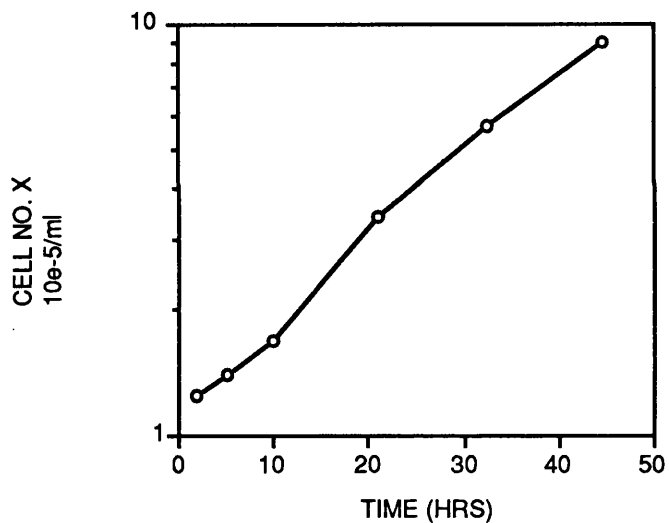


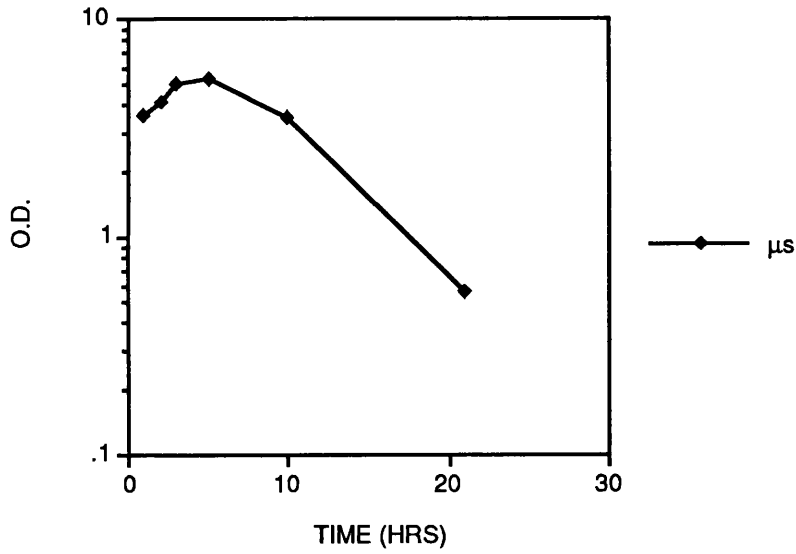
Figure 6.8: Decay of μ mRNA in B3 cells.

(A) The slopes of the linear regions of the graphs, like those shown in Fig. 6.6 for B8, were calculated, and the ratio μ NP/actin plotted for each time point. The mRNA species is indicated on the right; SV40 indicates the mRNA is polyadenylated at the SV40 site.

(B) shows the graph of B3 cell number against time for the experiment shown in (A). Doubling time was 14.4 hours.

FIGURE 6.9

A



B

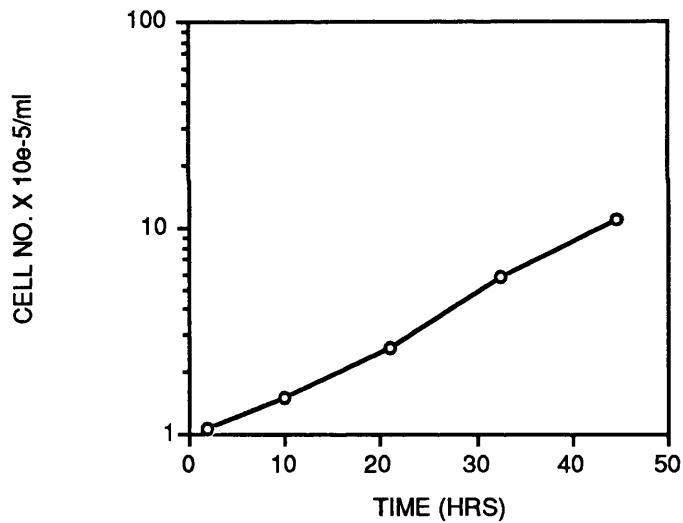


Figure 6.9: Decay of μ mRNA in D3 cells.

(A) The slopes of the linear regions of the graphs like those shown in Fig. 6.6 were calculated, and the ratio μ NP/actin has been plotted here for each time point. The mRNA species are indicated on the right; μ S indicates the mRNA with the μ S polyadenylation sequence.

(B) shows the graph of D3 cell number against time for the experiment shown in (A). Doubling time was 12.4 hours.

The derivation of this equation is outlined in the appendix, and the result is summarised in Table 6.1. The addition of the SV40 poly(A) addition sequence to the 3' end of the mRNA containing the *tk* leader sequence may shorten the half-life slightly, although this experiment would have to be repeated several times to determine whether or not the difference observed is significant.

Table 6.1

<u>mRNA</u>	<u>S1</u>	<u>S2</u>	<u>S1+S2</u>	<u>T_{1/2}</u>	<u>95% C.I.</u>
B8/ μ_s	-0.067571	0.037268	-0.030303	22.9 hrs	14.6-52.5
B8/SV40	-0.077664	0.037268	-0.040396	17.2 hrs	11.7-31.9

Table 6.1: The half-life of altered μ mRNA in B8 cells

Calculation of the half-life of μ mRNA in B8 cells, for the experiments shown in Fig. 6.7A and 6.7B. Linear regression analysis was used to calculate S1 and S2; S1 is the slope of the linear region of the graph obtained by plotting $\log_e(\mu/\text{actin})$ against time, and S2 is the slope of the graph obtained by plotting $\log_e(\text{cell no.})$ against time. The value of the t statistic in each case was significant to at least the 1% level. The half-lives were calculated from the equation $T_{1/2} = \log_e 2 / (S1+S2)$. The confidence intervals (C.I.) were calculated using the equation: $\log_e(95\%CI) = (S1+S2) \pm t s$, where t is the value of the t statistic at n-2 degrees of freedom for 95% confidence, and s is the combined standard error of the slopes S1 and S2.

6.2.4: Induction of μ mRNAs containing the mutated leader sequence.

Heat shock inductions were carried out as described above on the two cell lines 516 μ_s gpt/Z5 and 516DM μ_s gpt/D6, for brevity called Z5 and D6 respectively. Z5 cells contain the plasmid p516 μ_s gpt which leads to the production of wild-type μ mRNA (see Fig. 5.6A), and D6 cells contain the plasmid p516DM μ_s gpt (Fig. 5.6D). This plasmid produces μ mRNA with the mutated leader sequence, which is expected to localise onto cytoplasmic rather than membrane-bound polyribosomes. After heat shock induction of these two cell lines, cytoplasmic RNA was isolated at suitable time points and analysed by Northern blotting as described in Section 6.2.3, probing first with the μ NP probe and then with the actin probe. Hybridisation with the μ NP probe revealed that there

was a dramatic difference in the rate of decay of the mutant and wild-type mRNAs, the mutant mRNA being very short-lived compared to the wild-type (Fig. 6.10). This is true for μ mRNA with both the SV40 and μ_S polyadenylation sites, and hybridisation with the actin probe showed that the difference was not due to variation in loading (Fig.6.11).

Densitometric scanning was carried out on a series of exposures of these autoradiographs, and the slopes of the linear regions of the resulting graphs of optical density against exposure time were calculated, as was done for the experiments described in Section 6.2.3. Fig. 6.12A shows the result of plotting the ratio of slopes of μ NP/actin on a logarithmic scale against time for Z5, and Fig. 6.12B shows the graphs of cell number against time, also on a log scale. The half-lives of the two species of Z5 μ mRNA were calculated exactly as described in Section 6.2.3 for B8 μ mRNA, and these results are shown in Table 6.2.

Table 6.2

<u>mRNA</u>	<u>S1</u>	<u>S2</u>	<u>S1+S2</u>	<u>T_{1/2}</u>	<u>95% C.I.</u>
Z5/ μ_S	-0.064180	0.048284	-0.01594	43.6 hrs	20.8- ∞
Z5/SV40	-0.072427	0.048284	-0.024143	28.7 hrs	14.2- ∞

Table 6.2: The half-life of wild-type μ mRNA in Z5 cells

Calculation of the half-life of μ mRNA in Z5 cells, for the experiments shown in Fig. 6.12A and 6.12B. Linear regression analysis was used to calculate S1 and S2; S1 is the slope of the linear region of the graph obtained by plotting $\log_e(\mu/\text{actin})$ against time, and S2 is the slope of the graph obtained by plotting $\log_e(\text{cell no.})$ against time. The value of the t statistic in each case was significant to at least the 0.1% level. The half-lives were calculated from the equation $T_{1/2} = \log_2 / (S1+S2)$. The confidence intervals (C.I.) were calculated using the equation: $\log_e(95\%CI) = (S1+S2) \pm t s$, where t is the value of the t statistic at n-2 degrees of freedom for 95% confidence, and s is the combined standard error of the slopes S1 and S2.

Consistent with the results obtained with B8 μ mRNA, the addition of the SV40 polyadenylation sequence to the 3' end of μ mRNA reduces its half-life, but again further experiments would be required to determine whether this was a reproducible effect. Due to the rapid disappearance of D6 μ

FIGURE 6.10

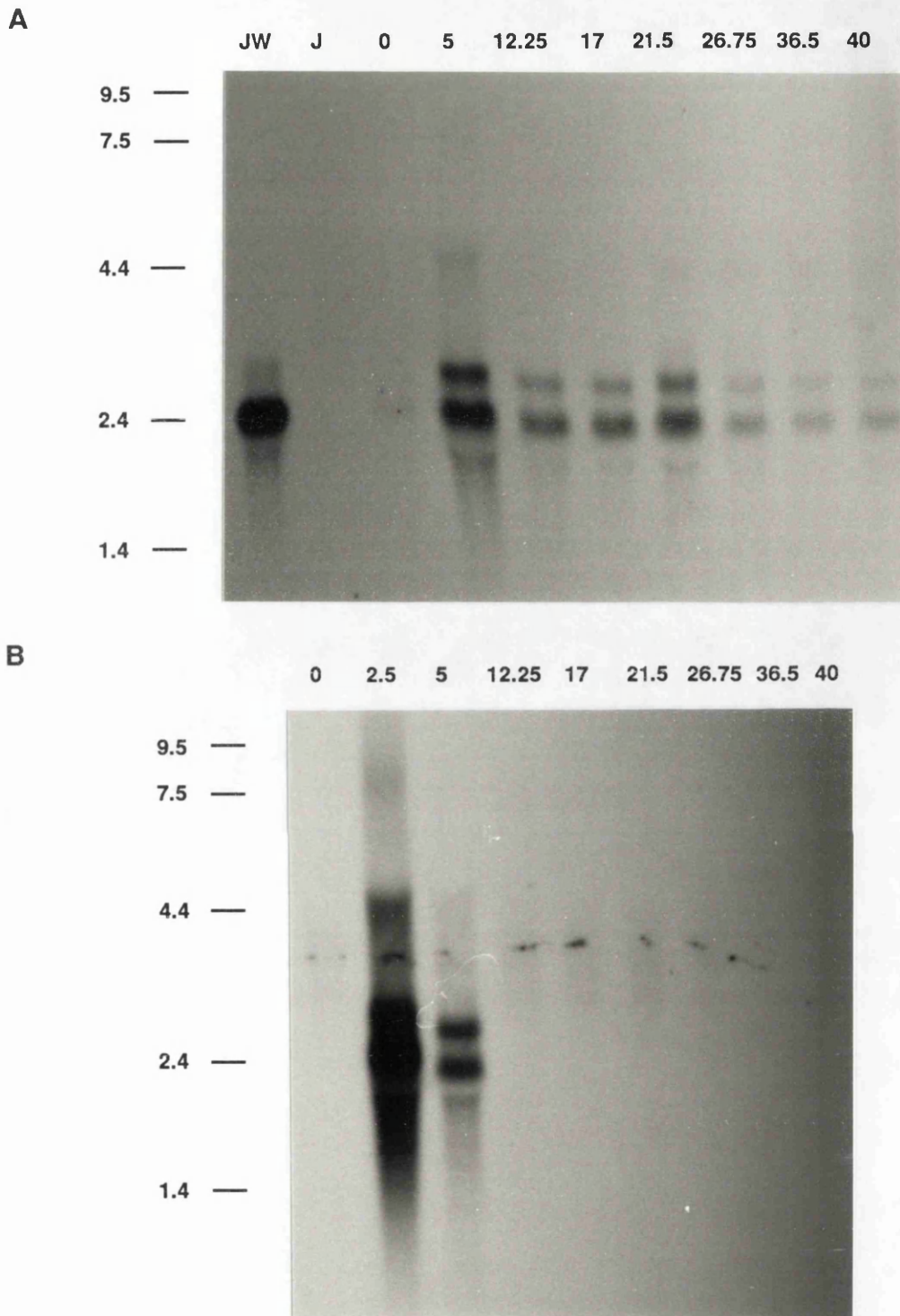


Figure 6.10: Northern blot analysis of total RNA after heat shock induction of Z5 and D6 cells: μ NP probe.

Northern blots were prepared as described in the text and probed with the μ NP probe. 8 μ g total RNA isolated at various times after heat shock induction was loaded on each track. The numbers above each track indicate the sampling time in hours, with time zero being the start of the heat shock. J indicates 2 μ g RNA from J558L cells, and JW indicates 2 μ g RNA from JW122 cells.

(A) RNA from Z5 cells

(B) RNA from D6 cells.

FIGURE 6.11

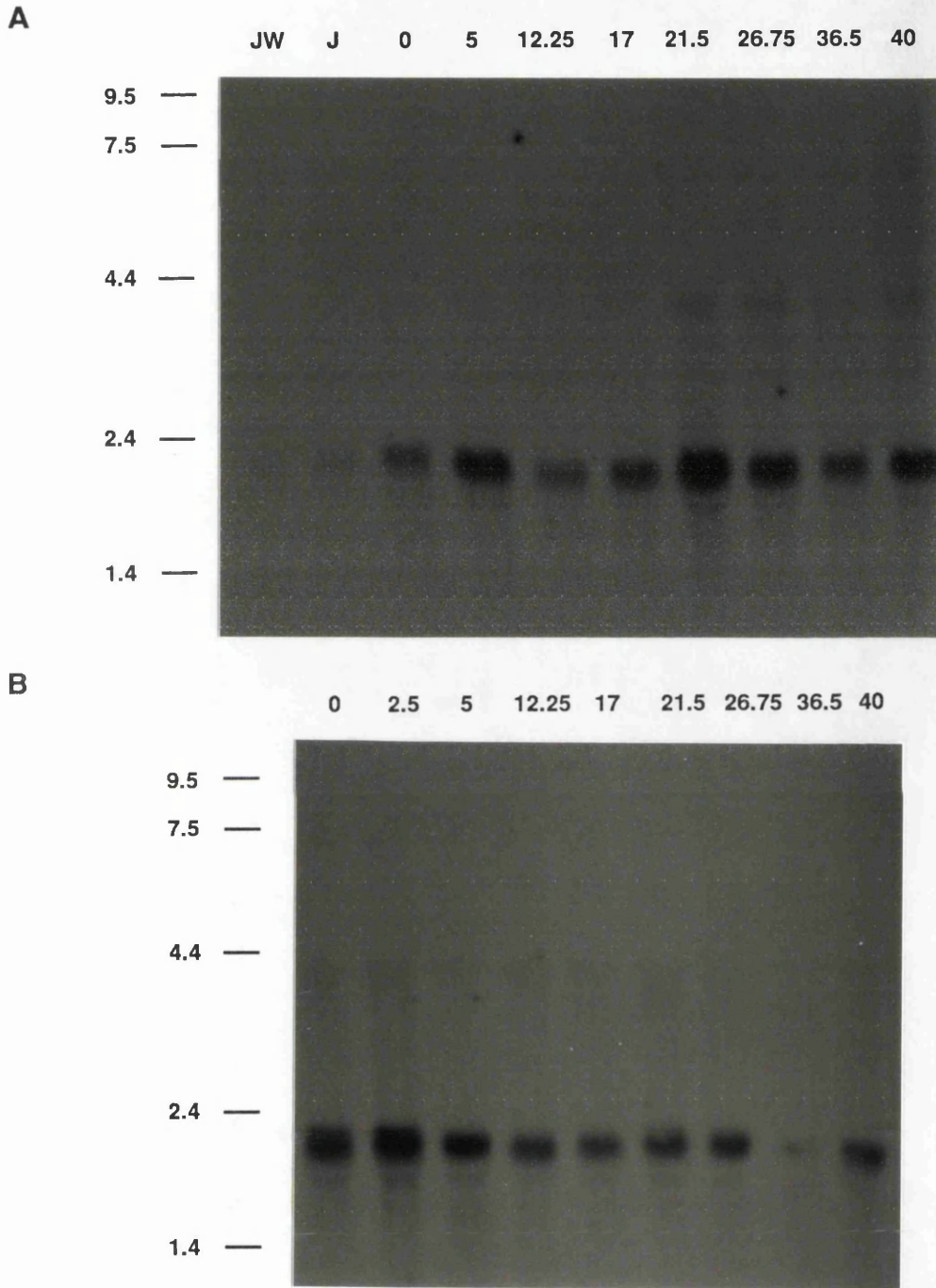


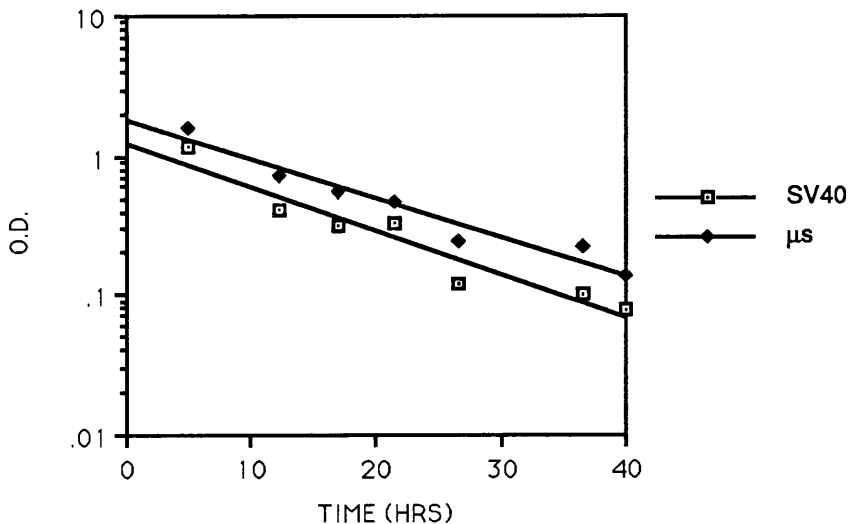
Figure 6.11: Northern blot analysis of total RNA after heat shock induction of Z5 and D6 cells; actin probe.

The Northern blots shown in Fig. 6.10 were stripped and reprobbed with the actin probe. For other details, see the legend to Fig. 6.10.

- (A) RNA from Z5 cells
- (B) RNA from D6 cells.

FIGURE 6.12

A



B

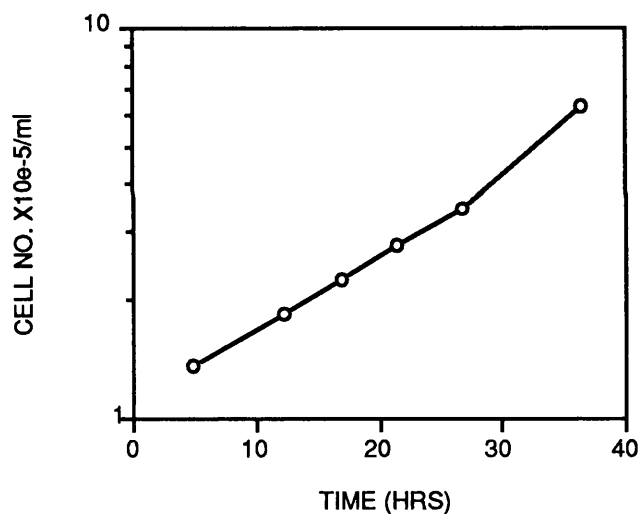


Figure 6.12: Decay of μ mRNA in Z5 cells.

(A) After densitometric scanning had been carried out on a series of exposures of the autoradiographs shown in Fig. 6.10A and 6.11A, the slopes of the linear regions of the graphs of optical density against exposure time were calculated. The ratio μ NP/actin for each time point has been plotted against sample time. The mRNA species are on the right; μ s indicates the mRNA with the μ s polyadenylation sequence and SV40 indicates the mRNA polyadenylated at the SV40 site. These graphs were used to calculate the half-lives shown in Table 6.2. The slopes of the lines were calculated from the lines of best fit, using time points from 5 hours onwards. The R-squared values were μ s: 0.953, SV40:0.925.

(B) shows the graph of Z5 cell number against time for the experiment shown in (A). The slope of this line was calculated from the line of best fit, and is shown in Table 6.2. R-squared was 0.990.

mRNAs, it was not possible to calculate their half-lives from this experiment, but clearly they are very short compared to the other mRNAs so far examined (see below).

To determine whether the latter result was an anomaly of the D6 cell line, the experiment was repeated using D6 and another transfectant, 516DM μ_s gpt/D1, containing the same plasmid construct (this cell line is abbreviated to D1). Cytoplasmic RNA was prepared from the cells at various times after the heat shock, and analysed by the Northern blot procedure. The results from this experiment, after probing the Northern blot with the μ NP and actin probes, are shown in Figs. 6.13 and 6.14 respectively, and it can be seen that the rates of decay of the μ mRNAs in the two cell lines are very similar. The μ mRNA decays so rapidly that more time points are required before 5 hours in order to be able to calculate the half-life, but the graphs shown in Fig. 6.15 indicate that it is likely to be of the order of one hour. Both the μ mRNA polyadenylated at the μ_s and SV40 sites are very unstable, indicating that the mutation in the signal sequence is a dominant factor in the control of stability.

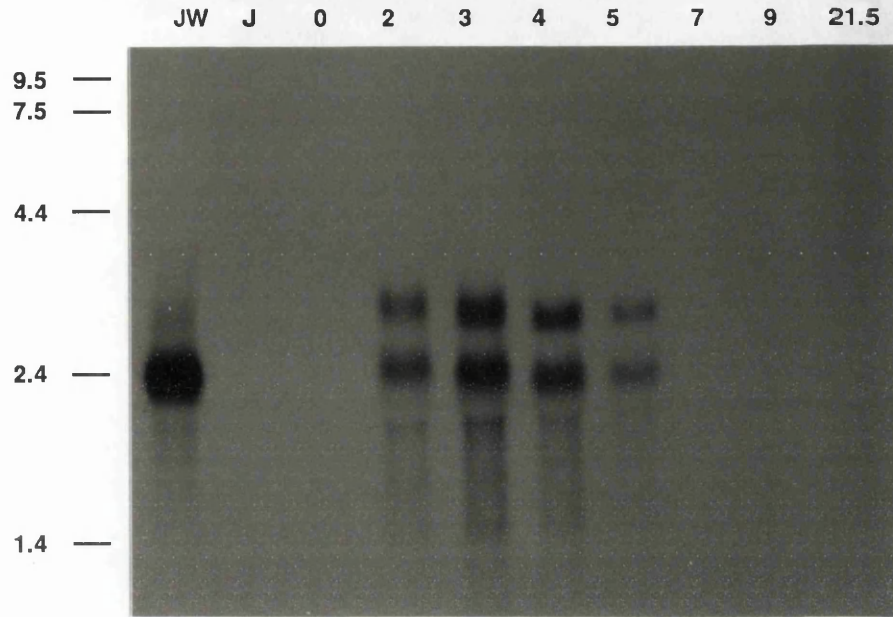
However, there are possible trivial reasons for the difference in half-life between wild-type and mutated mRNA. The longer apparent half-life of the wild-type could be caused by continued transport of transcripts from the nucleus after the end of the heat shock, maintaining the mRNA level in the cytoplasm. Another possibility is that the mutant mRNA may not be being translated, leading to its instability. These questions are addressed in the following Sections.

6.2.5: Nuclear RNA.

To investigate whether there was any difference in the rates of nuclear to cytoplasmic transport of μ mRNA between the D1 and D6 cell lines and Z5, nuclear RNA was analysed by Northern blotting. Nuclear RNA was isolated from equal volumes of Z5 cells, as described in Section 2.3.2, at various times after a heat shock induction, loaded onto a formaldehyde/agarose gel for Northern blotting, and the Northern blots were probed with the μ NP probe. Fig. 6.16A shows that the nuclear Z5 transcripts are much diminished by 4.5 hours, and have almost completely disappeared by 9 hours into the chase. As nuclear RNA from equal volumes of cells

FIGURE 6.13

A



B

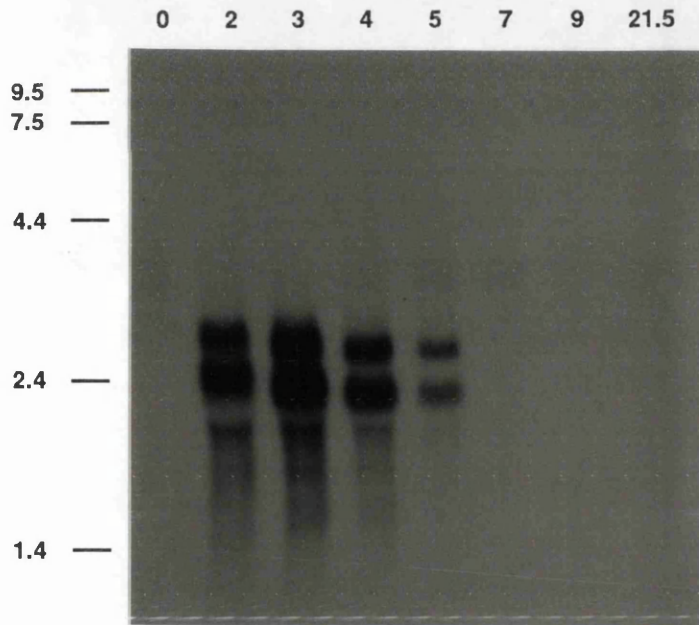


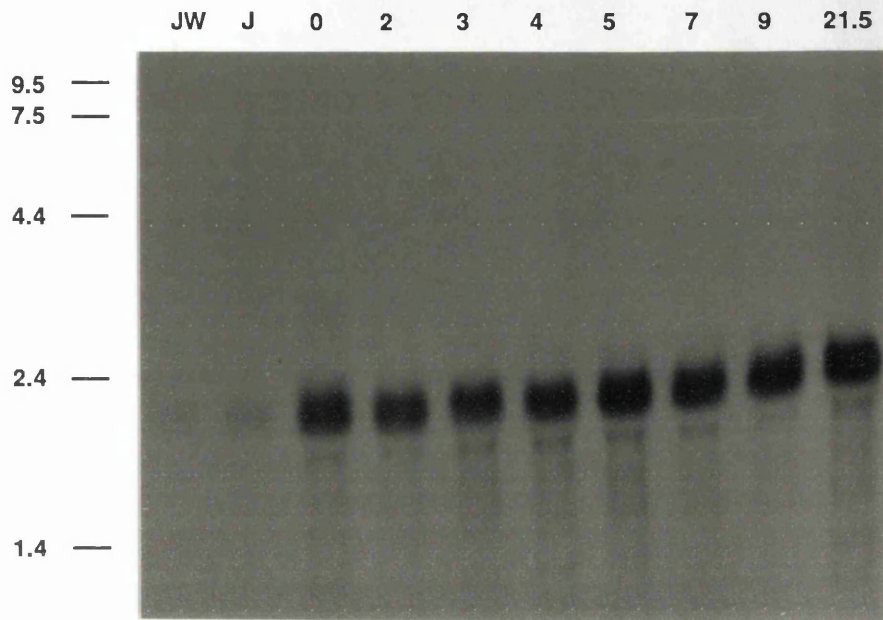
Figure 6.13: Northern blot analysis of RNA isolated after heat shock induction from D1 and D6 cells: μ NP probe.

8 μ g of cytoplasmic RNA isolated at various time points after heat shock induction was loaded on each track of a formaldehyde/agarose gel, which was then blotted as described in the text and probed with the μ NP probe. The numbers above each track indicate the time in hours, with time zero being the start of the heat shock. J indicates 2 μ g RNA from J558L cells, and JW indicates 2 μ g RNA from JW122 cells.

- (A) RNA from D1 cells
- (B) RNA from D6 cells.

FIGURE 6.14

A



B

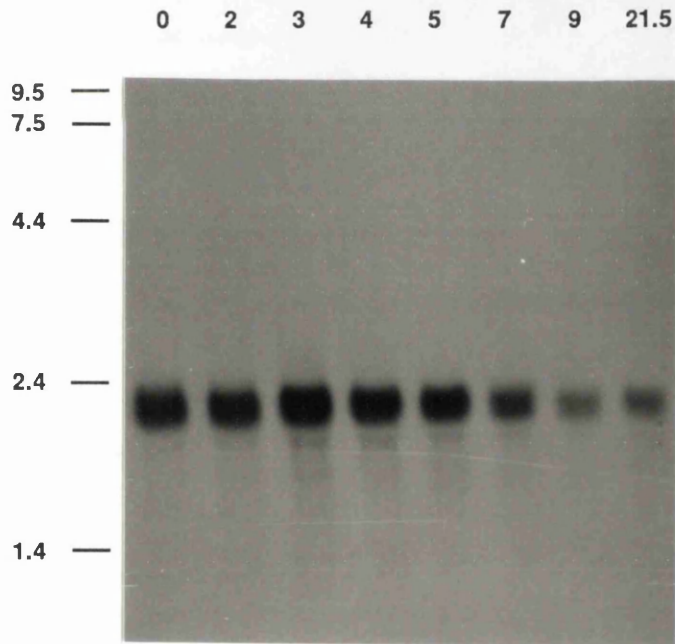


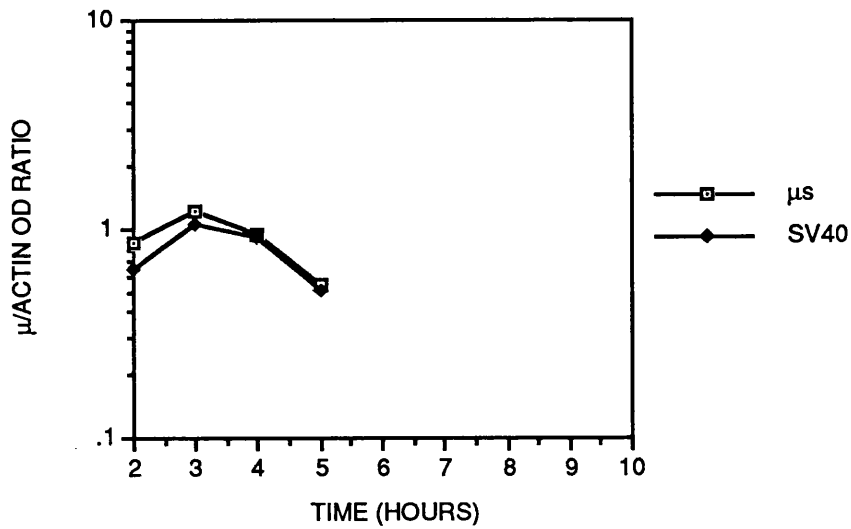
Figure 6.14: Northern blot analysis of RNA isolated after heat shock induction from D1 and D6 cells: actin probe.

The Northern blots shown in Fig. 6.13 were stripped and reprobated with the actin probe. For other details, see the legend to Fig. 6.13.

- (A) RNA from D1 cells
- (B) RNA from D6 cells.

FIGURE 6.15

A



B

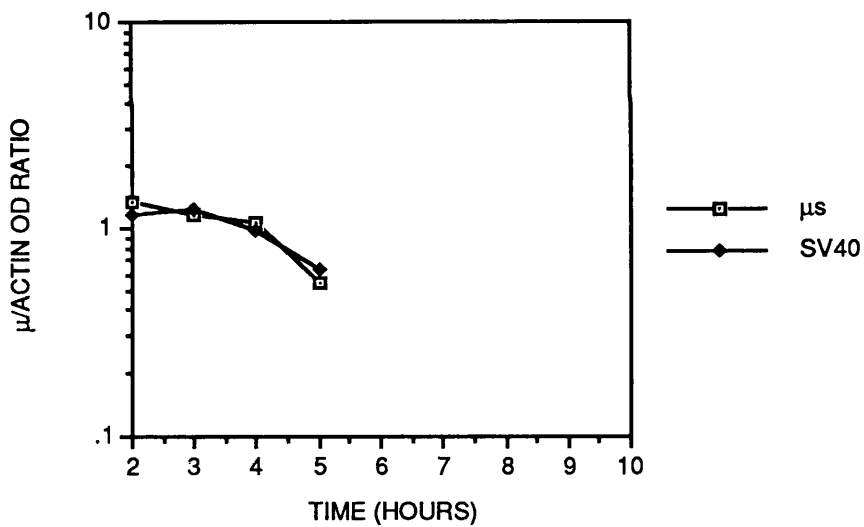


Figure 6.15: Decay of μ mRNA in D1 and D6 cells.

A series of autoradiographs of the Northern blots shown in Fig. 6.13 and 6.14 were scanned with a densitometer and the graphs obtained of OD against exposure time. The slopes of the linear regions of these graphs were used to calculate the ratio μ NP/actin for each time point of the experiment. The mRNA species are indicated on the right; μ s indicates the mRNA polyadenylated at the μ s site, and SV40 indicates the mRNA containing the SV40 polyadenylation sequence.

(A) D1 cells

(B) D6 cells.

FIGURE 6.16

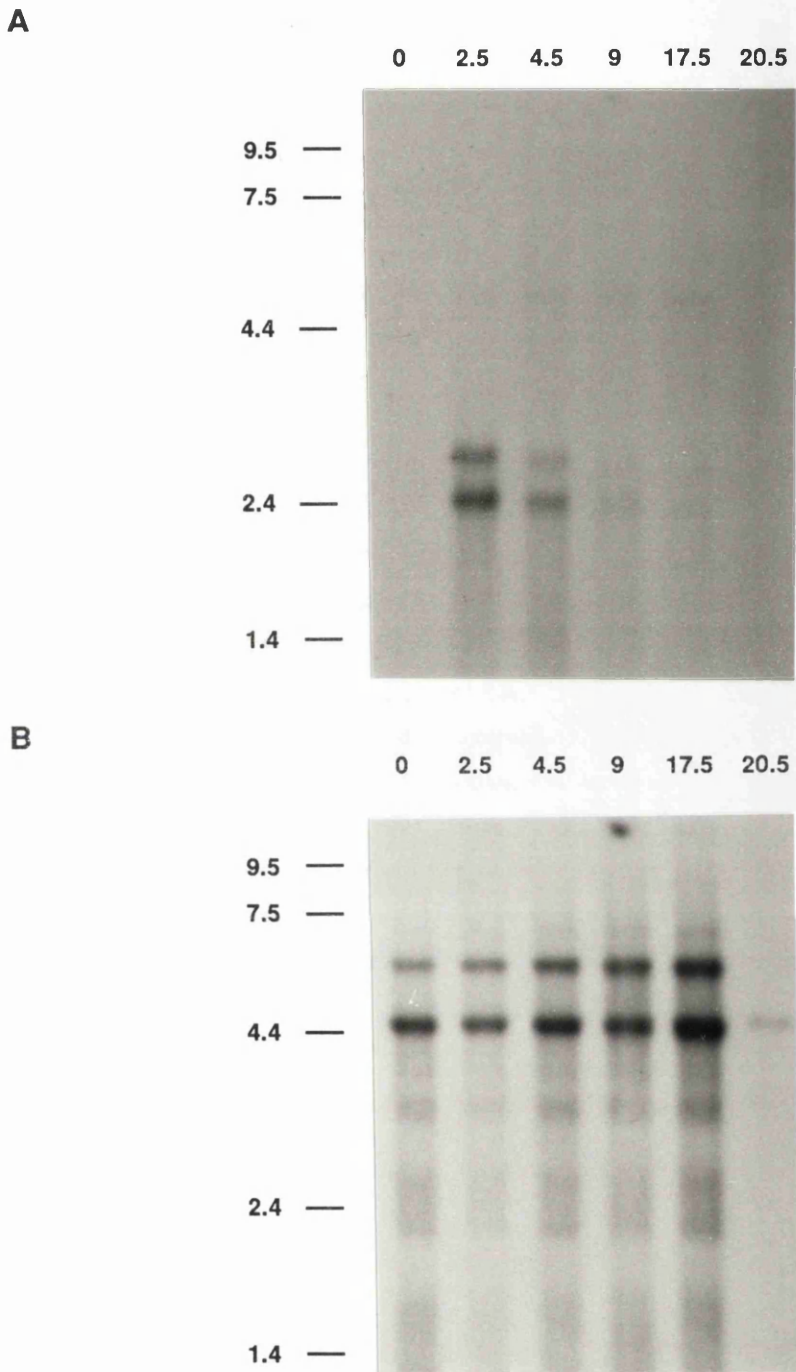


Figure 6.16: Northern blot of nuclear RNA from Z5 cells.

(A) Nuclear RNA isolated at various time points during a heat shock induction experiment on Z5 cells was loaded on a formaldehyde/agarose gel, which was then blotted as described in the text, and probed with the μ NP probe. The numbers above each track refer to the time in hours, times zero being the start of the heat shock.

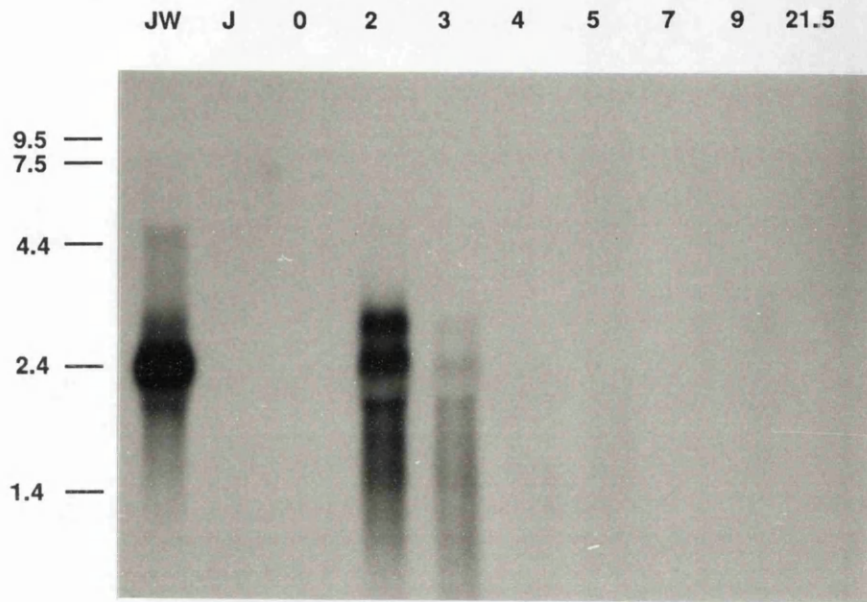
(B) The Northern blot shown in (A) was stripped and reprobed with the rRNA probe.

was loaded in each track, there should be no dilution effect of cell division on these samples, and this is confirmed by the result shown in Fig. 6.16B, where the same Northern blot has been probed using the ribosomal RNA probe pSP65rRNA. Two strong bands are evident in this autoradiograph, of sizes 4.5 and 6.6 kb, corresponding to mature 28S and precursor 32S ribosomal RNAs, respectively (Hassouna et al, 1984). The presence of a relatively high level of the precursor 32S rRNA is added confirmation that the RNA preparations contain predominantly nuclear RNA. In conclusion, it is clear that the contribution of nuclear μ NP transcripts to the cytoplasmic pool is very small after the 4.5 hour time point, and therefore unlikely to have a significant effect on the rate of decay of μ NP mRNA in the cytoplasm after this time.

The nuclear RNA from D1 and D6 cells was also examined, and the results of probing Northern blots containing nuclear RNA from these cells, with the μ NP probe, are shown in Fig. 6.17. The μ NP RNA disappears from the nucleus of D1 cells very rapidly, and is absent after the 3 hour time point. Nuclear RNA from D6 cells shows a more complex pattern, however. The normal μ NP bands show similar behaviour to those of D1 cells, but in addition, high molecular weight transcripts are present. These are observed before the heat shock, (zero time point), are absent at the 3-5 hour time points, and present again at the 7 hour time point. Such transcripts are not detected in the cytoplasmic RNA (see Figs 6.10B and 6.13B), and therefore are presumably not processed to form stable mRNA. It is probable that they arise from transcription from a chromosomal promoter adjacent to the integration site of the plasmid, and that when the heat shock promoter is activated, it interferes with the transcription from this chromosomal promoter. These transcripts behave in an analogous manner to the species observed in Chapter 5, in cell lines derived from plasmids linearised at the *Hind* III site (Fig. 5.14), in that they are present before the heat shock and absent immediately after. In the latter case, however, the transcripts were processed to mRNA found in the cytoplasm, and probably arose from the SV40 late region promoter in the plasmid. These nuclear D6 transcripts are much more heterogeneous, but their reappearance at 7 hours, probably indicates that by this time the cells have returned

FIGURE 6.17

A



B

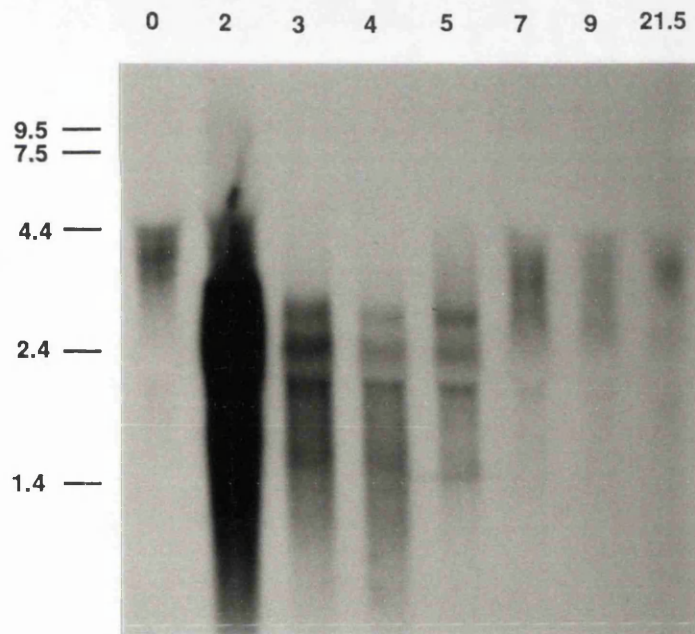


Figure 6.17: Northern blot of nuclear RNA from D1 and D6 cells: μ NP probe.

Nuclear RNA isolated at various time points after a heat shock induction of D1 and D6 cells was loaded on a formaldehyde/agarose gel, which was blotted as described in the text, and probed with the μ NP probe. The numbers above each track refer to the time in hours, with zero being the start of the heat shock. J indicates 2 μ g RNA from J558L cells, and JW indicates 2 μ g RNA from JW122 cells.

(A) Nuclear RNA from D1 cells.

(B) Nuclear RNA from D6 cells.

fully to normal after the heat shock. The main μ NP transcripts are undetectable at this time, which is consistent with this hypothesis.

Further evidence that the effects of the heat shock are completely over by approximately the 7 hour time point is provided by the autoradiograph shown in Fig. 6.18, which is the result of probing the Northern blot shown in Fig. 6.17 with the actin probe. In both the D1 and D6 cell lines, the actin mRNA is downregulated between the 3-5 hour time points, probably as a result of downregulation of transcription and/or RNA processing, as has been described after heat shock of mammalian cells (Yost and Lindquist, 1986; Labhart and Reeder, 1987; Sadis et al, 1988; reviewed in Yost et al, 1990). Interestingly, this effect is not at all evident in the cytoplasmic RNA shown in Fig. 6.14, indicating that actin mRNA in the cytoplasm is not noticeably affected by the heat shock, and its stability is such that the reduced input from the nucleus between 3-5 hours is not detected. The presence of high levels of actin mRNA in the nucleus from the 7 hour time point onwards also shows that the rapid disappearance of μ NP mRNA from the nucleus seen in Fig. 6.17 is not due to anomalous loading of the gels.

From these results, it does not appear likely that differential input of μ NP mRNA from the nucleus to the cytoplasm can account for the difference in the rate of decay observed between the wild type and mutant μ NP mRNAs. These experiments also give an indication of the duration of the effects of the heat shock on the cells, indicating that by the 7 hour time point at the latest, the cells have fully recovered from the heat shock. Recovery clearly begins even earlier than this, as the level of nuclear μ NP transcripts is already decreasing by the 3 hour time point, indicating that transcription from the heat shock promoter is being downregulated at this time.

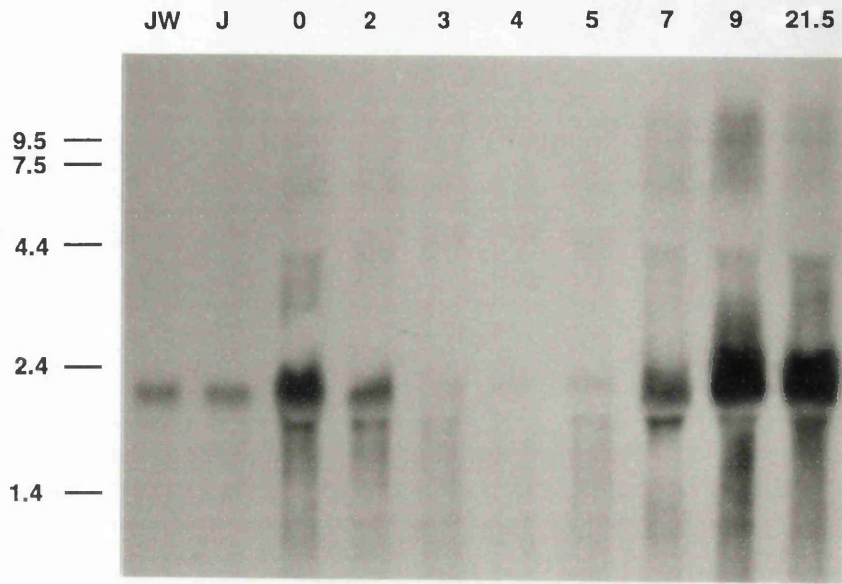
To determine whether the induced μ NP mRNAs were translated to μ polypeptide of the expected molecular weight, cell lysates were subjected to Western blot analysis as described in the following Section.

6.2.6: Protein produced from the heat shock-induced μ NP mRNA

The protein products from the various μ NP mRNAs were

FIGURE 6.18

A



B

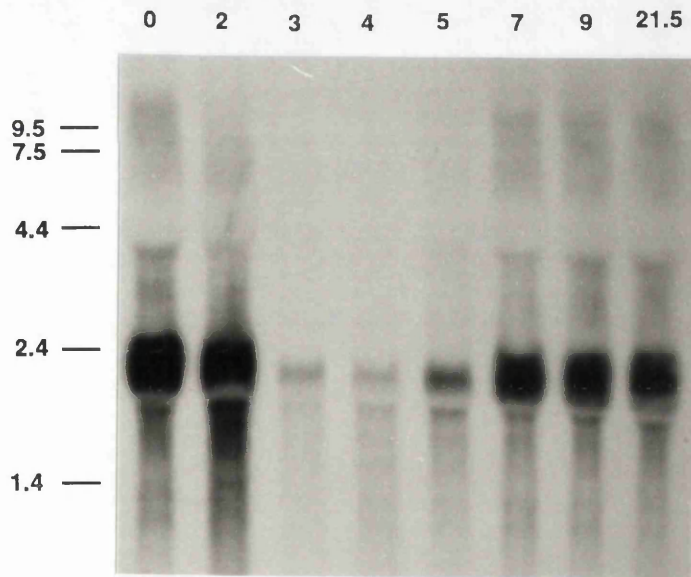


Figure 6.18: Northern blot of nuclear RNA from D1 and D6 cells: actin probe.

The Northern blots shown in Fig. 6.17 were stripped and reprobated with the actin probe.

(A) Nuclear RNA from D1 cells.

(B) Nuclear RNA from D6 cells.

analysed by Western blotting, which was carried out as described in Section 2.4.1, using a goat anti-mouse IgM antibody to detect μ chain. For each experiment, aliquots of cells were harvested at each time point and lysed in denaturing sample buffer (see Section 2.4.1). These were then diluted as appropriate in 1X sample buffer such that the equivalent of 5×10^5 cells could be loaded in each track of an SDS-PAGE gel. Culture supernatant samples were also diluted to take account of cell doubling, and each aliquot loaded corresponded to supernatant from approximately 1.5×10^3 cells, or $15 \mu\text{l}$ of neat supernatant at the start of the experiment. Samples from J558L cells were loaded on each gel as the negative control, and purified IgM from the B1-8 hybridoma (as used in the ELISA assays described in Section 6.2.3) was used as the positive control.

The results from B3, D3 and B8 inductions, corresponding to those described in Section 6.2.3, were all very similar, as would be expected from the fact that the μ coding regions in these cell lines are identical. Typical results, in this case from the B8 experiment, are illustrated in Fig. 6.19. Compared with the untransfected J558L cell line, the B8 cell lysates contain a significant amount of material which migrates just below the position of the B1-8 μ standard. This material is present before the heat shock and at all time points thereafter. In addition, a band of slightly higher molecular weight appears after the heat shock (see arrow). As terminal glycosylation is known to occur on fully assembled IgM, the induction of the higher molecular weight band, which migrates more closely with the standard, may represent this terminal glycosylation event (Tartakoff and Vassalli, 1979). The presence of the lower molecular weight material could be explained by the translation of the very small amount of μ mRNA present in un-heat shocked cells. This protein must be being turned over in the endoplasmic reticulum at a rate faster than that of pentamer assembly and secretion, as no secreted material is detected (Fig. 6.19, supernatant samples). Upon heat shock, when the amount of μ mRNA increases dramatically, protein is produced at a faster rate which overcomes the turnover rate, allowing terminal glycosylation and secretion to occur. This is confirmed by

FIGURE 6.19

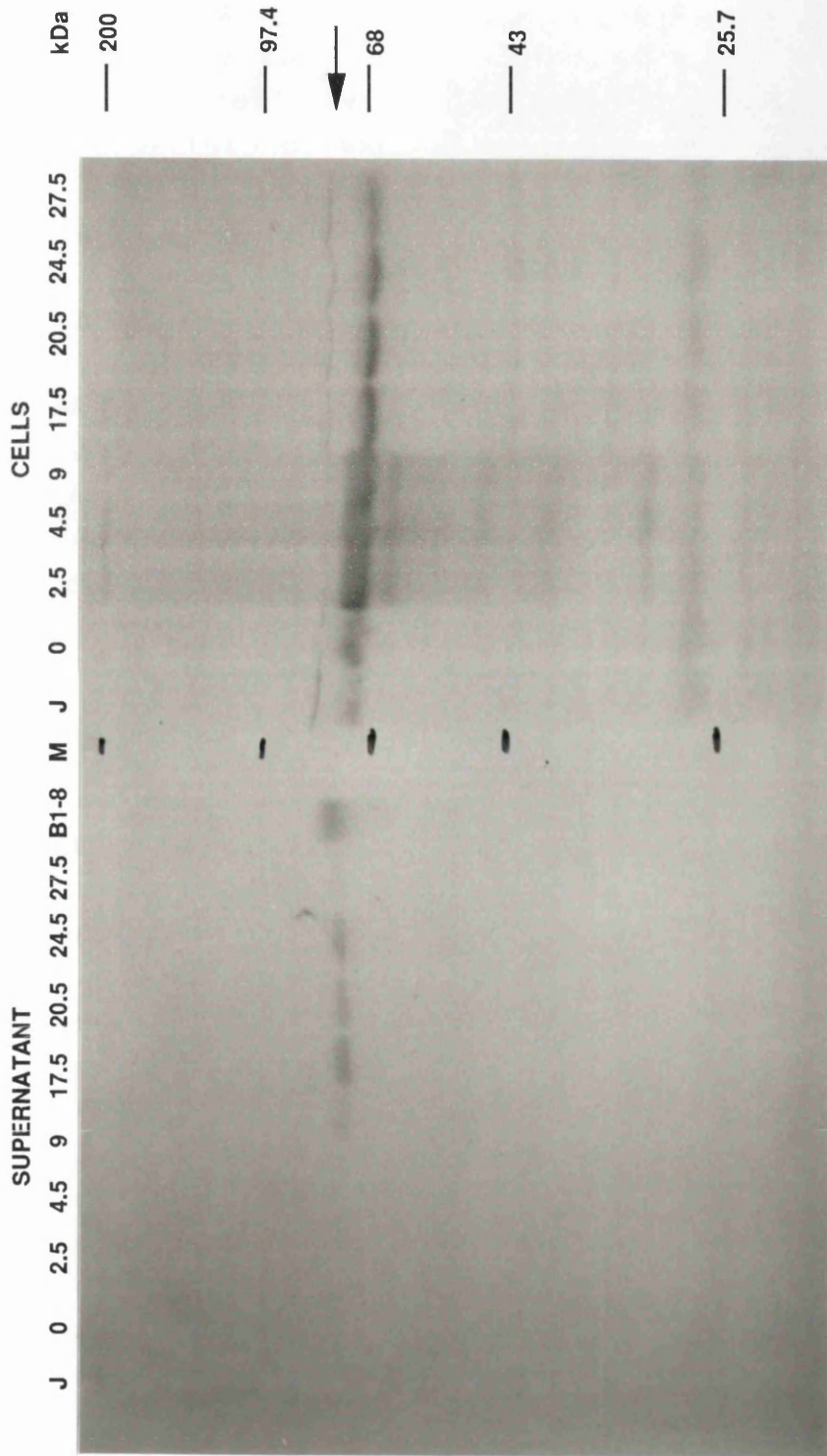


Figure 6.19: Western blot of cell lysates and culture supernatant from B3 cells.

Heat shock induction was carried out on B3 cells, and culture supernatant and cell pellet samples from equal volumes of cells were analysed by Western blotting as described in the text. The numbers above each track indicate the time in hours, time zero being the start of the heat shock. J indicates supernatant or cell sample from J558L cells, M indicates molecular weight markers, and B1-8 indicates antibody purified from B1-8 hybridoma supernatant.

the fact that μ chain is detected in supernatant samples after the heat shock by Western blotting and ELISA (Figs. 6.19 and 6.3B), indicating that secretion is occurring.

Western blot analysis was also carried out on cell lysates and supernatant samples from the Z5 and D6 cell lines. Fig. 6.20 shows that μ mRNA in Z5 cells directs synthesis of μ chain in a very similar manner to that described above for the B8 cell line. The μ polypeptide found in D6 cell lysates, on the other hand, shows a different pattern of expression and is a different molecular weight (Fig. 6.21). A low amount of material of a lower molecular weight than B1-8 μ is present at the zero time point, and this band increases in intensity after the heat shock, but returns to background level after the 5 hour time point. This band therefore closely reflects the expression of μ mRNA in these cells, indicating that the protein is unstable. Its instability may relate to its location in the cell (see below), or the addition of the methionine residue at the 5' end (see Chapter 5). Interestingly, the size of this protein is consistent with that of the unglycosylated form of μ chain, 62.5 kDa (Boss et al, 1984), and no μ chain is detected in the supernatant from these cells, by either ELISA assay (not shown) or Western blot (Fig. 6.21, supernatant samples). These observations are consistent with the expectation that the mutant μ mRNA is translated on free ribosomes in the cytoplasm. To confirm that this low molecular weight form was indeed the unglycosylated species of μ chain, an experiment was carried out in which cells were incubated in the presence of tunicamycin, an agent which inhibits N-linked glycosylation (Bettinger and Young, 1975; Tkacz and Lampen, 1975). This would be expected to result in a reduction in size of the μ chain produced from wild-type μ mRNA, with no change in the size of the μ chain produced from the mutant μ mRNA, if this hypothesis is correct.

Z5 and D6 cells were heat shocked by the normal method, and after the heat shock were resuspended in medium at 37°C, and divided between two flasks, as described in Section 2.5.9. To one flask, tunicamycin was added to a final concentration of 10 μ g/ml, and equal aliquots of cells were harvested from each flask after 3 and 6 hours incubation. The cells were lysed in denaturing sample buffer and analysed

FIGURE 6.20

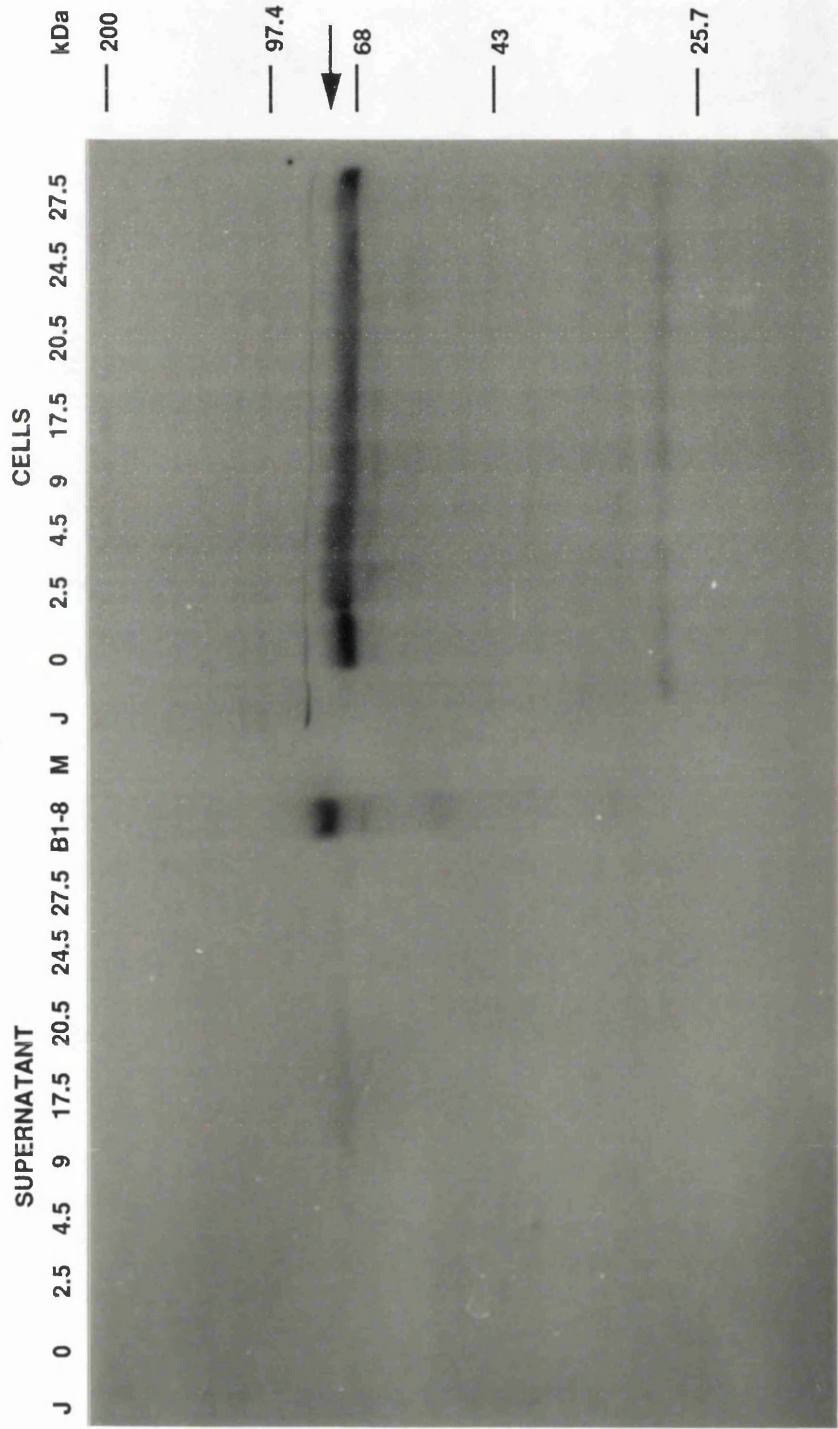


Figure 6.20: Western blot of cell lysates and culture supernatant from Z5 cells.

Heat shock induction was carried out on Z5 cells, and culture supernatant and cell pellet samples from equal volumes of cells were analysed by Western blotting as described in the text. The numbers above each track indicate the time in hours, zero being the start of the heat shock. J indicates supernatant or cell sample from J558L cells, M indicates molecular weight markers, and B1-8 indicates antibody purified from B1-8 hybridoma supernatant.

FIGURE 6.21

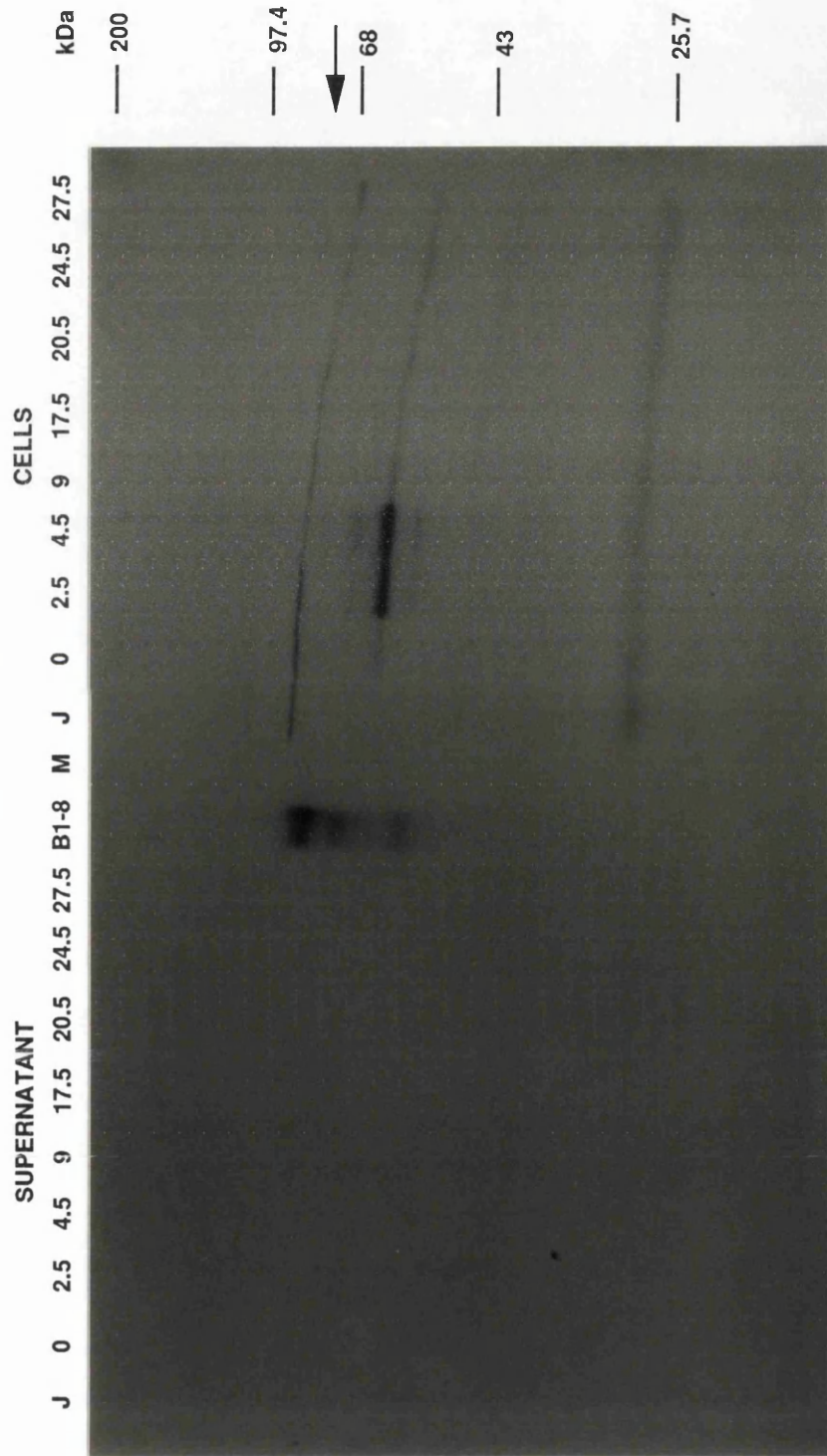


Figure 6.21: Western blot of cell lysates and culture supernatant from D6 cells.

Heat shock induction was carried out on D6 cells, and culture supernatant and cell pellet samples from equal volumes of cells were analysed by Western blotting as described in the text. The numbers above each track indicate the time in hours, zero being the start of the heat shock. J indicates supernatant or cell sample from J558L cells, M indicates molecular weight markers, and B1-8 indicates antibody purified from B1-8 hybridoma supernatant.

FIGURE 6.22

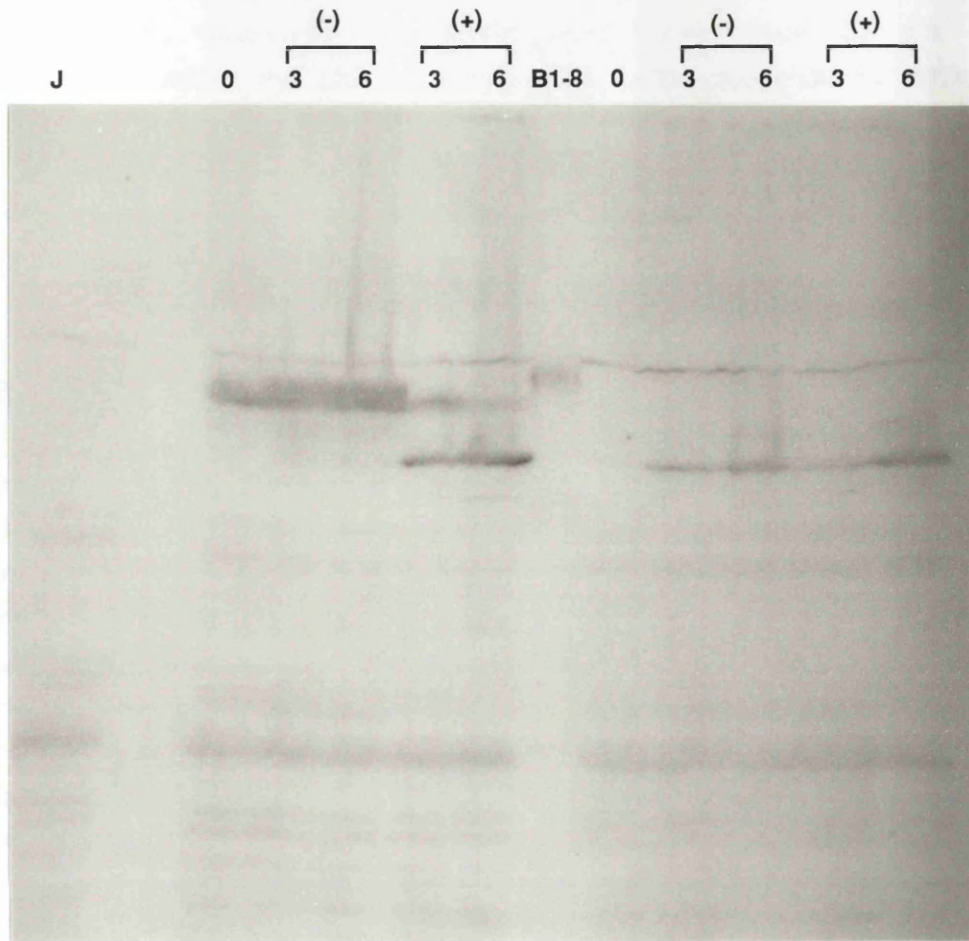


Figure 6.22: The effect of tunicamycin on α protein from Z5 and D6 cells.

Z5 and D6 cells were induced as before then incubated in the presence (+) or absence (-) of $10\mu\text{g/ml}$ tunicamycin, for the times indicated (in hours) above each track. Cell pellets from equal volumes of culture were then analysed by Western blotting. J indicates J558L negative control and B1-8 indicates antibody purified from B1-8 hybridoma supernatant.

by Western blotting as before. Fig. 6.22 shows that the size of the protein from D6 cells is unaffected by the presence of tunicamycin, whereas the μ protein from control Z5 cells is reduced in size in the presence of tunicamycin to that of the mutant. This is good evidence that the mutant mRNA leads to the production of unglycosylated protein, which in turn is consistent with it being translated on cytoplasmic polyribosomes.

6.3 Discussion

This Chapter describes data which demonstrate the use of the *Drosophila melanogaster* hsp 70 heat shock promoter to induce the production of specific μ mRNAs in an easily controllable manner. This has been used to measure the half-lives of specific mRNA species, and to analyse the protein which they produce. This system provides a convenient method for the systematic study of the effect of different mutations on the stability of the mRNA. The pulse-chase system used earlier (Chapters 3 and 4) is not suitable for these experiments, for two reasons. Firstly, it is not practical to carry out large numbers of pulse-chase experiments on different cell lines, as large amounts of radioactive precursor would have to be used, and the ribonuclease protection assay is cumbersome due to the long autoradiographic exposures required. Secondly, whilst it is relatively easy to detect ^3H -labelled mRNA from endogenous genes, this was not found to be the case with transfected genes (data not shown). This is presumably due to the lower transcription rate for transfected genes, making detection a problem due to the low level of incorporation of radioactive precursor. The heat shock promoter system, on the other hand, provides a high level induction, the results of which can easily be analysed by Northern blotting.

Several preliminary conclusions can be drawn from these experiments. Firstly, the addition of the SV40 polyadenylation signal to the 3' end of wild-type μ mRNA may reduce its half-life, when present as an addition to the μ_S polyadenylation sequence. This is demonstrated by the results with Z5 and B8 cell lines (see Tables 6.1 and 6.2), both of which contain two μ mRNA species, one with just the

μ_s polyadenylation site and one with both the μ_s and the SV40 sites. The differences in half-life, however, are relatively small, and the experiments would have to be repeated several more times to determine whether they are statistically significant. Unfortunately, it was not possible to determine a half-life from the B3 experiment, involving the μ mRNA containing just the SV40 polyadenylation sequence, but from the data in Fig. 6.8, it is evident that the half-life of this mRNA is likely to be similar to those in the B8 and Z5 cell lines. The second observation that can be made is that the addition of the 62 nucleotides of *tk* 5' untranslated leader to the 5' end of the mRNA also seems to cause a reduction in half-life, as both mRNA species with the *tk* leader (in B8 cells) have shorter half-lives compared to both species without it (in Z5 cells) (see Tables 6.1 and 6.2). Again, more experiments need to be done to determine whether this is a statistically significant result. The lack of any dramatic effect of the addition of sequences at the 5' and 3' ends of μ mRNA is perhaps not surprising, as the entire wild-type μ_s mRNA sequence is present in these constructs, and there would have to be a dominant stability determinant in the *tk* leader sequence or SV40 polyadenylation sequence to exert a significant effect.

A summary of the results obtained for the half-life of wild-type μ mRNA, from the experiments described in this Chapter and Chapter 4, are summarised in Table 6.3, together with the results of Mason and co-workers (1988). This table shows that consistent results were obtained between MXW231 and J558L cells, as would be predicted from their similarity in cell type, from the heat shock induction and pulse-chase experiments described in this Chapter and in Chapter 4. The results are also in reasonable agreement with those of Mason *et al* (1988) although the latter's results indicate a lower average value for the half-life. The reason for this is not clear, but one possible source of variability arises from the determination of the cell doubling time; small differences in this measurement have a large effect on the final half-life value. In the light of these results, it would be useful to transfect the MXW231 cell line with the heat shock promoter construct, to enable a direct comparison to be made of the heat shock and pulse-chase methods in the

Table 6.3

<u>Cell line</u>	<u>Half-life</u>	<u>Method</u>	<u>Reference</u>
MXW231	41 hours	pulse-chase	Chapter 4
MXW231	28 hours	pulse-chase	Chapter 4
J558L (Z5)	44 hours	heat shock	Chapter 6
MXW231	14 hours	heat shock	Mason <i>et al</i>
J558L	20 hours	heat shock	Mason <i>et al</i>
J558L	19 hours	heat shock	Mason <i>et al</i>
J558L	12 hours	heat shock	Mason <i>et al</i>

Table 6.3: Summary of heat shock induction data

Data presented here and by Mason *et al* (1988) is summarised.

same cell line. Attempts were made to do this, but unfortunately the transfection efficiency of MXW231 was found to be very low, and in the time available a clone which gave good induction could not be found (data not shown).

Whereas the experiments involving additions to the 5' and 3' ends of μ mRNA, discussed above, have not resulted in large alterations in μ mRNA half-life, the mutant μ mRNA in which the signal peptide is not translated has a dramatically reduced half-life when compared to the wild-type (Figs. 6.15, 6.12 and 6.9). This has been demonstrated with two independently isolated cell lines containing the plasmid with the mutant μ gene, D1 and D6, and therefore cannot be attributable to an effect unique to one clone.

The examination of nuclear μ mRNA from cell lines containing the control and mutant plasmids indicates that the difference in the rate of decay is not due to differential input from the nucleus to the cytoplasm, as after the 4-5 hour time points, very little μ mRNA is evident in the nucleus of either cell type (Figs. 6.16 and 6.17). Therefore the long half-life of μ mRNA in Z5 cells cannot be due to sustained pre-mRNA processing and nuclear to cytoplasmic transport. However, the presence of nuclear μ NP transcripts, particularly in D1 and D6 cells, over the time period up to 4 hours will undoubtedly affect the apparent rate of decay of

the mRNA in the cytoplasm. This is clearly evident from examination of the graphs in Fig. 6.15, where the significant decline in the intensity of the μ NP signal does not begin until the 4 hour time point, consistent with the drop in level of nuclear μ mRNA at this time. This does not cause a problem with the Z5 cells, as there are sufficient time points after 5 hours to allow determination of the half-life (Fig. 6.12), but the short half-life of the mutant mRNA in D1 and D6 cells precludes this. Nevertheless, the half-life of the mutant can be estimated to be at most one hour, from the graphs in Figs. 6.15.

Another aspect of this system which makes the determination of the short half-life more difficult, is that the effects of the heat shock itself are still in evidence during the time in which the mutant μ mRNA is decaying. This is indicated by the examination of the nuclear actin RNA levels, which begin to return to normal at around 5 hours (Fig. 6.18). It is therefore possible that the heat shock itself is affecting the mutant μ mRNA, (but not the wild-type), in some way which alters its half-life. Therefore to confirm the results presented here, it would be necessary to determine the half-life of the mutant μ mRNA by an alternative method, for example by using an inhibitor of transcription with a constitutive promoter, or an alternative inducible promoter.

Despite the difficulties in obtaining an accurate determination of the half-life of the mutant μ mRNA, it is clear that it is up to 40 fold less stable than the wild-type mRNA. Mason et al (1988) observed a half-life of 2.4-3.5 hours for the mutant with the hydrophilic signal sequence, which is a similar reduction (although less dramatic) to that observed here. The fact that both mutations cause a significant drop in half-life is good evidence that it is not the sequence changes *per se* which are having an effect, as different nucleotide changes are made in each case, although it cannot be ruled out that both the mutations are affecting a region of secondary structure which is an important stability determinant. However, it seems more likely that the effect of the sequence changes on the location, and/or mode of translation of the mRNA, is the overriding factor.

Strong circumstantial evidence indicates that both

mutant mRNAs are translated on free rather than membrane-bound polysomes. The μ protein produced comigrates under denaturing gel electrophoresis conditions with unglycosylated μ chain, and in addition, is not detectably secreted by the cells, despite the fact that the level of μ chain inside the cells is comparable to that in the wild-type (Figs. 6.21 and 6.22; Mason *et al*, 1988). To confirm the cellular location of the mutant mRNA, it would be necessary to isolate preparations of free and membrane-bound polysomes and determine the relative levels of μ mRNA in these two fractions. Attempts were made to do this, although these have been unsuccessful to date (not shown).

In summary, a mutant μ mRNA has been generated which is predicted to be translated on free cytoplasmic polysomes, and circumstantial evidence indicates that this is the case. This mutant has a drastically reduced half-life compared to the wild-type mRNA, which is likely to be due to an effect of the localisation of the message. Other factors, such as the rate of translation of the mRNA, have not been addressed in these experiments, but may be important. The relationships between the mRNA localisation, its rate of translation, and its stability, are discussed in Chapter 7, together with the possible implications with regard to the control of μ gene expression during B cell differentiation.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

The secretion of large amounts of specific immunoglobulin into the bloodstream plays a crucial role in the immune response in mammals. This event is triggered when resting B cells encounter antigen and are stimulated to divide and differentiate into antibody-secreting plasma cells, and involves a large increase in the cellular content of mRNA coding for immunoglobulin (Lamson and Koshland, 1984; Yuan and Tucker, 1984; Berger, 1986). The work described in this thesis was aimed at increasing our understanding of the mechanism by which this increase is brought about.

The starting point for the experiments was the observation of the discrepancy between the difference in steady state immunoglobulin μ mRNA levels and the difference in transcription rates of the μ gene between cell lines representing early and late stages of B cell differentiation (Perry and Kelley, 1979; Mather *et al*, 1984; Kelley and Perry, 1986; Gerster *et al*, 1986). The fact that the increase in transcription of the μ gene is insufficient to account for the increase in mRNA levels indicates that regulation must be occurring at the post-transcriptional level, and therefore experiments were embarked upon to determine whether a change in mRNA stability was involved.

Initially, a method for the determination of mRNA half-life in mammalian cells was developed, based on a conventional pulse-chase protocol using ^3H uridine, together with a ribonuclease protection assay for detection of specific transcripts. The reproducibility of this method was analysed using a model system, consisting of CBMG cells, a C127 cell line transfected with a high copy number BPV-based vector containing the hGH gene (Pavlakis and Hamer, 1983). The pulse-chase method was found to be reproducible, and yielded results that were in good agreement with those of Paek and Axel (1987), who carried out an analysis of the half-life of hGH mRNA in mouse L cells, which, like C127 cells, are a fibroblast cell type.

To study the rate of decay of μ mRNA, two B cell lines were used, WEHI231, which is a B cell lymphoma representative

of the immature B cell (Warner *et al*, 1975), and MXW231 which is a hybridoma representative of the plasma cell stage (Raschke *et al*, 1979). As MXW231 is derived from hybridisation of WEHI231 with a myeloma cell line (MPC 11), the half-life of mRNA from the endogenous μ gene of WEHI231 cells can be determined in two cellular environments which represent the different differentiation stages. Northern blot analysis showed that there is approximately an order of magnitude difference in the steady state μ mRNA levels between these two cell lines. When the pulse-chase and ribonuclease protection protocol was applied to these cell lines, a six fold difference in the half-life of μ mRNA was observed, when normalised to the half-life of ribosomal RNA. This difference was detected in the absence of any detectable difference in the rate of decay of total polyadenylated RNA, and was shown not to be due to any difference in chase conditions between the two cell lines. Combined with the 1.5 fold difference in the rates of transcription of the μ gene (Mason *et al*, 1988), the six fold increase in mRNA half-life is sufficient to account for the difference in steady-state mRNA levels between the two cell lines.

While this work was being carried out, other workers were engaged in similar experiments, using a variety of methods, and the data presented here are in general agreement with their results, which were discussed in Chapter 4. The overall conclusion that can be drawn from the various experiments in this area is that there is a significant difference in the half-life of μ mRNA between cell types representing early and late developmental stages, and that this makes a significant contribution to the difference in steady state mRNA levels. However, what is at present not completely clear is how directly this result applies to normal B cells; the difficulties inherent in working with primary B cells have been discussed in Chapter 4. The data available at present indicate that increases in transcription may make a more significant contribution to mRNA levels in normal B cells than in B cell lines (see Chapter 4).

The observation that control of the rate of decay of μ mRNA was important in the regulation of μ gene expression lead to the question of which part of the μ mRNA was important in the control of its stability, and in particular,

what factors were important in the stabilisation of μ mRNA in hybridoma and myeloma cell lines? The striking increase in endoplasmic reticulum in plasma cells prompted Neuberger and colleagues to formulate the hypothesis that the large amount of endoplasmic reticulum sequestered a greater proportion of the μ mRNA in membrane-bound polysomes, which had the effect of stabilising the mRNA (Mason *et al*, 1988). To test this hypothesis, these workers constructed a mutant which contained a μ leader sequence that encoded hydrophilic amino acids instead of the normal hydrophobic signal sequence, which thus would be predicted to be translated on cytoplasmic polysomes. Using the heat shock induction system to measure half-life, they found this mRNA to be unstable, and to have a similar half-life to normal μ mRNA in WEHI231 cells. Therefore the location of the mRNA could be involved in the control of its stability. Chapters 5 and 6 described work to investigate further this possibility, in which an alternative mutant was constructed. In this mutant, the normal initiation codon was removed and a new initiation codon was inserted after the signal sequence, at the start of the mature protein, thus precluding the translation of the signal peptide. This mutant was placed under the control of the heat shock promoter and transfected into the myeloma cell line J558L. In contrast to the wild-type μ mRNA, and other mutants analysed at the same time, this mutant had a dramatically reduced half-life of around one hour. This confirmed the result of Mason *et al* (1988), and indicated the sequence alterations themselves were not likely to be disrupting any short stability determining sequence that might be present, as different nucleotide changes were made in each case.

Strong evidence indicates that the sequence alterations made by Mason *et al* (1988) and those discussed here change the localisation of the μ mRNA from membrane-bound to free polysomes, although this remains to be proven directly. Therefore the experiments to isolate membrane-bound and free polysomes and examine the proportion of mutant and wild-type mRNA in each would be a first priority for further work in this area. In addition, the experiments thus far have not investigated the effect (if any) of the change in signal sequence on the rate of translation (and hence ribosome

loading) of the μ mRNA, although the Western blot experiments (Chapter 6) show that the mutant is translated at a reasonable efficiency.

It is important to distinguish between effects of mRNA location and effects of ribosome loading, as there is some evidence that reduced ribosome loading (as a result of premature termination of translation) can cause a decrease in stability of μ mRNA (Baumann *et al*, 1985; Jäck and Wabl, 1989), as it can for other mRNAs (Maquat *et al*, 1981; Albrecht *et al*, 1984; Baserga *et al*, 1988; Eccles *et al*, 1989). In addition, some observations indicate that the earlier the termination codon is encountered in translation, the more pronounced the effect on the rate of mRNA degradation (Baumann *et al*, 1985), although this is not always the case (Jäck and Wabl, 1989). Jäck and Wabl have postulated that an increase in ribosome loading when B lymphocytes differentiate is responsible for the increase in μ mRNA stability which is observed (Jäck and Wabl, 1989). According to this hypothesis, a specific secondary structure in the mRNA is a target for a rate-limiting endonucleolytic cut, and the presence of a high density of ribosomes prevents the formation of this secondary structure (Jäck and Wabl, 1989). The μ mRNAs that are not able to associate with the endoplasmic reticulum either because of limiting amounts of membrane (in resting B cells), or the mutated signal peptide, would not be completely translated, or would be translated inefficiently, and this would lead to instability via a mechanism such as that described above.

Two pieces of evidence argue against this hypothesis. Firstly, the Western blots shown in Chapter 6 show that significant amounts of the correct length μ polypeptide accumulate from the mutant mRNA, so despite the fact that it is located on free polysomes, the entire coding sequence is translated. Secondly, from studies of free and membrane-bound polysomes in myeloma cells, Mechler observed that μ -containing polysomes free in the cytoplasm (which comprised 10% of the total) were of the same size as those bound to the membrane, indicating that they contained the same number of ribosomes (Mechler, 1981a). The existence of these free polysomes in myeloma cells is consistent with the hypothesis that the number of mRNA molecules exceeds the capacity of the

endoplasmic reticulum ribosome binding sites, and therefore that the amount of endoplasmic reticulum is limiting (Mechler and Vassalli, 1975). At present, then, it is not clear whether ribosome loading is altered during B cell differentiation, or whether the signal peptide mutants have a reduced ribosome load. Therefore, to determine whether there is any correlation between ribosome density and mRNA stability, future work should be aimed at determining the ribosome loading of the mutant and wild-type mRNAs, and of wild-type μ mRNA in B cell lymphomas and myelomas.

A precedent for the localisation of the mRNA being an important factor in the control of its stability exists for histone mRNA, as mentioned in Section 1.6. In order for histone mRNA to be correctly degraded at the end of S phase, the histone polysomes must be associated with the cytoskeletal framework, rather than with the endoplasmic reticulum (Zambetti *et al*, 1987). Therefore the site of attachment of the polysomes may be an important part of the signal pathway by which the mRNA is degraded after DNA synthesis has ceased. In the case of immunoglobulin mRNA, it is possible to formulate a hypothesis whereby the attachment of the polysomes to the endoplasmic reticulum causes a conformational change in a factor associated with the ribosomes. This factor could be the ribonuclease itself, in which case the conformational change would be such as to inactivate it, or the factor could inactivate a ribonuclease associated with the mRNA. At present it is unclear which of these is more likely, as there is evidence in support of both possibilities. The nuclease responsible for the degradation of histone mRNA, for example, is loosely associated with the ribosomes (Ross *et al*, 1987), but in other cases the ribonuclease seems to be tightly bound to the mRNA (Bandyopadhyay *et al*, 1990). Appropriate cell fractionation experiments and the development of *in vitro* systems to assay ribonuclease activity will be necessary to answer these questions.

In conclusion, then, the high level of μ mRNA found in differentiated B cell lines is largely due to increased mRNA stability. The other major alteration in immunoglobulin synthesis which occurs when B cells differentiate, the switch from production of membrane-bound to secreted immunoglobulin,

is brought about by changes in mRNA processing, as well as by post-translational regulation (Sitia et al, 1987; Galli et al, 1988). It is of interest to note that the size of the endoplasmic reticulum is important for the post-translational down-regulation of μ_m heavy chain in plasmacytomas, causing the transit time of the μ_m polypeptide through the endoplasmic reticulum to exceed its half-life, and thus preventing cell surface expression (Sitia et al, 1987). In addition, from the work described here, the endoplasmic reticulum seems to be involved in the high level production of secreted immunoglobulin via stabilisation of its mRNA. Therefore, two phases are evident in the control of immunoglobulin gene expression. Initial commitment to immunoglobulin gene expression in the B cell lineage occurs early in development at the level of gene rearrangement and transcriptional activation, whereas post-transcriptional and post-translational factors become dominant later in development. The changes in cellular morphology associated with differentiation, in particular the increase in the size of the endoplasmic reticulum, play a major role in these latter processes.

REFERENCES

- Abelson, H.T., Johnson, L.F., Penman, S. and Green, H. (1974) *Cell* **1**, 161-165.
- Adam, S.A., Nakagaw, T., Swanson, M.S., Woodruff, T.K. and Dreyfuss, G. (1986) *Mol. Cell. Biol.* **6**, 2932-2943.
- Albrecht, G., Krowczynska, A. and Brawerman, G. (1984) *J. Mol. Biol.* **178**, 881-896.
- Allshire, R.C., and Bostock, C.J. (1986) *J. Mol. Biol.* **188**, 1-13.
- Alt, F.W., Bothwell, A.L.M., Knapp, M., Siden, E., Mather, E., Koshland, M. and Baltimore, D. (1980) *Cell* **20**, 293-301.
- Alt, F.W. and Baltimore, D. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4118-4122.
- Alt, F.W., Rosenberg, N.E., Enea, V., Siden and Baltimore, D. (1982) *Mol. Cell. Biol.* **2**, 386-400.
- Alt, F.W., Yancopoulos, G.D., Blackwell, T.K., Wood, C., Thomas, E., Boss, M., Coffman, R., Rosenberg, N., Tonegawa, S. and Baltimore, D. (1984) *EMBO J.* **3**, 1209-1219.
- Alt, F.W., Blackwell, T.K., DePinho, R.A., Reth, M.G. and Yancopoulos, G.D. (1986) *Immunol. Rev.* **89**, 5-30.
- Alt, F.W., Blackwell, K.T. and Yancopoulos, G.D. (1987) *Science* **238**, 1079-1087.
- Alterman, R.M., Sprecher, C.S., Graves, R.A., Marzluff, W.F. and Skoultchi, A.I. (1985) *Mol. Cell. Biol.* **5**, 2316-2324.
- Antmann, E., Muller, H. and Suaer, G. (1980) *J. Virol.* **35**, 962-964.
- Aviv, H. and Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408-1412.

References

- Aviv, H., Voloch, Z., Bastos, R. and Levy, S. (1976), *Cell* **8**, 495-503.
- Baker, E.J., Keller, L.R., Schloss, J.A. and Rosenbaum, J.L. (1986) *Mol. Cell. Biol.* **6**, 54-61.
- Baker, E.J., Diener, D.R. and Rosenbaum, J.L. (1989) *J. Mol. Biol.* **207**, 771-781.
- Bakker, O., Arnberg, A.C., Noteborn, M.H.M., Winter, A.J. and Ab, G. (1988) *Nucleic Acids Res.* **16**, 10109-10118.
- Bandyopadhyay, R., Stein, G. and Stein, J. (1987) *Biochemistry* **26**, 2938-2944
- Bandyopadhyay, R., Coutts, M., Krowczynska, A and Brawerman, G. (1990) *Mol. Cell. Biol.* **10**, 2060-2069.
- Bandziulis, R.J., Swanson, M.S. and Dreyfuss, G. (1989) *Genes. Develop.* **3**, 431-437.
- Banerji, J., Olsen, L. and Schaffner, W. (1983) *Cell* **33**, 729-740.
- Barton, H.A., Eisenstein, R.S., Bomford, A. and Munro, H.N. (1990) *J. Biol. Chem.* **265**, 7000-7008.
- Baserga, S.J. and Benz, E.J. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2056-2060.
- Baumann, B., Potash, M.J. and Köhler, G. (1985) *EMBO J.* **4**, 351-359.
- Baumbach, L.L., Marashi, F., Plumb, M., Stein, G. and Stein, J. (1984) *Biochemistry* **23**, 1618-1625.
- Belasco, J.G., Nilsson, G., von Gabain, A. and Cohen, S.N. (1986) *Cell* **46**, 245-251.
- Ben Ze'ev, A., Farmer, S.R. and Penman, S. (1979) *Cell* **17**,

319-325.

Bentley, D.L. and Groudine, M. (1986) *Nature* (London) **321**, 702-706.

Bentley, D.L. and Groudine, M. (1988) *Cell* **53**, 245-256.

Berberich, I. and Schimpl, A. (1990) *Eur. J. Immunol.* **20**, 445-448.

Berger, C.N. (1986) *EMBO J.* **5**, 85-93.

Berger, S.L. and Cooper, H.L. (1975) *Proc. Natl. Acad. Sci U.S.A.* **72**, 3873-3877.

Bernstein, P., Peltz, S.W. and Ross, J. (1989) *Mol. Cell. Biol.* **9**, 659-670.

Berton, M.T., Uhr, J.W. and Vitetta, E.S. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2829-2833.

Bettinger, G.E. and Young, F.E. (1975) *Biochem. Biophys. Res. Commun.* **67**, 16-21.

Binder, R., Hwang, S-P.L., Ratnasabapathy, R. and Williams, D.L. (1989) *J. Biol. Chem.* **264**, 16910-16918

Bird, R.C., Jacobs, F.A., Stein, G., Stein, J. and Sells, B.H. (1985) *Proc. Natl. Acad. Sci U.S.A.* **82**, 6760-6764.

Blackwell, T.K., Moore, M.W., Yancopoulos, G.D., Suh, H., Lutzker, S., Selsing, E. and Alt, F.W. (1986) *Nature* (London) **324**, 585-589.

Blackwell, T.K., Malynn, B.A., Pollock, R.R., Ferrier, P., Covey, L.R., Fulop, G.M., Phillips, R.A., Yancopoulos, G.D. and Alt, F.W. (1989) *EMBO J.* **8**, 735-742.

Blanchard, J-M., Piechaczyk, M., Dani, C., Chambard, J-C., Franchi, A., Pouyegur, J. and Jeanteur, P. (1985) *Nature* (London) **317**, 443-445.

References

- Blattner, F.R. and Tucker, P.W. (1984) *Nature* (London) **307**, 417-422.
- Blobel, G. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 924-928.
- Blobel, G. and Dobberstein, B. (1975) *J. Cell. Biol.* **67**, 835-851.
- Blom van Assendelft, G., Hanscombe, O., Grosveld, F. and Greaves, D.R. (1989) *Cell* **56**, 969-977.
- Blume, J.E. and Shapiro, D.J. (1989) *Nucleic Acids Res.* **17**, 9003-9014.
- Blundell, M. and Kennell, D. (1974) *J. Mol. Biol.* **83**, 143-161.
- Bole, D.G., Hendershot, L.M. and Kearney, J.F. (1986) *J. Cell. Biol.* **102**, 1558-1566.
- Bonifer, C., Vidal, M., Grosveld, F. and Sippel, A.E. (1990) *EMBO J.* **9**, 2843-2848.
- Boss, M.A., Kenten, J.H., Wood, C.R. and Emtage, J.S. (1984) *Nucleic Acids Res.* **12**, 3791-3806.
- Bothwell, A.L.M., Paskind, M., Reth, M., Imanishi-Kari, T., Rajewsky, K. and Baltimore, D. (1981) *Cell* **24**, 625-637.
- Boyer, H.W. and Roulland-Dussoix, D. (1969) *J. Mol. Biol.* **41**, 459-472.
- Brawerman, G. (1975) *Cell* **5**, 271-280.
- Brawerman, G. (1981) *CRC Critical Reviews in Biochemistry* **10**, 1-38.
- Brawerman, G. and Diez, J. (1975) *Cell* **5**, 271-280.

References

- Brewer, G. and Ross, J. (1988) *Mol Cell Biol* **8**, 1697-1708.
- Brewer, G. and Ross, J. (1989) *Mol. Cell. Biol.* **9**, 1996-2006.
- Brock, M.L., and Shapiro, D.J. (1983a) *Cell* **34**, 207-214.
- Brock, M.L., and Shapiro, D.J. (1983b) *J. Biol. Chem.* **258**, 5449-5455.
- Brodeur, P.H. and Riblet, R. (1984) *Eur. J. Immunol.* **14**, 922-930.
- Burnet, F.M. (1957) *Aust. J. Sci.* **20**, 67-73.
- Burnette, W.N. (1981) *Anal. Biochem.* **112**, 195-203.
- Burrows, P., LeJeune, M. and Kearney, J.F. (1979) *Nature (London)* **280**, 838-841.
- Butler, E.T., and Chamberlin, M.J. (1982) *J. Biol Chem.* **257**, 5772-5778.
- Calame, K.L. (1985) *Annu. Rev. Immunol.* **3**, 159-195.
- Calame, K.L. (1989) *Trends Genet.* **5**, 395-399.
- Calvert, J.E., Kim, M.F., Gathings, W.E. and Cooper, M.D. (1983) *J. Immunol.* **131**, 1693-1697.
- Campisi, J., Gray, H.E., Pardee, A.B., Dean, M., and Sonnenschein, G.E. (1984) *Cell* **36**, 241-247.
- Capasso, O., Bleecker, G.C. and Heintz, N. (1987) *EMBO J.* **6**, 1825-1831.
- Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S. and Cerami, A. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1670-1674.
- Carlson, L.M., Oettinger, M.A., Schatz, D.G., Masteller, E.L., Hurley, E.A., McCormack, W.T., Baltimore, D. and

- Thompson, C.B. (1991) *Cell* **64**, 201-208.
- Carneiro, M. and Schibler, U. (1984) *J. Mol. Biol.* **178**, 869-880.
- Caron, J., Jones, A.L., Rall, L.B. and Kirscher, M.W. (1985) *Nature (London)* **317**, 648-650.
- Carter, P., Bedouelle, H., and Winter, G. (1985) *Nucleic Acids Res.* **13**, 4431-4443.
- Casey, J.L., Henzte, M.W., Koeller, D.M., Wright Caughman, S., Roualt, T.A., Klausner, R.D. and Harford, J.B. (1988) *Science* **240**, 924-928.
- Casey, J.L., Koeller, D.M., Ramin, V.C., Klausner, R.D. and Harford, J.B. (1989) *EMBO J.* **8**, 3693-3699.
- Cathala, G., Savouret, J-F., Mendez, B., West, B.L., Karin, M., Martial, J.A. and Baxter, J.D. (1983) *DNA* **2**, 329-335.
- Caton, A.J. (1990) *J. Exp. Med.* **172**, 815-825.
- Cattaneo, A. and Neuberger, M.S. (1987) *EMBO J.* **6**, 2753-2758.
- Cebra, J., Colberg, J.E. and Dry, S. (1966) *J. Exp. Med.* **123**, 547-557.
- Chen, U. (1988) *Gene* **72**, 209-217.
- Chen-Bettecken, U., Wecker, E. and Schimpl, A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7384-7388.
- Cheng, G., Nandi, A., Clerk, S. and Skoultchi, A.I. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7002-7006.
- Chu, G., Hayakawa, H. and Berg, P. (1987) *Nucleic Acids Res.* **15**, 1311-1326.
- Chun, J.J.M., Schatz, D.G., Oettinger, M.A., Jaenisch, R. and Baltimore, D. (1991) *Cell* **64**, 189-200.

References

- Cleveland, D.W., Lopata, M.A., Sherline, P. and Kirschner, M.W. (1981) *Cell* **25**, 537-546.
- Cleveland, D.W. and Havercroft, J.C. (1983) *J. Cell. Biol.* **97**, 919-924.
- Cochrane, A.W. and Deeley, R.G. (1988) *J. Mol. Biol.* **203**, 555-567.
- Cochrane, A.W. and Deeley, R.G. (1989) *J. Biol. Chem.* **264**, 6495-6503.
- Coffman, R.L. and Weissman, I.L. (1983) *J. Mol. Cell. Immunol.* **1**, 31-38.
- Cowan, N.J. and Milstein, C. (1974) *J. Mol. Biol.* **82**, 469-481.
- Croce, C.M. (1987) *Cell* **49**, 155-156.
- Dani, C., Blanchard, J.M., Piechaczyk, M., El Sabouty, S., Marty, L. and Jeanteur, P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7046-7050.
- Dani, C., Mechti, N., Piechaczyk, M., Lebleu, B., Jeanteur, P. and Blanchard, J.M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4896-4899.
- Danner, D. and Leder, P. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8658-8662.
- Darnell, J.E. Jr. (1982) *Nature (London)* **297**, 365-371.
- Dean, M., Levine, R.A. and Campisi, J. (1986a) *Mol. Cell. Biol.* **6**, 518-524.
- Dean, M., Levine, R.A., Ran, W., Kindy, M.S., Sonenschein, G.E. and Campisi, J. (1986b) *J. Biol. Chem.* **261**, 9161-9166.
- DeFranco, A.L., Davis, M.M. and Paul, W.E. (1982) in *B and T*

References

- Cell Tumours: Biological and Clinical Aspects (Vitetta, E., ed), pp. 445-450, Academic Press Inc., New York.
- DeLisle, A.J., Graves, R.A., Marzluff, W.F., Johnson, L.F. (1983) *Mol. Cell. Biol.* **3**, 1920-1929.
- Denniston, K.J., Yoneyama, T., Hoyer, B.H. and Gerin, J.L. (1984) *Gene* **32**, 357-368.
- DeNoto, F.M., Moore, D.D. and Goodman, H.M. (1981) *Nucleic Acids Res.* **9**, 3719-3730.
- Desiderio, S.V., Yancopoulos, G.D., Paskind, M., Thomas, E., Boss, M.A., Landau, N., Alt, F.W. and Baltimore, D. (1984) *Nature (London)*, **311**, 752-755.
- D'Eustachio, P., Pravtcheva, D., Marcu, K. and Ruddle, F.H. (1980) *J. Exp. Med.* **151**, 1545-1550.
- Deutscher, M.P. (1985) *Cell* **40**, 731-732.
- Deutscher, M.P. (1988) *Trends Biochem. Sci.* **13**, 136-139.
- Diamond, D. J. and Goodman, H.M. (1985) *J. Mol. Biol.* **181**, 41-62.
- Doel, M.T. and Carey, N.H. (1976) *Cell* **8**, 51-58.
- Donovan, W.P. and Kushner, S.R. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 120-124.
- Dony, C., Kessel, M. and Gruss, P. (1985) *Nature (London)*, **317**, 636-639.
- Dulis, B.H., Kloppel, T.M., Grey, H-M. and Kubo, R.T. (1982) *J. Biol. Chem.* **257**, 4369-4374.
- Dulis, B.H. (1983) *J. Biol. Chem.* **258**, 2181-2187.
- Dvoretzky, I., Shoher, R., Chattopadhyay, S.K. and Lowy, D.R. (1980) *Virology* **103**, 369-375.

References

- Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R. and Hood, L. (1980) *Cell* **20**, 313-319.
- Eberhardt, N.L., Selby, M., Cathala, G., Karin, M., Gutierrez-Hartmann, A., Mellon, S.H., Lan, N.C., Gardner, D. and Baxter, J.D. (1982) *UCLA Symposia in Molecular and Cellular Biology New Series* **26**, 235-251.
- Eccles, S.J., Vidal, M.A., Wrighton, C.J., Grosveld, F.G. and Burke, J.F. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1297-1301.
- Eccles, S., Sarnier, N., Vidal, M., Cox, A. and Grosveld, F. (1990) *The New Biologist* **2**, 801-811.
- Eick, D., Piechaczyk, M., Henglein, B., Blanchard, J-M., Traub, B., Kofler, E., Wiest, S., Lenoir, G.M. and Bornkamm, G.W. (1985) *EMBO J.* **4**, 3717-3725.
- Eick, D. and Bornkamm, G.W. (1986) *Nucleic Acids Res.* **14**, 8331-8346.
- Emerson, C.P. (1971) *Nature New Biology* **232**, 101-106.
- Engvall, E. and Perlmann, P. (1971) *Immunochemistry* **8**, 871-879.
- Engvall, E. and Perlmann, P. (1972) *J. Immunol.* **109**, 129-135.
- Eshhar, Z., Blatt, C., Bergamn, Y. and Haimovich, J. (1979) *J. Immunol.* **122**, 2430-2434.
- Esser, C. and Radbruch, A. (1989) *EMBO J.* **8**, 483-488.
- Evans, R.M., Birnberg, N.C. and Rosenfeld, M.G. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7659-7663.
- Favoloro, J., Treisman, R., and Kamen, R. (1981) *Methods Enzymol.* **65**, 718-728.

References

- Fazekasde St. Groth, S. and Scheidegger, D. (1980) *J. Immunol. Methods* **35**, 1-21.
- Folger, K.R., Wong, E.A., Wahl, G. and Capechi, M.R. (1982) *Mol. Cell. Biol.* **2**, 1372-1387.
- Forrester, W.C., Takegawa, S., Papayannopoulou, T., Stamatoyannopoulos, G. and Groudine, M. (1987) *Nucleic Acids Res.* **15**, 10159-10177.
- Forster, A., Hobart, M., Hengartner, H. and Rabbitts, T.H. (1980) *Nature (London)* **286**, 897-899.
- Fort, P., Rech, J., Vie, A., Piechaczyk, M., Bonniou, A., Jeanteur, P. and Blanchard, J-M. (1987) *Nucleic Acids Res.* **15**, 5657-5667.
- Fox, C.A. and Wickens, M. (1990) *Genes. Develop.* **4**, 2287-2298.
- Furuichi, Y., LaFiandra, A. and Shatkin, A.J. (1977) *Nature (London)* **266**, 235-239.
- Galli, G., Guise, J.W., McDevitt, M.A., Tucker, P.W. and Nevins, J.R. (1987) *Genes. Develop.* **1**, 471-481.
- Galli, G., Guise, J.W., Tucker, P.W. and Nevins, J.R. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2439-2443.
- Garcia, J.V., Bich-Thuy, L., Stafford, J. and Queen, C. (1986) *Nature (London)* **322**, 383-385.
- Gathings, W.E., Lawton, A.R. and Cooper, M.D. (1977) *Eur. J. Immunol.* **7**, 804-810.
- Gay, D.A., Yen, T.J., Lau, J.T.Y. and Cleveland, D.W. (1987) *Cell* **50**, 671-679.
- Gay, D.A., Sisodia, S.S. and Cleveland, D.W. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5763-5767.

References

- Genovese, C. and Milcarek, C. (1990) *Mol. Immunol.* **27**, 733-743.
- Gerster, T., Picard, D. and Schaffner, W. (1986) *Cell* **45**, 45-52.
- Gillies, S.D., Morrison, S.L., Oi, V.T. and Tonegawa, S. (1983) *Cell* **33**, 717-728.
- Gilmore, R. and Blobel, G. (1983) *Cell* **35**, 677-685.
- Gitelman, D.K. and Apirion, D. (1980) *Biochem. Biophys. Res. Commun.* **96**, 1063-1070.
- Glozak, M.A., Riley, R.L. and Blomberg, B.B. (1990) *J. Cell. Biochem. Supl.* **14D**, 203
- Gong, Z. and Brandhorst, B.P. (1988) *Mol. Cell. Biol.* **8**, 3518-3525.
- Gordon, D.A., Schelness, G.S., Nicosia, M. and Williams, D.A. (1988) *J. Biol. Chem.* **263**, 2625-2631.
- Gorski, K., Roch, J-M., Prentki, P. and Krisch, H.M. (1985) *Cell* **43**, 461-469.
- Graham, F.L. and Van Der Eb, A.J. (1973) *Virology* **52**, 456-467.
- Grange, T., Martins de Sa, C., Oddos, J. and Pictet, R. (1987) *Nucleic Acids Res.* **15**, 4771-4787.
- Graves, R.A. and Marzluff, W.F. (1984) *Mol. Cell. Biol.* **4**, 351-357.
- Graves, R.A., Wellman, S.E., Chiu, I-M. and Marzluff, W.F. (1985) *J. Mol. Biol.* **183**, 179-194.
- Graves, R.A., Pandey, N.B., Chodchoy, N. and Marzluff, W.F. (1987) *Cell* **48**, 615-626.

References

- Green, L.L. and Dove, W.F. (1988) *J. Mol. Biol.* **200**, 321-328.
- Green, M.R., Maniatis, T., and Melton, D.A. (1983) *Cell* **32**, 681-694.
- Greenberg, J.R. (1972) *Nature (London)* **240**, 102-104.
- Greenberg, J.R. and Perry, R.P. (1972) *Biochim. Biophys. Acta* **287**, 361-366.
- Greenberg, M.E. and Ziff, E.B. (1984) *Nature (London)* **311**, 433-438.
- Gronenborn, B. and Messing, J. (1978) *Nature (London)* **272**, 375-377.
- Grosschedl, R. and Baltimore, D. (1985) *Cell* **41**, 885-897.
- Grossi de Sa, M.F., Standort, N., Martins de Sa, C., Akhayat, O., Huesca, M. and Scherrer, K. (1988) *Eur. J. Biochem.* **176**, 521-526.
- Grosveld, F., Blom van Assendelft, G., Greaves, D.R. and Kollias, G. (1987) *Cell* **51**, 975-985.
- Grunstein, M. and Hogness, D.S. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3961-3965.
- Guaneros, G., Mantanez, C., Hernandez, T. and Court, D. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 238-242.
- Guise, J.W., Lim, P.L., Yuan, D. and Tucker, P.W. (1988) *J. Immunol.* **140**, 3988-3994.
- Guise, J.W., Galli, G., Nevins, J.R. and Tucker, P.W. (1989) in *Immunoglobulin Genes* (Honjo, T., Alt, F.W. and Rabbitts, T.H., eds), pp. 275-301, Academic Press, London and New York.
- Gutman, G.A., Warner, N.L. and Harris, A.W. (1981) *Clin. Immunol. Immunopathol.* **18**, 230-244.

References

- Guyette, W.A., Matusik, R.J. and Rosen J.M. (1979) *Cell* **17**, 1013-1023.
- Haas, I.G. and Wabl, M. (1983) *Nature (London)* **306**, 387-389.
- Hagmann, J., Rudin, C.M., Haash, D., Chaplin, D. and Storb, U. (1990) *Genes. Develop.* **4**, 978-992.
- Haile, D.J., Hentze, M.W., Rouault, T.A., Harford, J.B. and Klausner, R.D. (1989) *Mol. Cell. Biol.* **9**, 5055-5061.
- Hamm, J. and Mattaj, I.W. (1990) *Cell* **63**, 109-118.
- Harpold, M.M., Evans, R.M., Salditt-Georgieff, M. and Darnell, J.E. (1979) *Cell* **17**, 1025-1035.
- Harrison, T.M., Brownlee, G.G. and Milstein, C. (1974) *Eur. J. Biochem.* **47**, 613-620.
- Hassouna, N., Michot, B. and Bachellerie, J-P. (1984) *Nucleic Acids Res.* **12**, 3563-3583.
- Hay, A.J., Abraham, G., Skehel, J.J., Smith, J.C., and Fellner, P. (1977) *Nucleic Acids Res.* **4**, 4197-4209.
- Heintz, N., Sive, H.L. and Roeder, R.G. (1983) *Mol. Cell. Biol.* **3**, 539-550.
- Hendershot, L., Bole, D., Köhler, G. and Kearney, J.F. (1987) *J. Cell. Biol.* **104**, 761-767.
- Hendershot, L., Kerr, W., Kubagawa, H., Feng, L., Cooper, M.D. and Burrows, P. (1988) *J. Biol. Chem. Supl.* **12B**, 92.
- Hentschel, C.C. and Birnstiel, M.L. (1981) *Cell*, **25**, 301-313.
- Hentze, M.W., Rouault, T.A., Harford, J.B. and Klausner, R.D. (1989) *Science* **244**, 357-359.
- Hilshmann, N. and Craig, L.C. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **53**, 1403-1409.

References

- Huez, G., Bruck, C. and Cleuter, Y. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 908-911.
- Humphrey, T. and Proudfoot, N.J. (1988) Trends Genet. **4**, 243-245.
- Hwang, S-P.L., Eisenberg, M., Binder, R., Shelness, G.S. and Williams, D.L. (1989) J. Biol. Chem. **264**, 8410-8418.
- Jäck, H-M. and Wabl, M. (1988) EMBO J. **7**, 1041-1046.
- Jacobsen, A. and Favreau, M. (1983) Nucleic Acids Res. **11**, 6353-6358.
- Jeffery, W. and Brawerman, G. (1974) Biochemistry **13**, 4633-4637.
- Jones, T.R. and Cole, M.D. (1987) Mol. Cell. Biol. **7**, 4513-4521.
- Kabnick, K.S. and Housman, D.E. (1988) Mol. Cell. Biol. **8**, 3244-3250.
- Kadesch, T. and Berg, P. (1986) Mol. Cell. Biol. **6**, 2593-2601.
- Kakkis, E. Prehn, J. and Calame, K. (1986) Mol. Cell. Biol. **6**, 1357-1361.
- Kakkis, E., Mercola, M. and Calame, K. (1988) Nucleic Acids Res. **16**, 77-96.
- Kaplan, R. and Apirion, D. (1974) J. Biol. Chem. **249**, 149-151.
- Karasuyama, H., Kudo, A. and Melchers, F. (1990) J. Exp. Med. **172**, 969-972.
- Kearney, J.F., Cooper, M.D., Klein, J. and Abney, E.R. (1977) J. Exp. Med. **146**, 297-301.

- Keath, E.J., Kelekar, A. and Cole, M.D. (1984) *Cell* **37**, 521-528.
- Kelley, D.E. and Perry, R.P. (1986) *Nucleic Acids Res.* **14**, 5431-5447.
- Kemp, D.J., Harris, A.W., Cory, S. and Adams, J.M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2876-2880.
- Kincade, P.W. (1981) *Adv. Immunol.* **31**, 177-245.
- King, L.B. and Corley, R.B. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2814-2818.
- Klein, S., Sablitzky, F. and Radbruch, A. (1984) *EMBO J.* **3**, 2473-2476.
- Knight, E.Jr., Anton, E.D., Fahey, D., Friedland, B.K. and Jonak, G.J. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1151-1154.
- Koeller, D.M., Casey, J.L., Hentze, M.W., Gerhardt, E.M., Chan, L-N.L., Klausner, R.D. and Harford, J.B. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3574-3578.
- Koshland, M.E. (1975) *Adv. Immunol.* **20**, 41-69.
- Kostura, M. and Craig, N. (1986) *Biochemistry* **25**, 6384-6391.
- Kozak, M. (1983) *Microbiol. Rev.* **47**, 1-45.
- Kozak, M. (1984) *Nucleic Acids Res.* **12**, 857-872.
- Kozak, M. (1987) *J. Mol. Biol.* **196**, 947-950.
- Kramer, W., Schugart, K. and Fritz, H-J. (1982) *Nucleic Acids Res.* **10**, 6475-6485.
- Kramer, W., Drutsa, V., Jansen, H-W., Kramer, B., Pflugfelder, M. and Fritz, H-J. (1984) *Nucleic Acids Res.* **12**,

9441-9456.

Krisch, H.M. and Allet, B. (1982) Proc. Natl. Acad. Sci. **79**, 4937-4941.

Krowczynska, A., Yenofsky, R. and Brawerman, G. (1985) J. Mol. Biol. **181**, 231-239.

Kruijer, W. Cooper, J.A., Hunter, T. and Verma, I.M. (1984) Nature (London) **312**, 711-720.

Kudo, A. and Melchers, F. (1987) EMBO J. **6**, 2267-2272.

Kudo, A., Sakaguchi, N. and Melchers, F. (1987) EMBO J. **6**, 103-107.

Kurosawa, Y., Von Boehmer, H., Haas, W., Sakano, H., Trauneker, A. and Tonegawa, S. (1981) Nature (London) **290**, 565-570.

Kurosawa, Y. and Tonegawa, S. (1982) J. Exp. Med. **155**, 201-218.

Kwan, S.W. and Brawerman, G. (1972) Proc. Natl. Acad. Sci. U.S.A. **69**, 3247-3250.

Labhart, P. and Reeder, R.H. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 56-60.

Laemmli, E.K. (1970) Nature (London) **227**, 680-685.

Laird-Offringa, I.A., Elfferich, P., Knaken, H.J., de Rooter, J. and van der Eb, A.J. (1989) Nucleic Acids Res. **17**, 6499-6514.

Laird-Offringa, I.A., de Wit, C.L., Elfferich, P. and van der Eb, A.J. (1990) Mol. Cell. Biol. **10**, 6132-6140.

Lamson, G. and Koshland, M.E. (1984) J. Exp. Med. **160**, 877-892.

- Lancaster, W.D. (1981) *Virology* **108**, 251-255.
- Law, M-F., Lowy, D.R., Dvoretzky, I., and Howley, P.M. (1981) *Biochemistry* **78**, 2727-2731.
- Law, M-F., Byrne, J.C and Howley, P.M. (1983) *Mol. Cell. Biol.* **3**, 2110-2115.
- Law, R., Kuwabara, M.D., Briskin, M., Fasel, N., Hermanson, G., Sigman, D.S. and Wall, R. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 9160-9164.
- Layton, J.E., Johnson, G.R., Scott, D.W. and Nossal, G.J.V. (1978) *Eur. J. Immunol.* **8**, 325-330.
- Lee, W.M.F., Lin, C. and Curran, T. (1988) *Mol. Cell. Biol.* **8**, 5521-5527.
- Lehrach, H., Diamond, D., Wozney, J., and Boedtke, H. (1977) *Biochemistry* **16**, 4743-4751.
- Leibold, E. and Munro, H.N. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2171-2175.
- Leibold, E.A., Laudano, A. and Yu, Y. (1990) *Nucleic Acids Res.* **18**, 1819-1824.
- Lennon, G.G. and Perry, R.P. (1985) *Nature (London)*, **318**, 475-478.
- Lernhardt, W., Karasuyama, H., Rolink, A. and Melchers, F. (1987) *Immunol. Rev.* **99**, 241-262.
- Levine, B.J., Chodchoy, N., Marzluff, W.F and Skoultchi, A.I. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6189-6193.
- Levis, R. and Penman, S. (1977) *Cell* **11**, 105-113.
- Levitt, D. and Cooper, M.D. (1980) *Cell* **19**, 617-625.
- Lieber, M.R., Hesse, J.E., Mizuichi, K. and Gellert, M.

- (1987) *Genes. Develop.* **1**, 751-761.
- Lieberson, R., Gianni, S.L., Birshtein, B.K. and Eckhardt, L.A. (1991) *Nucleic Acids Res.* **19**, 933-937.
- Lim, S., Mullins, J.J., Chen, C-M., Gross, K.W. and Maquat, L.E. (1989) *EMBO J.* **8**, 2613-2619.
- Lin, W., and Kasamatsu, H. (1983) *Anal. Biochem.* **128**, 302-311.
- Linial, M., Gunderson, N. and Groudine, M. (1985) *Science* **230**, 1126-1132.
- Livant, D., Blatt, C. and Hood, L. (1986) *Cell* **47**, 461-470.
- Losson, R. and Lacroute, F. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5134-5137.
- Lowenhaupt, K., and Lingrel, J.B. (1978) *Cell* **14**, 337-344.
- Lowy, D.R., Rands, E. and Solnick, E.M. (1978) *J. Virol.* **26**, 291-298.
- Lowy, D.R., Dvoretzky, I., Shober, R., Law, M-F., Engel, L. and Howley, P.M. (1980) *Nature (London)* **287**, 72-74.
- Lundberg, U., von Gabain, A. and Melefors, O. (1990) *EMBO J.* **9**, 2731-2741.
- Lüscher, B., Stauber, C., Schindler, R. and Schümperli, D. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4389-4393.
- Lusky, M. and Botchan, M.R. (1985) *J. Virol.* **53**, 955-965.
- Lusky, M. and Botchan, M.R. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3609-3613.
- Lutzker, S., Rothman, P., Pollock, R., Coffman, R. and Alt, F.W. (1988) *Cell* **53**, 177-184.

References

- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning (A laboratory manual)*, Cold Spring Harbor, New York.
- Maquat, L.E., Kinniburgh, A.J., Rachmilewitz, E.A. and Ross, J. (1981) *Cell* **27**, 543-553.
- Martial, J.A., Hallewell, R.A., Baxter, J.D. and Goodman, H.M. (1979) *Science* **205**, 602-607.
- Martin, D.J. and Van Ness, B.G. (1989) *Mol. Cell. Biol.* **9**, 4560-4562.
- Mason, J.O., Williams, G.T. and Neuberger, M.S. (1985) *Cell* **41**, 479-487.
- Mason, J.O., Williams, G.T. and Neuberger, M.S. (1988) *Genes Develop.* **2**, 1003-1011.
- Mather, E.L. (1987) *Mol. Immunol.* **24**, 661-666.
- Mather, E.L. and Perry, (1981) *Nucleic Acids Res.* **9**, 6855-6867.
- Mather, E.L., Alt, F.W., Bothwell, A.L.M., Baltimore, D. and Koshland, M.E. (1981) *Cell* **23**, 369-378.
- Mather, E.L., Nelson, K.J., Haimovich, J. and Perry, R.P. (1984) *Cell* **36**, 329-338.
- Matsui, K., Nakanishi, K., Cohen, D.I., Hada, T., Furuyama, J-I., Hamaoka, T. and Higashino, K. (1989) *J. Immunol.* **142**, 2918-2923.
- Mattia, E., den Blaauwen, J.D., Ashwell, G. and van Renswoude, J.O.S. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1801-1805.
- Mattia, E., den Blaauwen, J. and van Renswoude, J.O.S. (1990) *Biochem. J.* **267**, 553-555.
- McDonnell, M.W., Simon, M.N. and Studier, F.W. (1977) *J. Mol.*

Biol. **110**, 119-146.

McGrew, L.L., Dworkin-Rastl, E., Dworkin, M. and Richter, J.D. (1989) Genes Develop. **3**, 803-815.

McGrew, L.L. and Richter, J.D. (1990) EMBO J. **9**, 3743-3751.

Mechler, B. (1981a) J. Cell Biol. **88**, 37-41.

Mechler, B. (1981b) J. Cell Biol. **88**, 42-50.

Mechler, B. and Vassalli, P. (1975) J. Cell biol. **67**, 25-37.

Mechler, B. and Rabbitts, T.H. (1981) J. Cell Biol. **88**, 29-36.

Meijlink, F., Curran, T., Miller, A.D. and Verma, I.M. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4987-4991.

Meischke, H.R.C. (1979) J. Gen. Virol. **43**, 473-487.

Melchers, F. and Andersson, J. (1974) Eur. J. Immunol. **4**, 181-188.

Melefors, Ö. and von Gabain, A. (1988) Cell **52**, 893-901.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Nucleic Acids Res. **12**, 7035-7056.

Mendecki, J., Lee, S.Y. and Brawerman, G. (1972) Biochemistry **11**, 792-798.

Mercer, J.F.B. and Wake, S.A. (1985) Nucleic Acids Res. **13**, 7929-7943.

Messing, J., Gronenborn, B., Müller-Hill, B. and Hofschneider, P.H. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 3642-3646.

Messing, J. (1979) Recombinant DNA Technical Bulletin **2**, 43-

48.

Messing, J., Crea, R. and Seeburg, P.H. (1981) *Nucleic Acids Res.* **2**, 309-321.

Mestecky, J., Zihán, J. and Butler, W.T. (1971) *Science* **171**, 1163-1165.

Meyer, K.B. and Neuberger, M.S. (1989) *EMBO J.* **8**, 1959-1964.

Michot, B., Bachellerie, J.P., Raynal, F. and Renalier, M-H. (1982) *FEBS Lett.* **140**, 193-197.

Miller, A.D., Curran, T. and Verma, I.M. (1984) *Cell* **36**, 51-60.

Milstein, C., Brownlee, G.G., Harrison, T.M. and Mathews, M.B. (1972) *Nature New Biology* **329**, 117-120.

Minty, A.J., Caravatti, M., Robert, B., Cohen, A., Daubas, P., Weydert, A., Gros, F. and Buckingham, M.E. (1981) *J. Biol. Chem.* **256**, 1008-1014.

Misener, V., Jongstra-Bilen, J., Young, A.J. Atkinson, M.J., Wu, G.E. and Jongstra, J. (1990) *J. Immunol.* **145**, 905-909.

Mitchell, R.L., Henning-Chubb, C., Huberman, E. and Verma, I.M. (1986) *Cell* **45**, 497-504.

Mitrani-Rosenbaum, S., Maroteaux, L., Mory, Y., Revel, M. and Howley, P.M. (1983) *Mol. Cell. Biol.* **3**, 233-240.

Moore, D.D., Marks, A.R., Buckley, D.I., Kapler, G., Payvar, F. and Goodman, H.M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 699-709.

Morris, T.D., Weber, L.A., Hickey, E., Stein, G. and Stein, J.L. (1991) *Mol. Cell. Biol.* **11**, 544-553.

Morse, D.E. and Yanofsky, C. (1969) *Nature (London)* **224**, 329-331.

References

- Mudd, E.A., Carpousis, A.J. and Krisch, H.M. (1990) *Genes Develop.* **4**, 873-881.
- Mulligan, R. and Berg, P. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2072-2076.
- Müllner, E.W. and Kühn, L.C. (1988) *Cell* **53**, 815-825.
- Munro, S. and Pelham, H.R.B. (1986) *Cell* **46**, 291-300.
- Munroe, D. and Jacobsen, A. (1990) *Gene* **91**, 151-158.
- Murphy, W. and Attardi, G. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 115-119.
- Nelson, K. J., Haimovich, J. and Perry, R.P. (1983) *Mol. Cell. Biol.* **3**, 1317-1332.
- Nepveu, A. and Marcu, K.B. (1986) *EMBO J.* **5**, 2859-2865.
- Neuberger, M.S. (1983) *EMBO J.* **2**, 1373-1378.
- Neuberger, M.S., Williams, G.T. and Fox, R.O. (1984) *Nature (London)*, **312**, 604-608.
- Neuberger, M.S. and Williams, G.T. (1986) *Phil. Trans. R. Soc. Lond.* **317**, 425-432.
- Neuberger, M.S., Caskey, H.M., Pettersson, S., Williams, G.T. and Surani, M.A. (1989) *Nature (London)*, **338**, 350-352.
- Neumann, E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P.H. (1982) *EMBO J.* **1**, 841-845.
- Newbury, S.F., Smith, N.H., Robinson, E.C., Hiles, I.D. and Higgins, C.F. (1987a) *Cell* **48**, 297-310.
- Newbury, S.F., Smith, N.H. and Higgins, C.F. (1987b) *Cell* **51**, 1131-1143.

References

- Nilsson, G., Belasco, J.G., Cohen, S.N. and von Gabain, A. (1984) *Nature* (London) **312**, 75-77.
- Nilsson, G., Belasco, J.G., Cohen, S.N. and von Gabain, A. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4890-4894.
- Nilsson, G., Lundberg, U. and von Gabain, A. (1988) *EMBO J.* **7**, 2269-2275.
- Noelle, R.J. and Snow, E.C. (1990) *Immunol. Today* **11**, 361-368.
- Nossal, G.J.V. and Pike, B.L. (1973) *Immunology* **25**, 33-45.
- Nossal, G.J.V. and Pike, B.L. (1975) *J. Exp. Med.* **141**, 904-917.
- Nussenzweig, M.C., Shaw, A.C., Sinn, E., Danner, D.B., Holmes, K.L., Morse, H.C.III and Leder, P. (1987) *Science*, **236**, 816-819.
- Oettinger, M.A., Schatz, D.G., Garka, C. and Baltimore, D. (1990) *Science* **248**, 1517-1523.
- Oi, V.T., Morrison, S.L., Herzenberg, L.A. and Berg, P. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 825-829.
- Osley, M.A. and Hereford, L. (1981) *Cell* **24**, 377-384.
- Owen, J.J.T., Cooper, M.D. and Raff, M.C. (1974) *Nature* (London) **249**, 361-363.
- Pachter, J.S., Yen, T.J. and Cleveland, D.W. (1987) *Cell* **51**, 283-292.
- Paek, I. and Axel, R. (1987) *Mol. Cell. Biol.* **7**, 1496-1507.
- Page, D.M. and DeFranco, A.L. (1990) *Mol. Cell. Biol.* **10**, 3003-3012.
- Palmiter, R.D. and Carey, N.H. (1974) *Proc. Natl. Acad. Sci.*

References

U.S.A. **71**, 2357-2361.

Paris, J. and Richter, J.D. (1990) *Mol. Cell. Biol.* **10**, 5634-5645.

Pavlakakis, G.N. and Hamer, D.H. (1983a) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 397-401.

Pavlakakis, G.N. and Hamer, D.H. (1983b) *Recent Progress in Hormone Research* **39**, 353-385.

Pear, W.S., Wahlstrom, G., Nelson, S.F. Axelson, H., Szeles, A., Wiener, F., Bazin, H., Klein, G. and Sumegi, J. (1988) *Mol. Cell. Biol.* **8**, 441-451.

Pelham, H.R.B. (1982) *Cell* **30**, 517-528.

Pelsey, F. and Lacroute, F. (1984) *Curr. Genet.* **8**, 277-282.

Peltz, S.W. and Ross, J. (1987) *Mol. Cell. Biol.* **7**, 4345-4356.

Peltz, S.W., Brewer, G., Kobs, G. and Ross, J. (1987) *J. Biol. Chem.* **262**, 9382-9388.

Peltz, S.W., Brewer, G., Groppi, V. and Ross, J. (1989) *Mol. Biol. Med.* **6**, 227-238.

Perlman, D. and Halvorson, H.O. (1983) *J. Mol. Biol.* **167**, 391-409.

Pernis, B.G., Chiapino, G., Kelus, A.S. and Gell, P.G.H. (1965) *J. Exp. Med.* **122**, 853-875.

Perry, R.P. and Kelley, D.E. (1973) *J. Mol. Biol.* **79**, 681-696.

Perry, R.P. and Kelley, D.E. (1979) *Cell* **18**, 1333-1339.

Perry, R.P., Kelley, D.E., Coleclough, C. and Kearney, J.F. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 247-251.

References

- Peterson, M.L. and Perry, R.P. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 8883-8887.
- Peterson, M.L. and Perry, R.P. (1989) Mol. Cell. Biol. **9**, 726-738.
- Petterson, S., Sharpe, M.J., Gilmore, D.R., Surani, M.A. and Neuberger, M.S. (1989) Int. Immunol. **1**, 509-516.
- Petterson, S., Cook, G.P., Brüggermann, M., Williams, G.T., and Neuberger, M.S. (1990) Nature (London), **344**, 165-168.
- Picard, D. and Schaffner, W. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 417-421.
- Picard, D. and Schaffner, W. (1984a) EMBO J. **3**, 3031-3035.
- Picard, D. and Schaffner, W. (1984b) Nature (London), **307**, 80-82.
- Piechaczyk, M., Yang, J-Q., Blanchard, J-M., Jeanteur, P. and Marcu, K.B. (1985) Cell **42**, 589-597.
- Pillai, S. and Baltimore, D. (1987) Nature (London) **329**, 172-174.
- Pittenger, M.R. and Cleveland, D.W. (1985) J. Cell. Biol. **101**, 1941-1952.
- Proudfoot, N.J. (1986) Nature (London) **322**, 562-568.
- Puckett, L., Chambers, S. and Darnell, J.E. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 389-393.
- Puckett, L., and Darnell, J.E. (1976) J. Cell. Physiol. **90**, 521-534.
- Purvis, I.J., Bettany, A.J.E., Loughlin, L. and Brown, A.J.P. (1987) Nucleic Acids Res. **15**, 7951-7962.

References

- Putnam, F.W. and Easley, C.W (1965) *J. Biol. Chem.* **240**, 1626-1638.
- Queen, C. and Baltimore, D. (1983) *Cell* **33**, 741-748.
- Queen, C. and Stafford, J. (1984) *Mol. Cell. Biol.* **4**, 1042-1049.
- Rabbitts, P.H., Watson, J.V., Lamond, A., Forster, A., Stinson, M.A., Evan, G., Fischer, W., Atherton, E., Sheppard, R. and Rabbitts, T.H. (1985a) *EMBO J.* **4**, 2009-2015.
- Rabbitts, P.H., Forster, A., Stinson, M.A. and Rabbitts, T.H. (1985b) *EMBO J.* **4**, 3727-3733.
- Raff, M.C., Owen, J.J.T., Cooper, M.D., Lawton, A.R., Megson, M. and Gathings, W.E. (1975) *J. Exp. Med.* **142**, 1052-1064.
- Raff, M.C., Megson, M., Owen, J.J.T. and Cooper, M.D. (1976) *Nature (London)*, **259**, 224-226.
- Rahmsdorf, H.J., Schönthal, A., Angel, P., Litfin, M., Rütger, U. and Herrlich, P. (1987) *Nucleic Acids Res.* **15**, 1643-1659.
- Raschke, W.C., Mather, E.L. and Koshland, M.E. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3469-3473.
- Ratnasabapathy, R., Hwang, S-P.L. and Williams, D.L. (1990) *J. Biol. Chem.* **265**, 14050-14055.
- Reth, M.G. and Alt, F.W. (1984) *Nature (London)*, **312**, 418-423.
- Reth, M., Petrac, E., Wiese, P., Lobel, L. and Alt, F.W. (1987) *EMBO J.* **6**, 3299-3305.
- Rogers, J., Early, P., Carter, C., Calame, K., Bond, M., Hood, L., and Wall, R. (1980) *Cell* **20**, 303-312.
- Ross, J. and Kobs, G. (1986) *J. Mol. Biol.* **188**, 579-593.

References

- Ross, J., Peltz, S.W., Kobs, G. and Brewer, G. (1986) Mol. Cell. Biol. **6**, 4362-4371.
- Ross, J., Kobs, G., Brewer, G. and Peltz, S.W. (1987) J. Biol. Chem. **262**, 9374-9381.
- Roth, D.B., Porten, T.N. and Wilson, J.H. (1985) Mol. Cell. Biol. **5**, 2599-2607.
- Roth, R.A. and Koshland, M.E. (1981) J. Biol. Chem. **256**, 4633-4639.
- Roualt, T.A., Hentze, M.W., Caughman, S.W., Harford, J.B. and Klausner, R.D. (1988) Science **241**, 1207-1210.
- Roualt, T.A., Hentze, M.W., Haile, D.J., Harford, J.B. and Klausner, R.D. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5768-5772.
- Roualt, T.A., Tang, C.K., Kaptain, S., Burgess, W.H., Haile, D.J., Samaniego, F., McBride, O.W., Harford, J.B. and Klausner, R.D. (1990) Proc. natl. Acad. Sci. U.S.A. **87**, 7958-7962.
- Sachs, A.B., Bond, M.W. and Kornberg, R.D. (1986) Cell **45**, 827-835.
- Sachs, A.B., Davis, R.W. and Kornberg, R.D. (1987) Mol. Cell. Biol. **7**, 3268-3276.
- Sachs, A.B. and Davis, R.W. (1989) Cell **58**, 857-867.
- Sachs, A.B. and Davis, R.W. (1990) Science **247**, 1077-1079.
- Sadis, S., Hickey, E. and Weber, L.A. (1988) J. Cell. Physiol. **135**, 377-386.
- Saito, H. and Richardson, C.C. (1981) Cell **27**, 533-542.
- Sakaguchi, N. and Melchers, F. (1986) Nature (London) **324**,

579-582.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463-5467.

Santiago, T.C., Purvis, I.J., Bettany, A.J.E. and Brown, A.J.P (1986) Nucleic Acids Res. **14**, 8347-8360.

Santiago, T.C., Bettany, A.J.E., Purvis, I.J. and Brown, A.J.P. (1987) Nucleic Acids Res. **15**, 2417-2429.

Sariban, E., Wu, R.S., Erickson, L.C. and Bonner, W.M. (1985) Mol. Cell. Biol. **5**, 1279-1286.

Sarver, N., Byrne, J.C. and Howley, P.M. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 7147-7151.

Sauter, H. and Paige, C.J. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 4989-4993.

Schatz, D.G., Oettinger, M.A. and Baltimore, D. (1989) Cell **59**, 1035-1048.

Scher, I., Sharrow, S.O., Wistar, R.Jr. and Asofsky, R. (1976) J. Exp. Med. **144**, 494-506.

Scher, I., Berning, A. K., Kessler, S. and Finkelman, F.D. (1980) J. Immunol. **125**, 1686-1693.

Schibler, U., Marcu, K.B. and Perry, R.P. (1978) Cell **15**, 1495-1509.

Schimke, R.T. (1970) Mammal. Prot. Metab. **4**, 117-227.

Schlissel, M.S. and Baltimore, D. (1989) Cell **58**, 1001-1007.

Schmeissner, U., McKenney, K., Rosenberg, M. and Court, D. (1984) J. Mol. Biol. **176**, 39-53.

Schneider, Blundell, and Kennell, (1978) Mol. Gen. Genet. **160**, 121-129.

References

- Scholtissek, C. (1971) *Eur. J. Biochem.* **24**, 358-365.
- Schuler, G.D. and Cole, M.D. (1988) *Cell* **55**, 1115-1122.
- Searle, P.F., Davison, B.L., Stuart, G.W., Wilkie, T.M., Norstedt, G. and Palmiter, R.D. (1984) *Mol. Cell. Biol.* **4**, 1221-1230.
- Sen, R. and Baltimore, D. (1989) in *Immunoglobulin Genes*, (Honjo, T., Alt, F. and Rabbitts, eds.) Academic Press Ltd., 327-342.
- Shapiro, D.J. and Brock, M.L. (1985) *Biochemical Actions of Hormones* **12**, 139-172.
- Shatkin, A.J. (1976) *Cell* **9**, 645-653.
- Shaw, G. and Kamen, R. (1986) *Cell* **46**, 659-667.
- Sheiness, D. and Darnell, J.E. (1973) *Nature (London)* **241**, 265-268.
- Shen, V., Cynamon, M., Daugherty, B., Kung, H-F. and Schlessinger, D. (1981) *J. Biol. Chem.* **256**, 1896-1902.
- Shyu, A-B., Greenberg, M.E. and Belasco, J.G. (1989) *Genes Develop.* **3**, 60-72.
- Singer, R.H. and Penman, S. (1972) *Nature (London)* **240**, 100-102.
- Singer, R.H. and Penman, S. (1973) *J. Mol. Biol.* **78**, 321-334.
- Sitia, R., Neuberger, M.S. and Milstein, C. (1987) *EMBO J.* **6**, 3969-3977.
- Sitia, R., Albeini, C., Biassoni, R., Rubartelli, A., DeAmrosis, S. and Vismara, D. (1988) *Mol. Immunol.* **25**, 189-197.

References

- Sitia, R., Neuberger, M., Alberini, C., Bet, P., Fra, A., Valetti, C., Williams, G. and Mistein, C. (1990) *Cell* **60**, 781-790.
- Sittman, D.B., Graves, R.A. and Marzluff, W.F. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1849-1853.
- Sive, H.L., Heintz, N. and Roeder, R.G. (1984) *Mol. Cell. Biol.* **4**, 2723-2734.
- Slater, E.P., Rabenau, O., Karin, M., Baxter, J.D. and Beato, M. (1985) *Mol. Cell. Biol.* **5**, 2984-2992.
- Sobell, H.M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5328-5331.
- Soler, A.P., Gozalbo, D., Zueco, J and Sentandreu, R. (1987) *Biochem. J.* **246**, 575-581.
- Southern, E.M. (1975) *J. Mol. Biol.* **98**, 503-517.
- Southern, P. and Berg, P. (1982) *J. Mol. Appl. Genet.* **1** 327-341.
- Spear, P.G., Wang, A-L., Rutishauser, U. and Edelman, G.M. (1973) *J. Exp. Med.* **138**, 557-572.
- Stanssens, P., Remaut, E. and Fiers, W. (1986) *Cell* **44**, 711-718.
- Stauber, C., Lüscher, B., Eckner, R., Lotscher, E. and Schümperli, D. (1986) *EMBO J.* **5**, 3297-3303.
- Stavnezer-Nordgren, J. and Sirlin, S. (1986) *EMBO J.* **5**, 95-102
- Stephens, P.E. and Hentschel, C.C.G. (1987) *Biochem. J.* **248**, 1-11.
- Stern, M.J., Ames, G.F-L., Smith, N.H., Robinson, E.C. and Higgins, C.F. (1984) *Cell* **37**, 1015-1026.

References

- Stevens, A. (1980) *J. Biol. Chem.* **255**, 3080-3085.
- Storb, U. (1973) *Biochem. Biophys. Res. Commun.* **52**, 1483-1491.
- Storb, U. (1987) *Annu. Rev. Immunol.* **5**, 151-174.
- Su, L-K. and Kadash, T. (1990) *Mol. Cell. Biol.* **10**, 2619-2624.
- Suggs, S.V., Wallace, R.B., Hirose, T., Kawashima, E.H. and Itakura, K. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6613-6617.
- Swartout, S.G. and Kinniburgh, A.J. (1989) *Mol. Cell. Biol.* **9**, 288-295.
- Tartakoff, A. and Vassalli, P. (1979) *J. Cell. Biol.* **83**, 284-299.
- Thelander, L. and Reichard, P. (1979) *Annu. Rev. Biochem.* **48**, 133-158.
- Thomas, P.S. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **9**, 5201-5205.
- Thomas, K.R., Folger, K.R. and Capecchi, M.R. (1986) *Cell* **44**, 419-428.
- Tkacz, J.S. and Lampen, J.O. (1975) *Biochem. Biophys. Res. Commun.* **65**, 248-257.
- Tonegawa, S. (1983) *Nature (London)* **302**, 575-581.
- Towbin H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350-4354.
- Treisman, R. (1985) *Cell* **42**, 889-902.
- Tsubata, T. and Reth, M. (1990) *J. Exp. Med.* **172**, 973-976.

References

- Tsuda, M., Honjo, T., Shimizu, A., Mizuno, D. and Natori, S. (1978) *J. Biochem.* **84**, 1285-1290.
- Tsurushita, N., Avdalovic, N.M. and Korn, L.J. (1987) *Nucleic Acids Res.* **15**, 4603-4615.
- Ullrich, S.J., Appella, E. and Mercer, W.E. (1988) *Exp. Cell Res.* **178**, 273-286.
- Van Ness, B.G., Weigert, M., Coleclough, C., Mather, E.L., Kelley, D.E. and Perry, R.P. (1981) *Cell* **27**, 593-602.
- Vassalli, P., Tedghi, R., Lisowska-Bernstein, B., Tartakoff, A. and Jaton, J-C. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5515-5519.
- Verladi, A. and Cooper, M.D. (1984) *J. Immunol.* **133**, 672-677.
- Vitetta, E.S., Baur, S. and Uhr, J.W. (1971) *J. Exp. Med.* **134**, 242-264.
- Volloch, V. and Housman, D. (1981) *Cell* **23**, 509-514.
- Von Gabain, A., Belasco, J.G., Schottel, J.L., Chang, A.C.Y. and Cohen, S.N. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 653-657.
- Von Heijne, G. (1983) *Eur. J. Biochem.* **133**, 17-21.
- Wallace, R.B., Johnson, M.J., Hirose, T., Miyake, T., Kawashima, E.H. and Itakura, K. (1981) *Nucleic Acids Res.* **9**, 879-894.
- Wang, J.C., Caron, P.R. and Kim, R.A. (1990) *Cell* **62**, 403-406.
- Warner, J.R., Soeiro, R., Birnboim, H.C., Girard, M. and Darnell, J.E. (1966) *J. Mol. Biol.* **19**, 349-361.
- Warner, N.L., Harris, A.W. and Gutman, G.A. (1975) in

References

- Membrane Receptors of Lymphocytes (Seligmann, M., Preud'homme, J.L. and Kowilsky, F.M. eds), pp. 203-216, Am. Elsevier, New York.
- Weber, M.J. (1972) *Nature New Biology* **235**, 58-61.
- Weiss, E.A., Michael, A. and Yuan, D. (1989) *J. Immunol.* **143**, 1046-1052.
- Wertz, G.W. (1975) *J. Virol.* **16**, 1340-1344.
- Wickens, M. and Stephenson, P. (1984) *Science* **226**, 1045-1051.
- Wickner, W.T. and Lodish, H.F. (1985) *Science*, **230**, 400-407.
- Williams, G.T., Venkitaraman, A.R., Gilmore, D.J. and Neuberger, M.S. (1990) *J. Exp. Med.* **171**, 947-952.
- Wilson, M.C., Sawicki, S.G., White, P.A. and Darnell, J.E.Jr. (1978) *J. Mol. Biol.* **126**, 23-36.
- Wilson, T. and Treisman, R. (1988) *Nature (London)*, **336**, 396-399.
- Winter, E., Radbruch, A. and Krawinkel, U. (1985) *EMBO J.* **4**, 2861-2867.
- Wiskocil, R., Bensky, P., Dower, W., Goldberger, R.F., Gordon, J.I. and Deeley, R.G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4474-4478.
- Wood, C. and Tonegawa, S. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3030-3034.
- Wood, C.R., Boss, M.A., Patel, T.P. and Emtage, J.S. (1984) *Nucleic Acids Res.* **12**, 3937-3950.
- Wu, T.T. and Kabat, E.A. (1970) *J. Exp. Med.* **132**, 211-250.
- Wu, K.S. and Bonner, W.M. (1985) *Mol. Cell. Biol.* **5**, 2959-2966.

References

- Yaffe, B.M. and Samuels, H.H. (1984) *J. Biol. Chem.* **259**, 6284-6291.
- Yaffe, D., Nudel, U., Mayer, Y. and Neuman, S. (1985) *Nucleic Acids Res.* **13**, 3723-3737.
- Yagle, M.K. and Palmiter, R.D. (1985) *Mol. Cell. Biol.* **5**, 291-294.
- Yancopoulos, G.D., and Alt, F.W. (1985) *Cell* **40**, 271-281.
- Yancopoulos, G., Blackwell, T., Suh, H., Hood, L. and Alt, F. (1986) *Cell* **44**, 251-259.
- Yang, Y. and Ames, G.F-L. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8850-8854.
- Yen, T.J., Machlin, P.S. and Cleveland, D.W. (1988) *Nature (London)* **334**, 580-584.
- Yost, H.J. and Lindquist, S. (1986) *Cell* **45**, 185-193.
- Yost, H.J., Peterson, R.B. and Lindquist, S. (1990) *Trends Genet.* **6**, 223-227.
- Yuan, D. (1984) *J. Immunol.* **132**, 1566-1570.
- Yuan, D. (1986) *Mol. Cell. Biol.* **6**, 1015-1022.
- Yuan, D. and Tucker, P.W. (1982) *J. Exp. Med.* **156**, 962-974.
- Yuan, D. and Tucker, P.W. (1984a) *J. Exp. Med.* **160**, 564-583.
- Yuan, D. and Tucker, P.W. (1984b) *J. Immunol.* **132**, 1561-1565.
- Yuan, D. and Dang, T. (1989) *Mol. Immunol.* **26**, 1059-1067.
- Yuan, D., Dang, T. and Sanderson, C. (1990) *J. Immunol.* **145**, 3491-3496.

References

- Zahringer, J., Baliga, B.S. and Munro, H.N. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 857-861.
- Zaller, D.M. and Eckhardt, L.A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 5088-5092.
- Zambetti, G., Schmidt, W., Stein, G. and Stein, J. (1985) J. Cell. Physiol. **125**, 345-353.
- Zambetti, G., Stein, J. and Stein, G. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 2683-2687.
- Zeevi, M., Nevins, J.R. and Darnell, J.E. Jr. (1982) Mol. Cell. Biol. **2**, 517-525.
- Zelus, B.D., Giebelhaus, D.H., Eib, D.W. Kenner, K.A. and Moon, R.T. (1989) Mol. Cell. Biol. **9**, 2756-2760.
- Zinn, K., Mellon, P., Ptashne, M. and Maniatis, T. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 4897-4901.
- Zinn, K., DiMaio, D. and Maniatis, T. (1983) Cell **34**, 865-879.
- Zúñiga, M.C., D'Eustachio, P. and Ruddle, N.H. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 3015-3019.

APPENDIX

For a molecule undergoing exponential decay, the rate of change in concentration of the molecule is proportional to its concentration at any one time. The half-life of an RNA molecule, i.e. the time taken for its concentration to decay to half its original value, can be determined by plotting the log of RNA concentration against time, as follows.

Let $[R]$ be the concentration of RNA at time t .

Then the rate of loss of $[R]$ is given by

$$\frac{-d[R]}{dt} = k[R]$$

where k is the rate constant for the reaction.

Integrate this equation with respect to t :-

$$\int_{[R]_0}^{[R]_t} \frac{-1d[R]}{[R]} = \int_0^t k dt$$

$$\therefore \log_e R = -kt + \text{a constant}$$

$$\text{When } R = R_0 \quad t = 0$$

$$\therefore \text{constant} = \log_e R_0$$

Therefore it follows that

$$\log_e R - \log_e R_0 = -kt \quad \{1\}$$

$$\therefore \log_e \left[\frac{R}{R_0} \right] = -kt \quad \{2\}$$

$$\therefore \frac{R}{R_0} = e^{-kt}$$

and

$$R = R_0 e^{-kt} \quad \{3\}$$

To calculate the half-life, we need to calculate the time taken for the concentration of [R] to fall from $[R]_0$ to $1/2 [R]_0$

This can be calculated from equation {2}

$$\log_e \left[\frac{1/2 R_0}{R_0} \right] = -kt_{1/2}$$

$$\therefore \log_e [1/2] = -kt_{1/2}$$

$$\therefore -\log_e 2 = -kt_{1/2}$$

or

$$t_{1/2} = \frac{\log_e 2}{k} \quad \{4\}$$

Equation {1} is the equation of the line obtained by plotting the log of [R] against time, where k is the slope of the line. Therefore the half-life can be calculated by substituting this value into equation {4}.

Where correction for the dilution effect of cell division is necessary, each observed value of R must be multiplied by the value of the cell number/ml at that time point, to obtain the true rate of decay of R with time. This is mathematically equivalent to the addition of the log values, so to calculate the half-life, $S1+S2$ can be substituted in place of k in equation 4, where S1 and S2 are the values of the slopes of the lines obtained by plotting $\log[R]$ and $\log[\text{cell number/ml}]$ against time respectively.