TUMOUR ANTIGENS AND HERPES SIMPLEX VIRUS ONCOGENESIS

by

© ROBERT EDWIN PATRICK HEWITT

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Church Street
Glasgow G11 5JR

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SUMMARY

Herpes simplex virus (HSV) has been implicated in the aetiology of human cervical cancer, but despite intensive investigation the oncogenic properties of HSV are still not well understood. Studies reported here, explore one possible molecular mechanism by which this virus may cause oncogenic transformation; namely, the induction of cellular polypeptides which play a role in oncogenesis.

A group of cellular polypeptides specific to cells showing the immortalized phenotype were previously detected in a range of cell lines, including the Bn5T cell line which is derived from rat embryo fibroblasts transformed by a fragment of the HSV type 2 (HSV-2) genome. These polypeptides which will be referred to as Bn5T:TBS polypeptides, are not detectable in control rat embryo fibroblasts. Bn5T:TBS polypeptides are immunoprecipitated by tumour bearing serum (TBS) and studies presented in this thesis confirm that they are also immunoprecipitated by the monoclonal antibody TG7A. The TG7A monoclonal was raised against affinity purified DNA binding proteins from HSV-2 infected cells and recognizes cellular polypeptides induced on infection by the virus. This suggests that Bn5T:TBS polypeptides are related to polypeptides induced in HSV-2 infection.

Another feature of Bn5T:TBS polypeptides is that they all show oncofoetal expression, suggesting that they have a physiological role in cell proliferation and differentiation. Members of the Bn5T:TBS set have always been found to be either co-expressed or not expressed at all, in a range of different cell types and culture conditions. This suggests that these polypeptides may have a common precursor or be induced by the same factor(s) and it also suggests that they

may have a common function. Three of the six members of the Bn5T:TBS set give similar peptide maps, suggesting that they possess similar or shared domains. In addition, pulse labelling and pulse-chase labelling experiments suggest that at least two members of the set result from post-translational processing events.

Immunological and peptide mapping studies indicate that a Bn5T:TBS polypeptide of 97kDa molecular weight, is related to the 90kDa heat shock protein (HSP90) and that it may share a domain with an HSV-2-induced form of HSP90. The TG7A monoclonal recognizes a highly conserved epitope present on a bacterial heat-shock protein, an HSV-2-induced 90kDa polypeptide (also recognized by a monoclonal against HSP90) and a 97kDa transformed cell polypeptide belonging to the Bn5T:TBS set. These findings suggest that an HSV-2-induced form of the HSP90 may play a role in oncogenic transformation by the virus.

The synthesis of Bn5T:TBS polypeptides has not been detected in the rabbit reticulocyte lysate in vitro translation system and possible reasons for this have been investigated. Further, data from an amino acid sequencing study suggests that the 97kDa polypeptide in the Bn5T:TBS set has a domain with homology to one form of bovine casein; likely explanations are discussed in detail.

ABBREVIATIONS

The following abbreviations are used in this thesis:

AFP	alpha-foetoprotein
ATP	adenosine triphosphate
BHK	baby hamster kidney
b.p.	base pairs
CEA	carcinoembryonic antigen
CEF	chick embryo fibroblasts
CIN	cervical intra-epithelial neoplasia
CMV	cytomegalovirus
c-onc	cellular oncogene
c.p.e.	cytopathic effect
c.p.m.	counts per minute
dATP	deoxyadenosine triphophate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
d.s.	double stranded
DW	distilled water
E	early gene
EBV	Epstein-Barr virus
EBNA	Epstein-Barr virus nuclear antigen
EC	embryonal carcinoma
EDTA	ethylenediamide tetra-acetic acid
GRP	glucose regulated protein
h	hour
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
HPLC	high performance liquid chromatography
HPV	human papillomavirus
HSP	heat-shock protein
HSV	herpes simplex virus

HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
IE	immediate-early HSV gene
i.p.	immunoprecipitation/ immunoprecipitate(d)
IVT	*in vitro* translation
k.b.	kilobases
L	late gene
LTR	long terminal repeat
MEF	mouse embryo fibroblast
MHC	major histocompatibility complex
min.	minutes
m.o.i.	multiplicity of infection
MTR	morphological transformation region
m.u.	map unit
MuLV	murine leukaemia virus
MW	molecular weight
NK	natural killer cell
NPT	nonpermissive temperature
OFA	oncofoetal antigen
ori	origin of DNA replication
p.f.u.	plaque forming units
p.i.	post infection
pI	isoelectric point
PT	permissive temperature
RE	rat embryo
RNA	ribonucleic acid
RNase	ribonuclease
r.p.m.	revolutions per minute
RR	ribonucleotide reductase
RSV	Rous sarcoma virus
S	Svedberg unit of sedimentation

SD	standard deviation
SDEA	stage-dependent embryonic antigen
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SV40	simian virus 40
syn	syncytial
syn+	non-syncytial
TBS	tumour bearing serum
TCA	trichloroacetic acid
TK	thymidine kinase
ts	temperature sensitive mutant
TSTA	tumour-specific transplantation antigen
u	units
UV	ultraviolet
Vmw(X)	virus-specific polypeptide of apparent molecular weight(X)
v-onc	viral oncogene
wt	wild type
ug	microgram

1 INTRODUCTION

The aim of this project was to explore one possible mechanism by which herpes simplex virus may induce oncogenic transformation. Studies described in this thesis have further characterized certain HSV-induced cellular polypeptides, which may have an important role in viral oncogenesis. As an introduction to this subject, an overview of the molecular biology of herpes simplex virus is presented in section 1.1; emphasis being given to genome organization, transcriptional regulation and virus-host interactions. Potential mechanisms of oncogenic transformation by HSV are then reviewed in relation to other DNA tumour viruses in section 1.2 and evidence for the transcriptional activation of certain cellular genes is described. Results presented in this thesis suggest that HSV-induced polypeptides are related both to a stress protein and to certain cellular tumour antigens. The properties of stress proteins are therefore outlined in section 1.3, while the characteristics of tumour antigens are reviewed in section 1.4.

1.1 Herpes simplex virus: general molecular biology

1.1.1 Classification

Herpes simplex virus (HSV) is a member of the herpesvirus genus or family. This family of viruses is subdivided into three sub-families on the basis of biological properties: The alpha-, beta- and gammaherpesviruses (Roizman *et al.*, 1978; Matthews, 1982; Roizman, 1982). Alphaherpesviruses show a relatively short replicative cycle, a variable host range and frequently establish latent infections in ganglia. HSV type 1

(HSV-1), HSV type 2 (HSV-2), varicella-zoster virus and pseudorabies virus are members of this group.
Betaherpesviruses have a relatively long replicative cycle, a narrow host range and may establish a latent state in secretory glands and lymphoreticular cells, and included in this group are human and mouse cytomegaloviruses.
Gammaherpesviruses have a variable replicative cycle, are restricted to either T or B lymphocytes *in vitro* and frequently give rise to a latent infection in lymphoid tissue. Examples from this group are Epstein-Barr virus, Marek's disease virus and herpesvirus saimiri.

1.1.2 Structure of the virion

Members of the herpesvirus genus have been recognized primarily by the architecture of the virion (Fenner *et al.*, 1974). They are large DNA viruses of 150-170nm in diameter and are composed of the following structures (see figure 1.1):

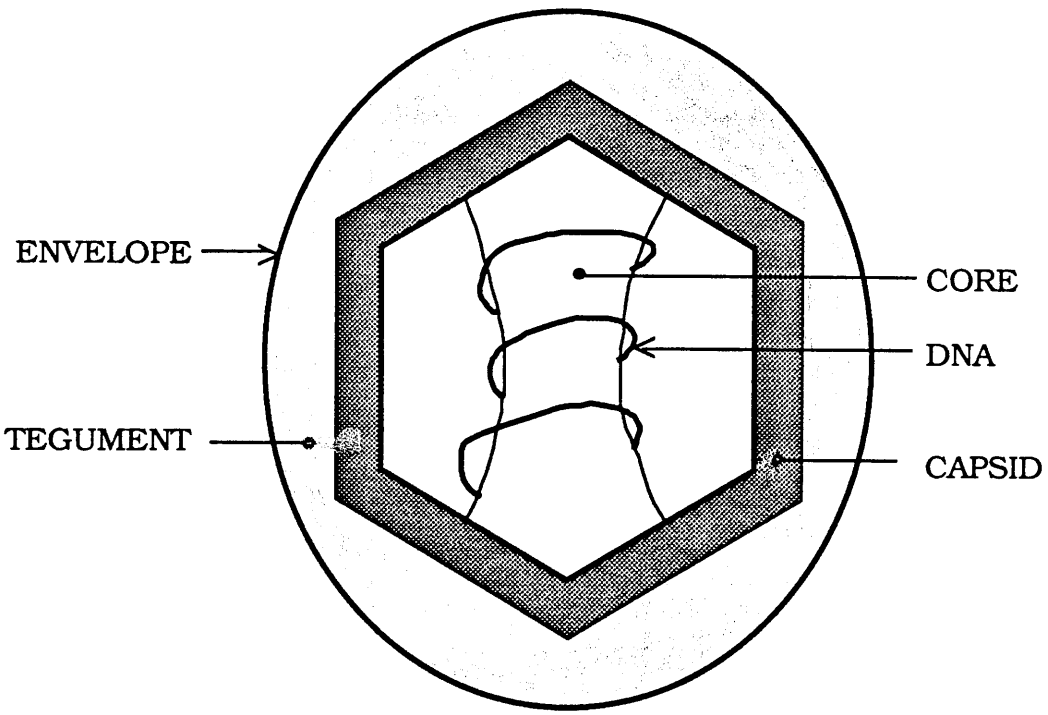
(a) Core: This is a proteinaceous structure, around which the double-stranded (d.s.) DNA genome is arranged (Epstein, 1962; Ben-Porat and Kaplan, 1962; Furlong *et al.*, 1972).

(b) Capsid: An icosahedral structure (20 equilateral triangular faces), composed of 162 protein capsomeres which surrounds the core (Wildy *et al.*, 1960). It is approximately 100nm diameter.

(c) Tegument: An amorphous layer, between the capsid and the envelope (Roizman and Furlong, 1974).

(d) Envelope: A lipid membrane which forms the outer margin

Figure 1.1; Diagramatic representation of a section through a herpesvirus particle.


ENVELOPE
TEGUMENT
CORE
DNA
CAPSID

of the virus particle (Wildy et al., 1960) and is derived from the inner nuclear membrane of the cell (Darlington and Moss, 1968).

1.1.3 Lytic cycle of HSV

(a) Adsorption and penetration: Adsorption of HSV to the cell surface occurs maximally within 30 min. of addition of virus to cells (Hochberg and Becker, 1968) by a process which is thought to involve cell receptors for the virus. HSV-1 and HSV-2 do not appear to have the same receptors as they interfere with the adsorption of strains of the homologous, but not heterologous, serotype (Vahlne et al., 1979; Addison et al., 1984). Electron microscopy studies suggest that penetration occus mainly by fusion of the viral envelope with the cell membrane (Abodeely et al., 1970) but there is evidence that virions are internalized by endocytosis as well (Hummeler et al., 1969).

(b) Uncoating of viral genome: Uncoating of the HSV genome is a poorly understood process. De novo RNA and protein synthesis do not appear to be required for virus uncoating, which suggests that either cellular enzyme(s) or viral capsid component(s) are responsible (Hochberg and Becker, 1968). Nuclease-sensitive input virus DNA can be detected in the cell nucleus 15-60 min. post infection (p.i.) (Hummeler et al., 1969).

(c) Transcription, Translation and Replication: Transcription of viral DNA and viral DNA replication occur in the cell nucleus. Translation of viral polypeptides occurs in

the cell cytoplasm. These processes are described in detail below, as are the concurrent alterations in cellular metabolism.

(d) Assembly and release: Herpesvirus capsids are assembled in the cell nucleus (Morgan et al., 1954). As viral protein synthesis occurs in the cytoplasm, viral structural proteins must be transported into the nucleus for encapsidation to occur. The process of cleavage of concatameric viral DNA into unit length genomes is closely linked with the packaging of DNA into capsids (Vlazny et al., 1982). Nucleocapsids are enveloped at the inner lamella of the nuclear membrane and appear to become enveloped by budding outward into the perinuclear space from areas of the inner lamella that are enriched with viral antigens (Ben-Porat and Kaplan, 1971; Spear and Roizman, 1972). Egress of virus from the cell is first seen at approximately 8h p.i. at 37°C and seems to occur by exocytosis (Nii et al., 1968; Katsumoto et al., 1981).

1.1.4 HSV-encoded proteins

HSV-encoded proteins will be largely discussed in the context of their functions in the viral multiplication cycle. However, a brief summary is given here.

(a) Structural proteins: HSV encoded glycoproteins have been implicated in virus adsorption and penetration, cell to cell spread of infectious virus and cell fusion (reviewed by Marsden, 1987). They are present both on the virion envelope and on the plasma membrane of infected cells and are the major virus-specific proteins recognized by the host immune system following infection. Proteins present in the capsid and

tegument of the virion have been reviewed by Dargan (1986).

(b) Enzymes: Virus encoded enzymes involved in synthesis of viral DNA include thymidine kinase, ribonucleotide reductase, deoxyuridine triphosphatase, DNA polymerase, exonuclease and topoisomerase (reviewed by McGeoch, 1987). An HSV-1 encoded protein kinase has recently been identified, which is the product of the viral gene US3 (Purves et al., 1987; Frame et al., 1987). This is of particular interest in relation to viral oncogenesis and is discussed in section 1.2.6.

(c) Other proteins: Proteins that interact with viral DNA include the major DNA binding protein and the "ori$_S$" binding protein (reviewed by McGeoch, 1987). Proteins that regulate viral transcription are described in section 1.1.9.

1.1.5 Genome structure

Most studies on HSV genome arrangement have focussed on HSV-1. However, intertypic recombination and complementation data (Timbury and Subak-Sharpe, 1973; Esparza et al., 1976; Marsden et al., 1978; Schaffer et al., 1978) and DNA/DNA hybridization studies (Davison and Wilkie, 1983a) indicate that HSV-1 and HSV-2 genomes are essentially colinear with viral genes mapping at equivalent positions on both genomes.

The entire nucleotide sequence of HSV-1 has now been determined (Dr D. McGeoch, 12th International Herpesvirus Workshop).

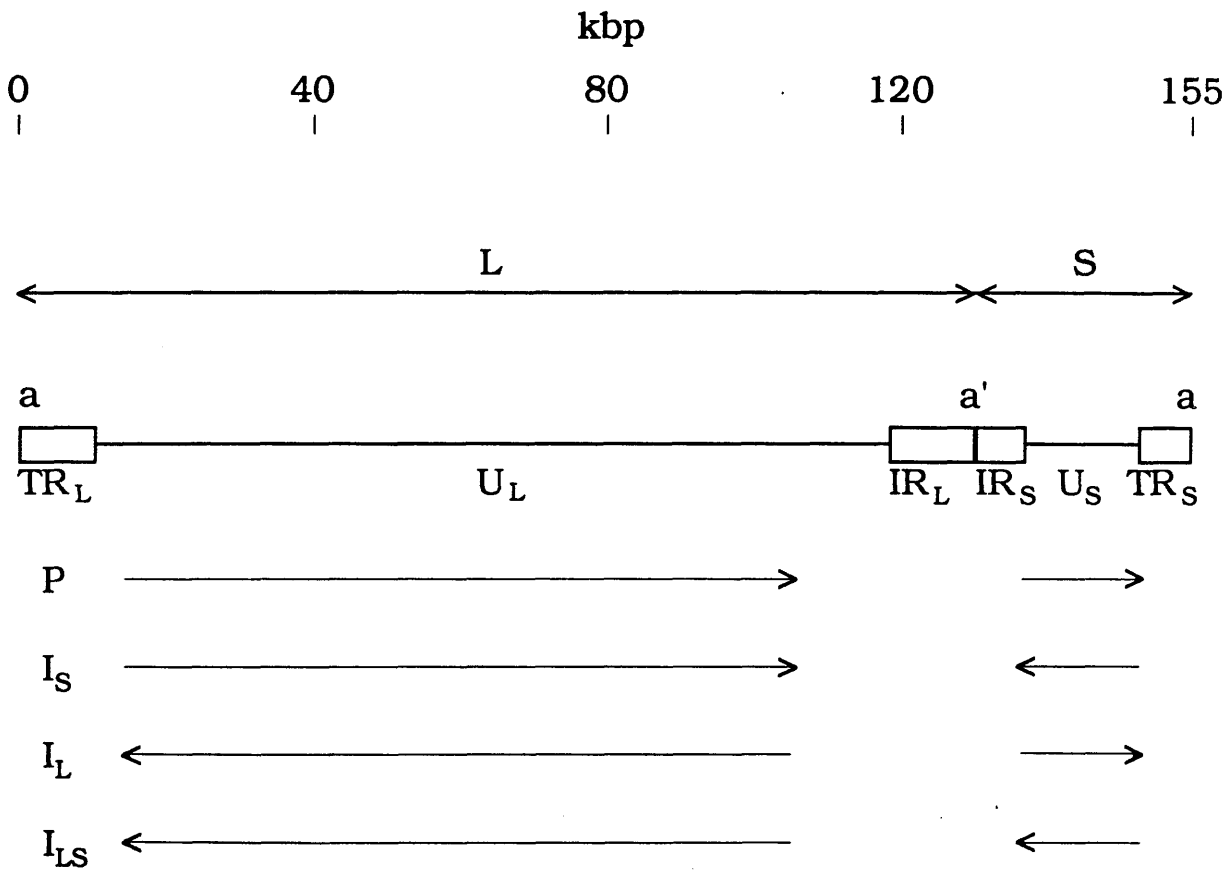
(a) Chemical composition: The HSV-1 genome is a linear double stranded DNA molecule of MW 95-100 X 10^6 daltons (Becker et al., 1968) and 152,260 residues in length with a

base composition of 68.3% G+C (McGeoch et al., 1988a).

(b) Long and short segments: Electron microscopy studies of structures produced by intramolecular hybridization of single-stranded DNA have shown that the HSV genome can be divided into long (L) and short (S) segments (see figure 1.2), each containing a unique sequence flanked by a pair of repeat sequences in opposing orientation (Sheldrick and Berthelot, 1974). The long unique sequence (U_L) of 110 k.b. is flanked by internal and terminal long repeats (IR_L and TR_L) both of 9.2 k.b. and the short unique sequence (U_S) of 13 k.b. by the internal and terminal short repeats (IR_S and TR_S) both of 6.6 k.b.. Sequences of R_L and R_S are distinct (Mc Geoch, 1987). HSV virion DNA contains equivalent amounts of 4 genome isomers in which U_L and U_S are in different orientations (Delius and Clements, 1976). These isomers are apparently functionally equivalent, each producing viable progeny (Davison and Wilkie, 1983b, 1983c). The biological significance of these different genome isomers is not yet known.

(c) "a" sequence: The "a" sequence is an element present at both genomic termini in the direct orientation and at the junction of IR_L and IR_S in the inverse orientation (Grafstrom et al., 1974, 1975; Wagner and Summers, 1978). The HSV-1 "a" sequence varies from 250 base pairs (b.p.) to 500 b.p. due to a variable number of tandemly reiterated sequences, while the HSV-2 sequence which does not contain a reiteration has a constant size of 251 b.p. (Davison and Wilkie, 1981; Mocarski and Roizman, 1981; Varmuza and Smiley, 1985). The "a" sequence is thought to play a role in circularization of the genome following infection, in DNA maturation and

Figure 1.2; Schematic representation of an HSV genome. The genome consists of long (**L**) and short (**S**) regions. Each of these regions consist of a unique segment (U_L and U_S) flanked on either side by segments that are inverted repeats of eachother (R_S and R_L regions). Inverted repeats may be either terminal (TR_L and TR_S) or internal (IR_L and IR_S). The "**a**" sequence is present as a direct repeat at the genomic termini and in an inverted orientation at the L-S junction. Inversion of L and S regions occurs between inverted copies of the "a" sequence and this yields four possible isomers of HSV, each differing in the relative orientation of UL and US. (McGeoch et al., 1988)

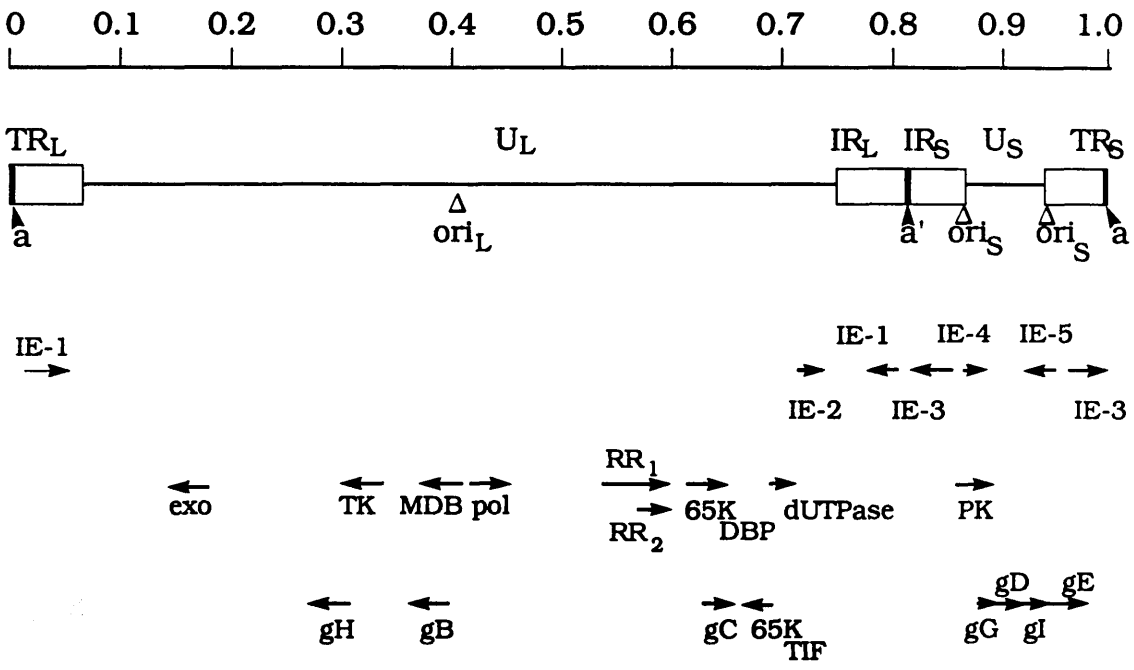

kbp
0
40
80
120
155
L
S
a
a'
a
TR$_L$
U$_L$
IR$_L$
IR$_S$
U$_S$
TR$_S$
P
I$_S$
I$_L$
I$_{LS}$

encapsidation (see below) and in the inversion of L and S segments. Inversion will occur if DNA fragments are flanked by "a" sequences in the inverted orientation but not if they are in the same orientation (Mocarski et al., 1980; Mocarski and Roizman, 1981; Smiley et al., 1981). Inversion could occur either by recombination or circularization of the genome followed by cleavage.

(d) Short tandem reiterations: Tandem sequence reiterations have been observed in U_S, R_S and R_L, with unit lengths of 5 to 54 b.p. (Rixon et al., 1984; McGeoch et al., 1985; Perry, 1986). Reiterations may serve to increase the level of genetic exchange between the repeat regions (Rixon et al., 1984). In support of this suggestion one set of reiterations in the R_S segments has homology with sequences at immunoglobulin class-switch recombination sites (Gomez-Marquez et al., 1985).

(e) Organization of genes: Figure 1.3 shows the arrangement of a number of HSV genes, discussed in this thesis. In all there are approximately 75 genes in the genome of HSV and these are found in both orientations and each gene has its own promoter. They are densely arranged, show some gene overlap and rarely have introns (reviewed by McGeoch, 1987). Families of 3' co-terminal mRNAs occur relatively frequently in HSV (Wagner, 1985). Transcription of such genes starts 5' to that gene coding sequence and continues through other genes of the family until a common distal polyadenylation site is reached. Only the 5'-proximal coding region of each transcript is translated (McGeoch, 1987).

Figure 1.3; Organization of the HSV-1 genome. This diagram consists of a schematic representation of the HSV genome, with locations of the following indicated below it: (a) origins of DNA replication (**ori**$_L$ and **ori**$_S$), (b) immediate early (**IE**) genes 1-5, (c) some virus induced enzyme activities including, ribonucleotide reductase subunits (**RR**$_1$ and **RR**$_2$), protein kinase (**PK**), thymidine kinase (**TK**), DNA polymerase (**pol**), (d) major DNA binding protein (**MDB**), (e) the seven known glycoproteins (**gB**, **gC**, **gD**, **gE**, **gG**, **gH**, and **gI**), and (f) Vmw65 or the 65kDa trans-inducing factor (**65K**$_{TIF}$). The scale above the representation of the HSV-1 genome shows genome locations in map units (m.u.); (C. Maclean, thesis, University of Glasgow).


0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
TRL
UL
IRL
IRS
US
TRS
a
oriL
a'
oriS
oriS
a
IE-1
IE-1
IE-4
IE-5
IE-2
IE-3
IE-3
RR1
exo
TK
MDB
pol
65K
dUTPase
PK
RR2
DBP
gD
gE
gH
gB
gC
65K
TIF
gG
gI

1.1.6 Replication of viral DNA

Semi-conservative HSV DNA replication at 37°C can be detected in the BHK cell nucleus 3h p.i., reaches a maximum between 9h and 11h p.i. and is virtually completed by 16h p.i. (Rixon, 1977). In the newly infected cell, incoming virus DNA accumulates in the nucleus and it is likely that it is quickly converted to a circular form (Poffenberger and Roizman, 1985; McGeoch, 1987). Late in infection, replicated DNA is in a very rapidly sedimentable form (Jacob et al., 1979) and there is a decrease in the number of terminal fragments detectable as compared with virion DNA (Jacob et al., 1979; Ben-Porat and Rixon, 1979). This evidence suggests that circularized unit-length molecules are replicated by a rolling circle mechanism yielding head-to-tail concatemeric DNA. Concatameric DNA is further processed in the cell nucleus by packaging into nascent nucleocapsids and by cleavage to produce genome unit lengths (Vlazny et al., 1982).

(a) Origins of replication: Evidence for at least 2 separate origins of DNA replication in the viral genome, came originally from electron microscopy studies (Friedmann et al., 1977) and studies on the DNA of defective species of HSV (Frenkel et al., 1975). These studies suggested an origin of replication near the middle of U_L (ori_L) and also in the short repeat regions (ori_S). A plasmid replication system allowed detailed analysis of the cis-acting sequences needed for replication (Stow, 1982). A circular plasmid containing the HSV sequences under study was introduced into cells, together with virus/viral DNA to provide genes encoding proteins required for replication. Test plasmids containing sequences necessary for replication were amplified, and amplification

was detected by the use of labelled parental vector DNA as a hybridization probe. This system allowed identification of a short segment origin of replication, "ori_S" which was localized by deletion analysis to a region of 90b.p. in the R_S sequences of HSV-1 between divergently transcribed genes, IE3 and 4/5 (Stow and McMonagle, 1983). The ori_S sequence contains an imperfect palindrome with each arm consisting of 21 residues and at the centre of the palindrome is a sequence $(A\text{-}T)_6$ which is essential for ori function (Stow, 1985). In R_S of HSV-2 strain HG52 there are sequences closely similar to HSV-1 ori_S (Whitton and Clements, 1984). A similar element termed "ori_L" has been located in U_L between divergently transcribed genes for the major DNA binding protein and the DNA polymerase. Sequence analysis revealed a long perfect palindrome with arms of 72 residues bearing striking similarity to ori_S sequence (Gray and Kaerner, 1984; Quinn and McGeoch, 1985). The significance of the fact that both origins are located between divergently transcribed genes is unclear.

(b) Cleavage and packaging signals: The mechanism by which concatemers are cleaved into unit-length genome molecules and packaged into viral capsids is not clearly understood, but cleavage and packaging are tightly linked (Deiss and Frenkel, 1986; Stow et al., 1986). The "a" sequence contains signals for both cleavage (Varmuza and Smiley, 1985) and packaging (Stow et al., 1983).

(c) Trans-acting factors: The plasmid replication system has been used to identify viral genes required for DNA replication (Challberg, 1986), various cloned fragments of HSV DNA being

analysed by co-transfection with a ori_S- and ori_L- containing plasmids. Using this assay in combination with data from large scale sequence analysis of the HSV-1 genome, seven HSV genes have been identified which are necessary for transient replication of plasmids containing ori_S or ori_L (Wu et al., 1988). Two of the loci represent genes encoding DNA polymerase and the major DNA-binding protein, but the functions of the products of the remaining five genes are unknown (Wu et al., 1988). By analogy to well characterized prokaryotic replication systems it has been suggested that these proteins may include a primase, a helicase, a topoisomerase, a protein which specifically binds to the HSV origins of replication and an accessory factor that increases the efficiency of DNA polymerase (Wu et al., 1988). Sequence analysis of the five newly identified genes designated UL5, UL8, UL9, UL42 and UL52, showed that all have counterparts in VZV, but only UL5 and UL52 were detectably conserved in the more distantly related EBV (McGeoch et al., 1988). The UL42 gene product has recently been shown to be a previously recognized DNA-binding protein found in extracts of HSV-1 infected cells (Parris et al., 1988), but the remaining four genes do not correspond to any previously known proteins (McGeoch et al., 1988).

1.1.7 HSV transcription: viral and cellular transcription compared

HSV transcription occurs in the cell nucleus (Wagner and Roizman, 1969). The sensitivity of viral transcription to alpha-amanitin indicates the involvement of the cellular enzyme RNA polymerase II throughout all stages of infection (Alwine et al., 1974; In common with most eukaryotic mRNAs, HSV transcripts are capped at their 5' termini (Moss et al.,

1977) and most HSV transcripts are polyadenylated at 3' ends (Bachenheimer and Roizman, 1972). HSV genes have similar regulatory sequences to cellular genes, including 5' promoter elements such as the "TATA" box and "CAAT" box (Gannon et al., 1979; Efstratiadis et al., 1980) and the 3' sequence AATAAA important for cleavage and polyadenylation of pre-mRNAs (Proudfoot and Brownlee, 1974; Zarkower et al., 1986). In addition a consensus sequence "YGTGTTYY" has been identified about 10 b.p. downstream from the polyadenylation site in a number of eukaryotic genes and this sequence has been shown to be required for the efficient formation of mRNA 3' termini (McLauchlan et al., 1985). In contrast to cellular genes however, splicing is relatively rare for HSV genes (Wagner, 1985).

1.1.8 HSV transcription: sequential expression of viral genes

Infection of permissive cells with HSV results in the sequential expression of three sets of viral genes termed immediate-early (IE), early (E) and late (L), (Honess and Roizman, 1974; Clements et al., 1977).

(a) IE genes and gene products: IE genes are expressed immediately after virus infection and do not require de novo protein synthesis (Kozak and Roizman, 1974; Clements et al., 1977; Jones and Roizman, 1979). A summary of the nomenclature for these genes and the polypeptides they encode is shown in table 1.1.

With the exception of IE-5, all the IE genes encode phosphoproteins which are found mainly in the nucleus of infected cells and which are capable of binding to calf thymus

Gene number	Apparent MW	ICP number
IE1	Vmw110	ICP0
IE2	Vmw63	ICP27
IE3	Vmw175	ICP4
IE4	Vmw68	ICP22
IE5	Vmw12	ICP47

Table 1.1; Nomenclature of HSV-1 immediate-early (IE) gene products. The products of the five IE genes have been named according to their apparent MW as indicated by migration rate on SDS-PAGE (Preston et al., 1978; Watson et al., 1979). "Infectious cell polypeptide (ICP) numbers" (Honess and Roizman, 1974; Morse et al., 1978), are also shown.

DNA (Pereira et al., 1977; Hay and Hay, 1980). The major function of IE gene products appears to be the transcriptional activation of later classes of viral genes (O'Hare and Hayward, 1985a; Everett, 1986).

In early studies on IE-3 gene ts mutants, it was found that all showed the same phenotype at non-permissive temperature (NPT). IE mRNAs and polypeptides were overproduced, while later classes of viral genes were not expressed at NPT (Courtney et al., 1976; Marsden et al., 1976). The conclusion from these studies was that the functional IE-3 gene product, Vmw175, is required for transcription of later classes of viral genes and is also involved in autoregulation of IE gene transcription (Preston, 1979a; Dixon and Schaffer, 1980; Watson and Clements, 1980). Using a gel retardation assay Vmw175 has been demonstrated in complexes between infected cell polypeptides and the promoter/regulatory domains of 3 IE genes, a true L gene and the E thymidine kinase gene (Kristie and Roizman, 1986a). Vmw175 (and the HSV-2 equivalent) present in crude infected cell extracts, binds to both denatured and native DNA cellulose columns (Powell and Purifoy, 1976; Hay and Hay, 1980). Partial purification of the equivalent HSV-2 polypeptide causes loss of DNA binding, which is restored by mixing with infected or uninfected cell extracts (Freeman and Powell, 1982). These findings suggest that Vmw175 binds to DNA through an interaction with a host cell factor.

The IE-1 gene which encodes Vmw110 is diploid being encoded by both the TR_L and IR_L genomic regions. Virus mutants with deletions in both copies of this gene have been isolated (Stow and Stow, 1986; Sacks and Schaffer, 1987). Mutants were capable of replication in tissue culture, but

with a 10-100 fold reduction in yield compared with wild type (w.t.) virus (Stow and Stow, 1986; Sacks and Schaffer, 1987). At low multiplicity of infection (m.o.i.) this reduction was most marked (Stow and Stow, 1986). Thus, Vmw110 appears to be essential for normal virus growth, particularly at low m.o.i. A possible role for the protein is that it helps to ensure sufficient viral gene expression at low m.o.i. to commit the cell to a productive infection.

(b) E genes and gene products: E gene transcripts are readily detectable by 2h p.i. and increase in abundance until 6 to 8h p.i. after which the levels of individual early mRNAs either decrease or remain unaltered (McLauchlan and Clements, 1982). The expression of E genes depends on prior synthesis of IE gene products (Honess and Roizman, 1974; Watson and Clements 1980).

E polypeptides are synthesised prior to viral DNA replication and a number are involved in DNA synthesis. These include DNA polymerase, the major DNA-binding protein, thymidine kinase and ribonucleotide reductase (Maclean, 1987).

(c) L genes and gene products: L genes are expressed maximally following the onset of DNA replication about 2h p.i. and products reach peak levels by 10 - 16h p.i. (Swanstrom and Wagner, 1974; Holland et al., 1980). IE and E polypeptide synthesis as well as viral DNA replication are required for the efficient expression of L genes (Jones and Roizman, 1979; Holland et al., 1980). L genes can be divided into two groups. "Leaky late" genes (gamma 1 class) which are transcribed at low levels prior to DNA replication and "true late" genes (gamma 2 class) which are transcribed only after

its onset (Jones and Roizman, 1979; Holland et al., 1980).

A number of L gene products are structural proteins, including the major capsid protein (Vmw155, VP5), glycoprotein C (gC) and the US11 gene product, Vmw21 (Marsden et al., 1976; Frink et al., 1983).

1.1.9 HSV transcription: regulation by trans-acting factors and cis-acting elements

(a) IE gene regulation: IE gene transcription is specifically trans-activated by the Vmw65 protein or trans-inducing factor (TIF), which is a major tegument phosphoprotein (Post et al., 1981; Campbell et al., 1984). The consensus "TAATGARATTC" (R = purine) is found in the far-upstream region of all IE genes (up to -340 b.p.), but not E or L genes (Mackem and Roizman, 1982a; Whitton et al., 1983). The TAATGARATTC element has been shown to mediate responsiveness to Vmw65 (Preston et al., 1984; Gaffney et al.,1985) and to have enhancer-like properties (Bzik and Preston, 1985). Attempts to demonstrate direct binding of Vmw65 to either calf thymus DNA or to the TAATGARATTC sequence have been unsuccessful (Marsden et al., 1987). However, more recently, using gel retardation assays and specific antibodies, it has been demonstrated that Vmw65 interacts with the TAATGARATTC element via cellular polypeptides (Preston et al., 1988).

From the analysis of IE gene 5'-flanking sequences several cis-acting regulatory signals have been identified in addition to the TAATGARATTC consensus sequence (Gelman and Silverstein, 1987). These include a TATA homology located approximately -25 b.p. 5' to the RNA start site, multiple Sp1

binding sites (Jones and Tjian, 1985) and regions homologous to the SV40 enhancer core sequence.

IE genes are also subject to autoregulation and following the switch from IE to E mRNA synthesis, the abundance of IE polypeptides decreases (Dixon and Schaffer, 1980). IE, E and L proteins have been implicated in the negative regulation of IE polypeptide synthesis (Dixon and Schaffer, 1980; De Luca et al., 1984). Transfection assays and studies involving ts mutants suggest that Vmw175 has a strong suppressive effect on IE gene transcription (Dixon and Schaffer, 1980; Watson and Clements, 1980; Gelman and Silverstein, 1986), while in contrast Vmw110 appears to stimulate IE transcription (O'Hare and Hayward, 1985a; Gelman and Silverstein, 1986).

A binding site for partially purified Vmw175 has been identified by DNase I footprinting 100 nucleotides upstream from the HSV glycoprotein D (gD) mRNA cap site, as have two binding sites in the tet^R gene of the vector pBR322 (Faber and Wilcox, 1986). The authors propose that the sequence 5'-ATCGTCNNNNYCGRC-3' (N = any base, Y = pyrimidine, R = purine), forms an essential component of the binding site for Vmw175 or for complexes between Vmw175 and cellular transcription factors. Using a gel retardation assay and crude infected cell extracts it has been shown that Vmw175 can interact with the promoter/regulatory region of IE genes 1, 2 and 3 (Kristie and Roizman, 1986a, 1986b). The site of binding in IE1 and IE3 promoters was mapped by deletion analysis and exonuclease III protection. For IE1 the protected region is between 46 and 71 nucleotides upstream of the RNA start site and contains a sequence showing a high degree of homology to the consensus sequence of Faber and Wilcox. The complex with the IE3 promoter was found to be in

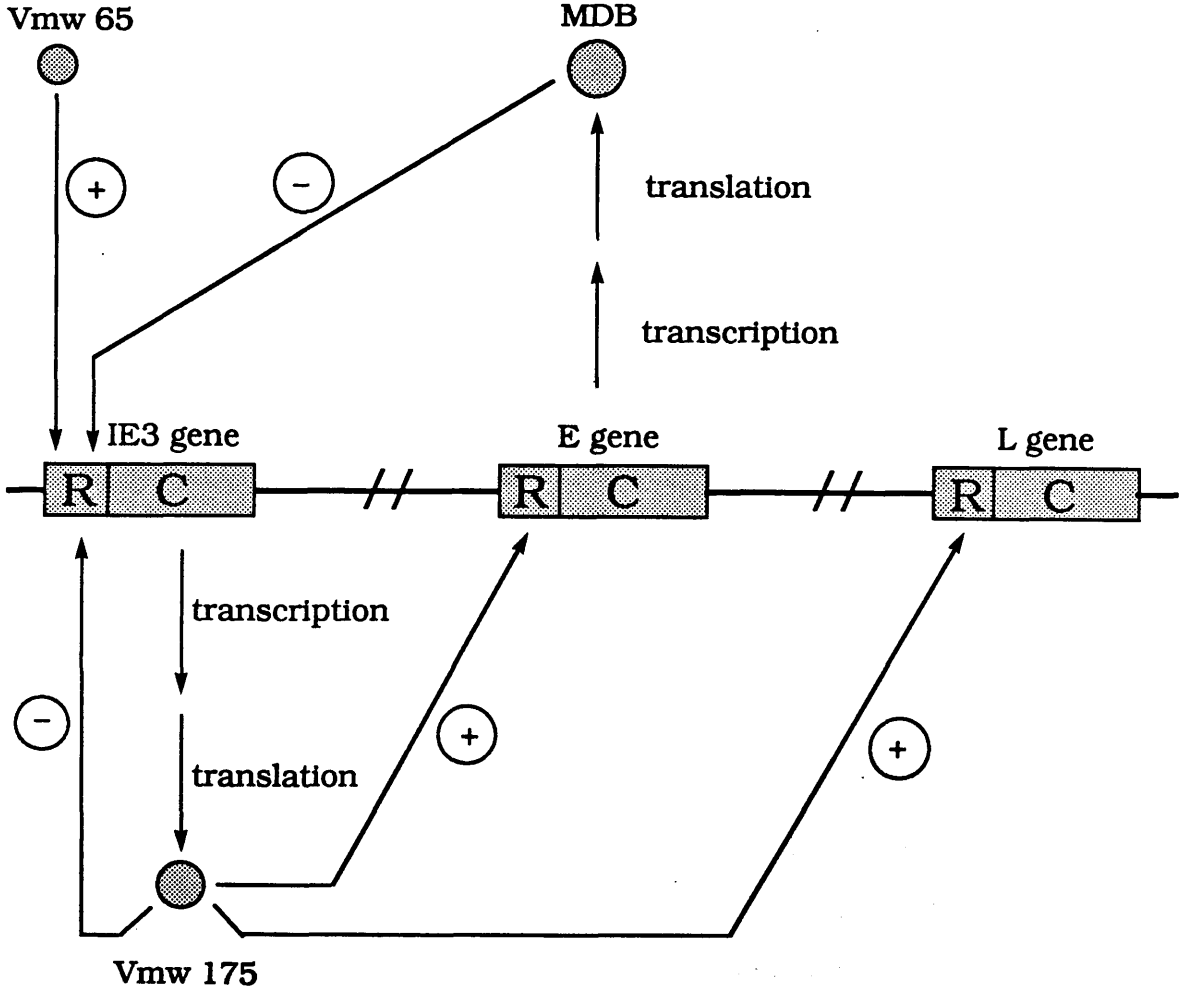
the upstream regulatory region and was mapped to a fragment which does not contain the consensus (Kristie and Roizman, 1986b). However, Muller (1987) has demonstrated Vmw175 binding to its own transcription start site and found that the target sequence fits the consensus sequence of Faber and Wilcox. Binding of Vmw175 at this site could account for negative autoregulation of the IE3 gene expression. IE3 gene regulation and Vmw175 activity are summarized in figure 1.4.

(b) E gene regulation: Vmw175 is essential for E gene expression, but is not necessarily sufficient. Of seven E genes examined only two were almost fully expressed in response to Vmw175 alone while the others varied in expression from intermediate to very low levels (Persson et al., 1985). Vmw175 and Vmw110 can both independently stimulate E gene transcription, while in combination they seem to have a synergistic action (Everett, 1986; Gelman and Silverstein, 1986; O'Hare and Hayward, 1985a).

The E gene tk has a promoter which contains binding sites for three cellular factors: TATA-box-binding protein, Sp1 and CAAT- binding protein (McKnight and Kingsbury, 1982; Coen et al., 1986). Promoters of the E genes gD and ICP8 also have sequence elements for binding of TATA-box-binding protein and Sp1, but not for the CAAT-binding protein (Su and Knipe, 1987). As the common promoter elements identified in HSV E genes all represent binding sites for cellular factors, it is likely that IE gene products interact with E gene promoters by modifying the binding properties of cellular transcription factors.

(c) L gene regulation: The greatly increased transcription

Figure 1.4; Regulation of IE3 gene expression. The closed boxes in this diagram represent viral genes, immediate early (**IE**), early (**E**) and late (**L**). Regulatory and coding region sequences are denoted **R** and **C** respectively. **MDB** is the major DNA binding protein, which is encoded by an E gene. Activating and inhibiting actions are represented by + and - signs.

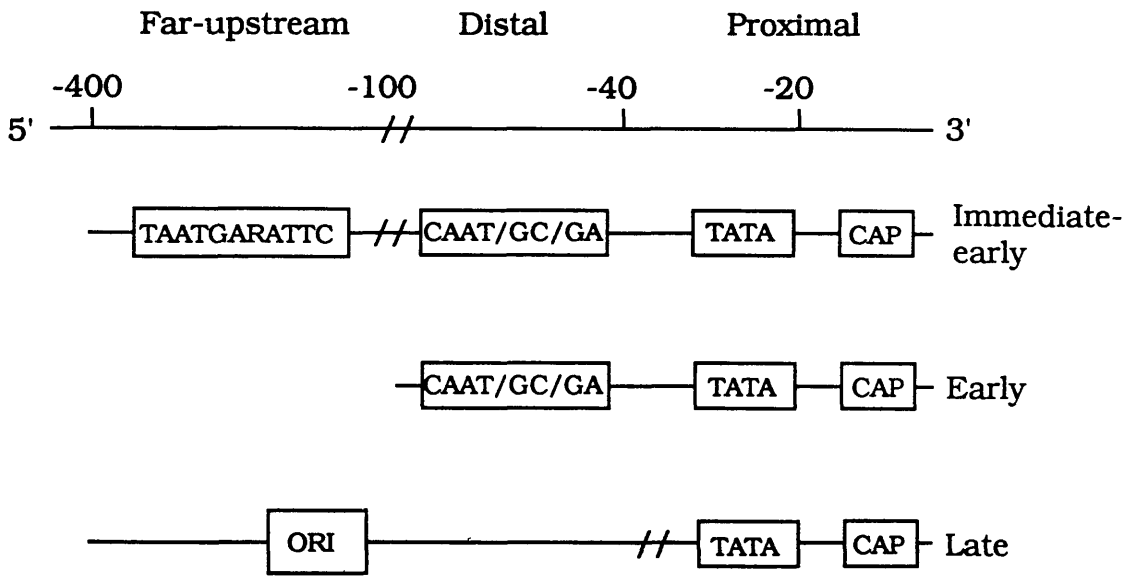

Vmw 65
MDB
+
−
translation
transcription
IE3 gene
E gene
L gene
R
C
R
C
R
C
transcription
translation
−
+
+
Vmw 175

of L genes following initiation of DNA replication may be due to the resulting increase in gene copy number, to changes in viral genome structure and factor binding, or to changes resulting from cellular gene induction (Silver and Roizman, 1985; Johnson and Everett, 1986a). Experiments with plasmid constructs containing the U_S11 promoter have shown that in the presence of a functional origin of replication, all sequences required for efficient expression of the L gene are contained within 31 b.p. of the RNA cap site. In addition, by deleting the distal promoter region of the E gene, gD and linking it to an origin of replication, its phenotype was changed to that of a L gene (Johnson and Everett, 1986b). These findings contrast with the upstream requirements of IE and E promoters and suggest that the three basic classes of HSV promoters may be distinguished by the extent of their sequence requirement, as shown in figure 1.5 (reviewed by Everett, 1987). Late promoters may consist simply of a TATA box and a capsite region on a replicating template.

Owing to the fact that many viral gene products are required for viral replication, it has not been possible to define the IE products required for late promoter activation in a normal replicating environment, but in the absence of DNA replication there is evidence that IE products are required for L promoter activation (reviewed by Everett, 1987).

(d) Conclusions: The understanding of eukaryotic gene expression has been greatly advanced by studies on viruses (Preston et al., 1988). HSV encodes approximately seventy polypeptides and while at least five of these regulate gene expression, transcription of HSV DNA requires the cellular enzyme RNA polymerase II (Costanzo et al., 1977). Evidence

Figure 1.5; A proposed scheme for differentiating between the DNA sequences required for IE, E and L promoter activity (Johnson and Everett, 1986). The top line indicates a scale in nucleotides upstream of the capsites.


Far-upstream
Distal
Proximal
-400
-100
-40
-20
5'
3'
TAATGARATTC
CAAT/GC/GA
TATA
CAP
Immediate-early
CAAT/GC/GA
TATA
CAP
Early
ORI
TATA
CAP
Late

discussed above suggests that in order for Vmw65 and Vmw175 to interact with HSV DNA they must form complexes with cellular components. The levels of cellular transcription factors in different cells might therefore determine the course of HSV infection. Thus, if cellular factors important for the trans-activating functions of Vmw65 and/or Vmw175 are present at low abundance, (in certain HSV infected cells), latent infection or oncogenic transformation may occur instead of lytic infection.

The requirement for complex formation between transcription factors before interaction with DNA, might be important in a much broader context and may form the basis of eukaryotic gene control (Preston et al., 1988). Complex formation between protein transcription factors could be sensitive to changes in the intracellular environment and could produce rapid changes in transcription rate in response to appropriate stimuli (Preston et al., 1988). This would provide an efficient mechanism for the control of cellular transcription.

1.1.10 Effects of HSV infection on cell metabolism

Infection of cultured cells with HSV-1 or HSV-2 results in a rapid inhibition of the synthesis of cell DNA, the mechanism for which is unknown (Roizman and Roane, 1964). The synthesis of most species of cell RNA and protein is also rapidly inhibited (Roizman et al., 1965), but there are notable exceptions as described in the next section.

In the light of the interactions between viral and cellular transcription factors discussed above, it is not surprising to find that HSV infection affects cellular transcription. Infection of the adenovirus transformed 293

cell line with HSV-1 has been shown to cause reduced transcription from the integrated adenovirus E1A gene and reduced synthesis of host ribosomal RNA (Stenberg and Pizer, 1982). These effects are not observed with ts mutants in IE gene 3, indicating that a functional Vmw175 may be required for decreased cell RNA synthesis following HSV infection (Stenberg and Pizer, 1982).

The shut-off of host protein synthesis can be divided into "early" and "delayed" phases. The early shut-off of host protein synthesis is mediated by an unidentified component of the infecting virion, while delayed shut-off depends on viral gene expression (Nishioka and Silverstein, 1977; Fenwick and Clarke, 1982). The delayed shut-off function is required for the full inhibitory effect on host protein synthesis and can occur in the absence of a functional virion component (Fenwick and Clark, 1982; Read and Frenkel, 1983). The mechanism(s) of shutoff of host protein synthesis are not clearly understood and appear to differ between cell types. Thus in HSV-infected Vero cells host mRNA degradation accompanies shutoff of host protein synthesis in the absence of viral gene expression (Schek and Bachenheimer, 1985), while in HSV-infected Friend erythroleukaemia cells, host mRNA degradation appears to require the expression of viral genes although globin synthesis is arrested by a virion function (Nishioka and Silverstein, 1978).

HSV-2 shut-off of host protein synthesis is generally more rapid and efficient than HSV-1 (Powell and Courtney, 1975; Pereira et al., 1977) and the virion function responsible for the diferential shut-off has been mapped using a battery of intertypic recombinant viruses to between 0.52 and 0.59 m.u. (Morse et al., 1978). Such functions have also

been mapped using virion-associated host shutoff (vhs) mutants like the vhs1 mutant of HSV-1 strain KOS. Unlike wt virus this is defective in function(s) responsible for the early phase of the shutoff of host protein synthesis but is normal with respect to the delayed shutoff function (Read and Frenkel, 1983). In cells infected with vhs1 viral mRNAs of IE, E and L classes also have a prolonged functional half-life (Read and Frenkel, 1983; Kwong et al., 1988). Mutation(s) affecting the functional half-lives of both host and viral mRNAs have been mapped by marker transfer assay to the same 265 b.p. NruI-XmaIII fragment spanning map coordinates 0.604 to 0.606 of the HSV-1 genome (Kwong et al., 1988). This suggests that the shutoff of host protein synthesis is mediated by the same function that decreases the half-life of viral mRNA. The rapid shutoff function of HSV-2 and the defective function of the vhs1 mutant may represent the same gene since the 265 b.p. NruI-XmaIII fragment lies at the right-hand border of the 11 k.b. region identified by Morse et al., (1978). The small discrepancy in map coordinates may reflect the way in which they were calculated (Kwong et al., 1988). Interestingly the 265 b.p. NruI-XmaIII fragment corresponds to one of the transforming regions of HSV-2 and the possible relationship between transformation and host shutoff functions will be discussed in section 1.2.6.

It has recently been proposed that HSV encodes a function which indiscriminately shortens the half-life of host and viral mRNAs (Kwong and Frenkel, 1987). Shutoff of host protein synthesis may allow the maximum translational capacity of the cell to be used in the expression of viral genes and the reduced viral mRNA half-life may lead to more economical use of the cell's translational machinery by IE and E mRNAs to

allow for maximum synthesis of structural proteins when virion maturation begins (Kwong et al., 1988).

1.1.11 Activation of cellular genes by HSV

It has been shown that a number of cellular RNAs are induced by a mechanism that requires IE gene expression (Patel et al., 1986), while others may be induced by mechanisms that parallel activation of the IE genes by Vmw65, or simply by adsorption of the virus to the cell surface (Kemp et al., 1986).

Certain cellular genes are activated by HSV IE products in transfection experiments. The rabbit beta-globin gene promoter responds to viral activation when in a plasmid (Everett, 1983) and when integrated into the host chromosome of a biochemically transformed cell line (Everett, 1985). However, the endogenous beta-globin gene of rabbit kidney cells is not activated by virus infection which may be due to a densely packed chromatin configuration making the promoter unavailable for viral trans-activation (Everett, 1985). Many other cellular genes may fail to be activated on viral infection for the same reason.

Heat-shock proteins are induced in chick embryo fibroblasts (CEF) by tsK at NPT (Notarianni and Preston, 1982). This cellular response has been shown to be due to the presence of abnormal Vmw175 protein rather than the overproduction of IE proteins (Russell et al., 1987). However natural isolates of HSV-1 frequently show mutations in the gene encoding Vmw175 (Knipe et al., 1981; Post et al., 1981). Thus, the induction of heat-shock proteins by HSV may be important in vivo.

Infection of human embryo fibroblasts with HSV-2 induces

the synthesis of a minor cellular stress protein of 57kDa MW, which is synthesised in a growth regulated manner (LaThangue et al., 1984). By the analysis of cDNA libraries made before and after HSV infection, it has been demonstrated that the gene encoding this protein is induced at the level of transcription (Patel et al., 1986).

The induction of stress proteins in viral infection may represent an attempt by the cell to protect itself. Alternatively, it may result from the viral induction of cellular functions which facilitate viral multiplication (Patel et al., 1986). Heat-shock proteins might be induced to mediate the repression of cellular biosynthesis which is seen in heat-shocked cells (Peterson and Mitchell, 1981)

The induction of heat-shock genes also occurs in cells infected with the DNA tumour viruses adenovirus (Nevins, 1982), polyoma virus and SV40 (Khandjian and Türler, 1983). This has led to the suggestion that the activation of such genes might be involved in transformation.

Western blotting studies have shown that TG7A recognizes a 35 - 40kDa polypeptide induced on HSV-1 infection and a 90kDa polypeptide induced on HSV-2 infection (La Thangue and Latchman, 1988). Cellular polypeptides investigated in this study have been immunoprecipitated from HSV-2 transformed cells using the same monoclonal antibody and have similar molecular weights (MW) to both HSV induced polypeptides (Macnab et al., 1985a). These authors suggested that induction of these cellular polypeptides in cells infected by HSV might be an important step in initiation of oncogenic transformation by the virus (see section 1.2.7).

1.1.12 Possible outcomes of infection with HSV

The possible outcomes of HSV infection are lytic infection with the production of infectious progeny, latent infection, cell transformation producing an oncogenic phenotype, abortive infection or persistent infection.

(a) Infection with HSV *in vitro*: Infection of permissive cells *in vitro* usually results in productive lytic infection, whilst abortive infection with defective virus can result in cell transformation as will be discussed in section 1.2.5.

(b) Clinical features of HSV infection: HSV-1 infections are usually first manifest as an acute stomatitis (inflammation of the mucous membrane of the mouth) contracted in early childhood (Burnet and Williams, 1939), although infection is usually asymptomatic. Typically, at intervals of months or years after recovery from the primary infection, vesicles from which the virus can be recovered appear around the lips and nose and are triggered by a variety of stimuli, including exposure to UV light, fever, menstruation, nerve injury and emotional disturbances (Fenner *et al.*, 1974). HSV-2 causes a sexually transmitted genital infection in man, characterized by the occurrence of vesicles which break down to give painful superficial ulcers persisting for two weeks or more. Genital lesions may occur in both sexes and like the "fever blisters" of HSV-1 infection, they may recur at intervals (Passmore and Robson, 1974). The link between HSV-2 infection and cervical cancer is discussed in section 1.2.

(c) HSV latency: The consistent failure of researchers to isolate HSV from biopsies of tissues at the site of recurrent

infections between the recurrences, as well as other studies, led Goodpasture to postulate that the virus persists in ganglia in a latent state after the local lesion has healed (Goodpasture, 1929). The persistence of latent HSV in the sensory neurones of trigeminal ganglia has since been demonstrated both in the mouse model (Stevens and Cook, 1971, 1973; MacLennan and Darby, 1980) and in man (Baringer and Swoveland, 1973; Lonsdale et al., 1979), latent virus being recoverable by co-cultivation of ganglionic tissue with susceptible cells in tissue culture.

HSV-1 DNA in latently infected mouse neurones has been found by Fraser and coworkers to exist as a non-linear endless form which may be either a circular molecule or a concatamer. Further, they have found that the viral DNA does not appear to be integrated into the host DNA (Mellerick and Fraser, 1987). It remains to be established whether the HSV-1 genome in latently infected neurones exists as a supercoiled plasmid as does latent EBV (Baichwal and Sugden, 1988). The search for HSV functions related to latency has involved the analysis of virus mutants for their ability to establish latent infection and the analysis of latently infected cells for the presence of viral gene products. Recent studies using the latter approach have yielded particularly interesting results. Transcripts from the HSV-1 genomic region encoding the IE protein Vmw110, but encoded by the opposite DNA strand, have been detected in latently infected mouse sensory neurones (Stevens et al., 1987). These were the only transcripts readily detected by in situ hybridization and were localized mainly in the neuronal nuclei. It has been suggested that the latency-associated transcript may function as an antisense RNA, regulating IE1 gene expression and thereby influencing

entry into the lytic cycle (Stevens et al., 1987).

(d) Conclusions: A subject of great importance in Herpesvirology is the molecular basis for the very different virus : cell interactions that can occur following infection, and recent advances in the understanding of HSV latency are therefore encouraging.

In the next chapter, current knowledge of the oncogenic properties of HSV will be reviewed, following a preliminary account of the properties of oncogenes and DNA tumour viruses other than HSV.

1.2 Oncogenes, DNA tumour viruses and HSV oncogenesis

1.2.1 Oncogenes

(a) The role of oncogenes in oncogenesis: Cellular oncogenes (c-onc) or protooncogenes and their viral counterparts, viral oncogenes (v-onc) which were first discovered as the transforming genes of retroviruses, have been established as critical elements in the neoplastic transformation of mammalian cells (reviewed by Bishop et al., 1984). In transformed cells oncogenes may be overexpressed or may express proteins that function abnormally or inappropriately (table 1.2), resulting in the by-passing of normal cellular controls that regulate cell division and differentiation.

The human ras oncogenes (Ha-ras, Ki-ras and N-ras) all code for highly related proteins of 189 amino acid residues known collectively as p21, and it has been demonstrated that these genes acquire oncogenic potential by single point

Table 1.2; Oncogenes and their products

A. Some viral oncogenes and their products. Abbreviations: sarcoma virus, **SV**; Murine leukemia virus, MuLV; protein kinase, **PK**; macrophage stimulating factor (**CSF-1**).

B. Some cellular homologues to viral oncogenes

A.

Viral oncogene	Viral origin	Subcellular location of v-onc product	Activity
v-src	Rous SV	plasma memb.	tyrosine kinase
v-yes	Y73 avian SV	plasma memb.?	tyrosine kinase
v-fps	Fujinami SV	cytoplasm/ plasma memb.	tyrosine kinase
v-fes	Snyder-Theilen feline SV	cytoplasm	tyrosine kinase
v-ros	UR2 avian SV	?	tyrosine kinase
v-abl	Abelson MuLV	plasma memb.	tyrosine kinase
v-mil	Avian SV, MH2	cytoplasm	serine/threonine kinase
v-fms	Feline SV, McDonough strain	plasma memb., ER and Golgi apparatus	activation of CSF-1 receptor PK domain
v-erb-A	Avian erythro-blastosis	cytoplasm	activation of oestrogen PK domain
v-erb-B	Avian erythro-blastosis	plasma memb.	activation of EGF receptor PK domain
v-sis	Simian SV	cytoplasm	PDGF activity
v-myc	Avian myelo-cytomatosis virus, MC29	nucleus	binds DNA, thought to activate transcription
v-myb	Avian myelo-blastosis virus	nucleus	binds DNA?

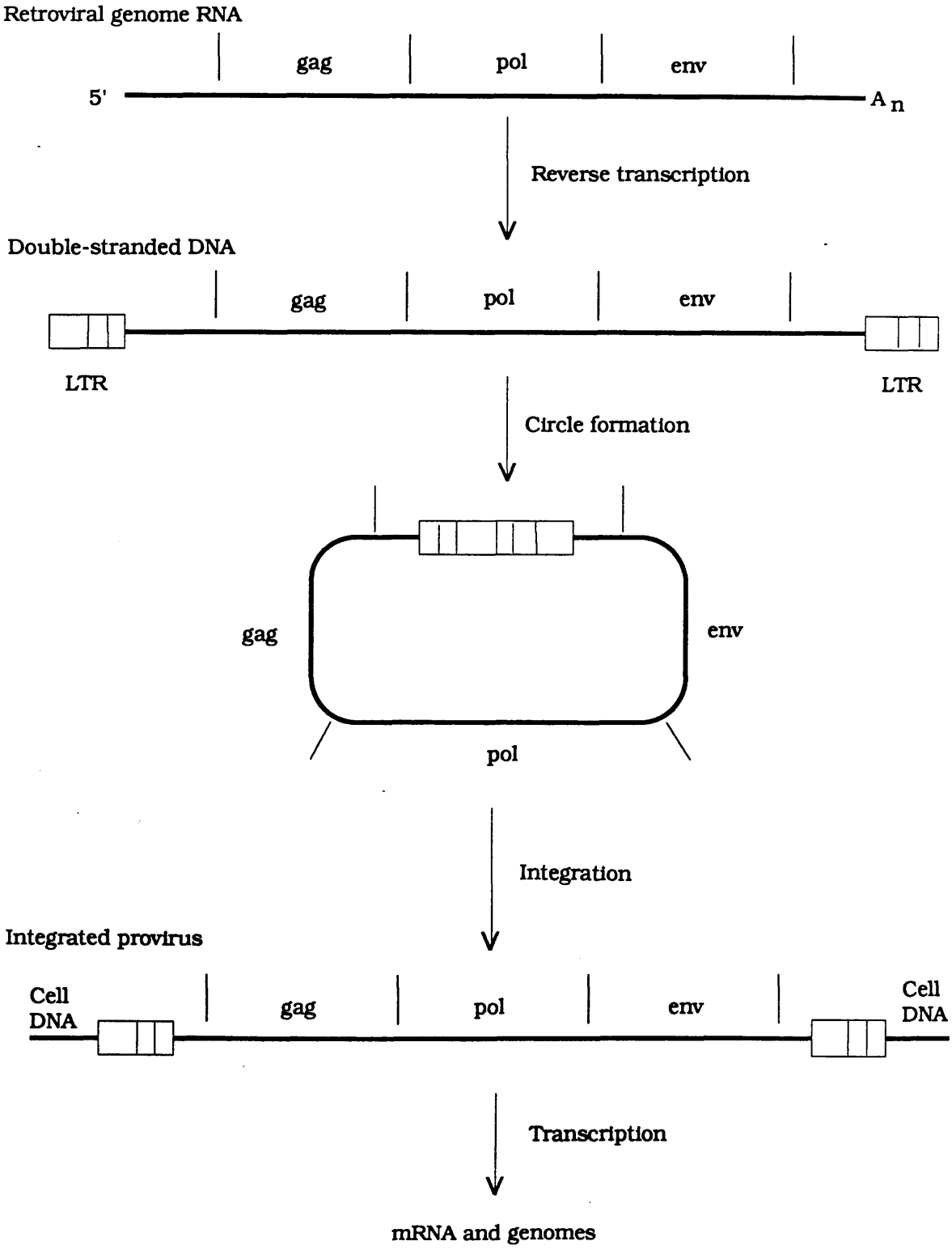
B.

Viral oncogene	v-onc product	cellular homologue
v-erb-A	$p75^{gag\text{-}erbA}$	oestrogen receptor
v-erb-B	$gp65^{erb\text{-}B}$	truncated EGF receptor
v-fms	gp^{120fms}	CSF-1 receptor
v-sis	$p28^{sis}$	PDGF B chain

mutations at two specific hot spots (codons 12 and 61). Further, it has been demonstrated that ras oncogenes can be activated in tumours induced by a variety of chemical carcinogens (Sukumar et al., 1984) however, the activation of oncogenesis by mutation does not appear to be a characteristic feature of spontaneous tumours (Slamon et al., 1984). A more common finding is the increased expression of c-onc genes due to changes like gene amplification (Dalla-Favera, 1982) or chromosome translocation. In some cases of Burkitt's lymphoma for example, the c-myc gene on chromosome 8 is translocated to the immunoglobulin heavy chain locus on chromosome 14, which contains enhancer elements which increase c-myc expression (Taub et al., 1984). Such changes are however, far from universal (Slamon et al., 1984).

"Acute" transforming retroviruses can deliver a dominant transforming v-onc gene to susceptible cells that can transform cells in one step (Klein and Klein, 1986). The genome structure and replication cycle of these viruses are shown in figure 1.6. They are assumed to have evolved by recombination between non-oncogene-containing retrovirus and c-onc genes. The recombined oncogenes exert their oncogenic effect in the viral genome where they are under the influence of a strong promoter in the long terminal repeat region (fig 1.6). Similarly, DNA constructs containing a mutated ras allele under the control of a strong promoter, can induce both immortalization and transformation of RE cells (Spandidos and Wilkie, 1984). While such rapid one-step transformations are interesting and important models for studying carcinogenesis, it must be remembered that naturally-occurring spontaneous cancers arise by slow multiple steps (Klein and Klein, 1985). In contrast to most retroviruses, oncogenic DNA viruses appear

Figure 1.6; Retrovirus life cycle. Following infection, reverse transcription of retroviral genomic RNA takes place and synthesis of the complementary DNA strand also occurs. The double-stranded DNA then circularizes and recombines into the cellular genome, where transcription occurs producing viral mRNA and genomes.


Retroviral genome RNA
gag
pol
env
5'
A n
Reverse transcription
Double-stranded DNA
gag
pol
env
LTR
LTR
Circle formation
gag
env
pol
Integration
Integrated provirus
Cell DNA
gag
pol
env
Cell DNA
Transcription
mRNA and genomes

in general to require more than one gene to establish and maintain neoplastic transformation (Glaichenhaus et al., 1985).

Retroviruses and their nonessential transforming v-onc genes, have been placed repeatedly under strong selection pressures yielding rare recombinational events that are probably encountered infrequently in the wild (Duesberg, 1985). In the wild selection pressures act against parasites which frequently cause fatal disease (e.g. malignant tumours) in members of the host species (Mims, 1977). A virus that is too pathogenic is a poorly adapted parasite, as by killing the host, it reduces its own chances of transmission.

(b) Classification of oncogenes: Oncogenes can be divided into two groups according to their ability to cooperate with either c-myc or c-ras in the transformation of rat embryo fibroblasts (Land et al., 1983). Group 1 oncogenes cooperate with c-ras, and include adenovirus E1a, polyomavirus large T and c-myc. These proteins which are found associated with nuclear structures have an immortalizing action on RE cells and are thought to act by modifying cellular transcription (Glaichenhaus et al.,1985). The gene encoding the cell protein p53 also belongs to this group and has been shown to cooperate with the ras oncogene in malignant transformation (Jenkins et al.,1984.).

Group 2 oncogenes, in contrast, cooperate with c-myc in the transformation of rat embryo fibroblasts, and include c-Harvey-ras, c-N-ras and polyoma middle T. The proteins encoded by this group are found attached to the inner surface of the plasma membrane. The ras gene product, p21 ras, is thought to be involved in signal transduction into the cell

following the binding of hormones to cell surface receptors (review, Newbold, 1984).

A group of viral oncogenes, not included in this classification are the tyrosine protein kinases, v-src, v-yes, v-fgr, v-abl and v-fps (review, Hunter, 1984). Sequence homologies provide strong evidence that these protein kinases comprise a single divergent gene family (Kamps et al., 1984; Hunter, 1984). The main substrate for viral protein kinases appears to be a 36kDa cellular polypeptide, which has been found in the terminal web of intestinal columnar epithelial cells and may have a structural function. In addition the v-src gene product, $p60^{v\text{-}src}$, has been shown to phosphorylate phosphatidyl inositol which is thought to lead to activation of a serine-specific protein kinase.

Sequence analysis of the viral oncogenes v-mil, v-mos, v-fms and v-erb-B suggests that they may also encode protein kinases (Kamps, 1984). The cellular homologue of v-erb-B encodes the epidermal growth factor receptor. Simian sarcoma virus encodes another interesting viral oncogene, v-sis, which encodes a product almost identical with platelet-derived growth factor (Waterfield, 1983). In addition, feline leukaemia virus transduction of the gene encoding the beta chain of the T cell antigen receptor has recently been detected (Fulton et al., 1987) and it has been suggested that this v-tcr gene is a novel viral oncogene.

(c) The immortalized phenotype: REF cells immortalized by group 1 oncogenes like c-myc are phenotypically similar but not identical to in vitro established cells (Glaichenhaus et al., 1985., Land et al.,1983.). In both cases cells show unlimited growth in culture and are able to grow as isolated

colonies at low cell density. However, in contrast to low passage cells established *in vitro*, oncogene-immortalized cells are also capable of growth at low serum concentration and show susceptibility to transformation by chemical promoters like TPA (Glaichenhaus *et al.*, 1985). At high passage however, *in vitro* established cells become less serum dependent and more like oncogene-immortalized cells, showing increased growth rate on treatment with TPA. (Glaichenhaus *et al.*, 1985).

Cells of a primary culture transformed by a combination of myc and ras oncogenes produce tumours that usually reach a large but static size. In contrast, established cells transformed by the ras oncogene produce tumours with apparently unlimited growth capacity (Land *et al.*, 1983). Thus, there may be a third distinct gene which cooperates with myc and ras to produce the full tumourigenic phenotype (Land *et al.*, 1983).

Thus, the phenotype of oncogene immortalized cells differs from that of low passage established cells, which suggests that the immortalized phenotype is complex and not always reached by a single step. There may be a number of undiscovered cellular genes responsible for induction of the immortalized phenotype, which have escaped detection because the assays used have only detected oncogenes which complete the entire immortalization process (Glaichenhaus *et al.*, 1985).

(d) Oncogene expression in early development: Differential transcriptional activity of c-onc genes, during the prenatal and early postnatal development of the mouse has been demonstrated (Muller *et al.*,1982). Slamon & Cline (1984)

reported appreciable levels of transcription of five c-onc genes, between days 7 and 18 of murine prenatal development. These included c-src, c-myc, c-Ha-ras, c-erb, and c-sis. The expression of c-src was not marked until after day 10, and at day 17 c-myc expression was 2-5 times greater than at other stages tested. These results indicate that c-onc gene products may have important physiological functions in the developmental processes of embryogenesis.

(e) Antioncogenes: The antioncogenes comprise a new class of genes important in oncogenesis, and they were detected through the study of hereditary human cancers like retinoblastoma and Wilm's tumour (reviewed by Knudson, 1985). For a given antioncogene locus, the presence of one normal allele is sufficient to protect a cell against neoplastic change. Therefore, in a heterozygous individual with one normal allele for a particular antioncogene, tumour formation only occurs if this normal allele is lost from a cell by somatic mutation or recombination (Knudson, 1985).

1.2.2 DNA tumour viruses: some oncogenic mechanisms

In this section, an account will be given of some oncogenic mechanisms of a few well characterized and intensively investigated DNA tumour viruses. This will provide a background against which possible oncogenic mechanisms for HSV will be described.

Both DNA and RNA tumour viruses are capable of causing tumours in animals, but while oncogenic potential among the RNA viruses is restricted to one genus (Leukovirus), oncogenic viruses are found in four of the six genera of DNA viruses. RNA tumour viruses are the causative agents of natural

leukaemias and sarcomas in a large number of animal species, whereas DNA tumour viruses with the exception of the Herpesviruses, do not in general cause tumours in their hosts under natural conditions (Fenner et al., 1974).

The oncogenic properties of the DNA tumour viruses SV40, polyoma virus and adenovirus have been thoroughly investigated in vitro and these properties will now be described briefly. In addition, the results of some interesting studies on transforming proteins of the Herpesvirus, Epstein-Barr virus (EBV) will be outlined.

(a) Transformation by SV40 and polyoma virus: SV40 and polyoma virus are members of the Polyomavirus genus and both are small viruses containing about $3X10^6$ daltons of dsDNA. Both viruses are oncogenic for rodents and cause transformation of cells in culture (Fenner et al., 1974). SV40 and polyoma virus may cause either productive or non-productive infection depending on whether the cell type is permissive for viral multiplication. The productive response yields viral progeny and results in cell death, while non-productively infected cells which produce little or no virus survive and may become stably transformed (reviewed by Sambrook, 1972).

In cells transformed by SV40 or polyoma virus, viral DNA containing a functional early region is invariably found integrated into cellular chromosomal DNA (Sambrook et al., 1968; Basilico et al., 1980; Lania et al., 1980a). No common sequence or structural features are found in either viral or cellular DNA sequences at integration sites. Transformation by polyoma virus and SV40 does not appear to be due to the integration event itself (Lania et al., 1980b), but

rather to the addition of a specific viral oncogene to the cellular genome. If a viral life cycle is completed, infectious virus particles are released and cell lysis occurs. However, rare transformation events are likely to arise in cells nonpermissive for viral DNA replication, when viral DNA happens by chance to become integrated into the host chromosome, thereby ensuring viral persistence in progeny cells. The expression of SV40 large T or polyoma middle T induces cell DNA synthesis and cell division (Stoker and Dulbecco, 1969; Fried, 1970). Thus, if these viral genes are integrated, cancer cells could result which would continue to divide under conditions where normal cell division is inhibited (Fried and Prives, 1986).

i. SV40 large T antigen: The large T antigen of SV40 can trans-activate certain genes. This was first demonstrated by the ability of SV40 large T to activate the late SV40 promoter in transient transfection assays (Keller and Alwine, 1984). More recently, it has been shown to activate a number of other promoters, including certain cellular promoters and the adenovirus E2 promoter. The fact that purified large T antigen shows no affinity for the E2 promoter DNA sequence, suggests an indirect mechanism of action (Alwine, 1985). In non-simian (non-permissive) cells, SV40 large T forms a tight complex with a cell-encoded nuclear phosphoprotein, p53 (Lane and Crawford, 1979). A lower affinity complex can be detected in simian (permissive) cells (Crawford et al., 1981). It has recently been shown that p53 competes with DNA polymerase alpha for binding to the SV40 T antigen (Gannon and Lane, 1987) and to inhibit SV40 origin-dependent DNA replication (Braithwaite et al., 1987). Further, p53 from non-permissive

species binds to the large T more tightly than p53 from permissive species (Lane et al., 1982). The outcome of competition between p53 and DNA polymerase alpha for the large T could be a determinant of host cell permissiveness (Gannon and Lane, 1987).

The oncogene encoding SV40 large T has properties of both group 1 and group 2 oncogenes. The large T antigen is a complex multifunctional protein which is found both in the nucleus where it binds to DNA and at the cell membrane. It is sufficient for both immortalization and transformation of primary RE cells (reviewed by Livingston and Bradley, 1987). SV40 mutants that have lost their nuclear location signal and whose large T is predominantly in the cytoplasm, can still efficiently transform cell lines but not primary cells (Kalderon et al., 1984. This indicates that either the amount of large T is required for transformation is small, or that transformation is mediated by a non-nuclear form of the large T.

A small proportion of SV40 large T antigen has been detected in association with cell membranes (Deppert et al., 1980; Gooding et al., 1984). Highly purified large T has an associated autokinase activity and can catalyse the phosphorylation of multiple, unrelated protein substrates (Griffin et al., 1979; Livingston and Bradley, 1987).

Thus, SV40 large T antigen is found both in nuclear and extranuclear locations, which would appear to be consistent with the general pattern, by which immortalizing oncogenes like c-myc are located mainly in the cell nucleus, and transforming oncogenes like the protein kinase c-src are located in the cytoplasm and plasma membrane (table 1.2).

ii. Polyoma virus T antigens: Three T antigens are expressed by integrated polyoma virus sequences in transformed rodent cell lines: the chromatin-associated large T, the membrane associated middle T and the cytosolic small T antigens (Ito, 1980). Polyoma large T like SV40 large T, represses early promoters and activates late gene expression (Brady et al., 1984). A tyrosyl-specific protein kinase activity is found associated with the middle T antigen, which is thought to be due to a small amount of c-src complexed to the middle T antigen (Courtneidge and Smith, 1984). However, it is possible that complex formation with the middle T antigen leads to activation of c-src by increased phosphorylation (Yonemoto et al., 1985). Expression of middle T antigen is sufficient to transform established cell lines (Triesman et al., 1981) and the small T antigen cooperates with middle T in this action. Polyoma virus large T antigen is not required for maintenance of the middle T antigen induced transformed phenotype in established cells, but is required for maintenance of transformation for primary cells. This is due to the immortalizing action of the polyoma virus large T antigen (Land et al., 1983a; Rassoulzadegan et al., 1983).

(b) Transformation by adenoviruses: Mammalian adenoviruses have a linear dsDNA of MW 20-25X10^6 daltons which for comparison, is approximately 1/4 the DNA content of Herpesviruses (Fenner et al., 1974). Like Polyomaviruses and Herpesviruses, adenovirus genes are expressed in temporally regulated groups during lytic, productive infections. Early (E) RNAs appear prior to DNA synthesis while late (L) RNAs only appear in significant amounts after viral DNA synthesis (reviewed by Green et al., 1970).

Different human adenovirus serotypes vary in their tumourigenic capacity in rodents and while some like adenovirus 12, 18 and 31 are highly tumourigenic, others like types 2 and 5 are non-tumourigenic. It is interesting to find that non-tumourigenic strains of human adenovirus transform rat cells *in vitro* as efficiently as the highly tumourigenic adenovirus 12 (reviewed by Fenner *et al.*, 1974).

Cell lines derived from adenovirus-transformed non-permissive cells do not usually maintain a complete copy of the viral genome, but only a subgenomic fragment from the left-hand 8-12% of the viral genome, which contains genetic information sufficient for transformation of primary cells in culture (Sambrook *et al.*, 1975). This region contains two transcription units, E1A and E1B which are both responsible for oncogenic transformation by the virus (Graham *et al.*, 1974; Gallimore *et al.*, 1974). E1A products transcriptionally activate other E genes in the adenovirus genome (Nevins, 1981) as well as certain cellular genes including HSP70 (Nevins, 1982). The E1A gene gives rise to three distinct mRNAs by differential splicing (Berk and Sharp, 1978) and the products of two of these are transcriptional activators, both being required for full transformation in combination with E1B (Winberg and Shenk, 1984). The E1B gene product, E1B-p58 complexes with p53 in adenovirus transformed cells (Sarnow *et al.*, 1982) as does large T antigen in SV40 transformed cells.

Transfection of primary RE cells with the E1A gene promotes the establishment of immortalized non-tumourigenic clones (Houwelling *et al.*, 1980), while E1B expressed in the absence of E1A produces no detectable effect (Van den Elsen *et al.*, 1983). These results suggest a two step transformation pathway, as do the studies with polyoma virus large and middle

T antigens.

(c) Transformation by EBV: Like HSV, EBV is a member of the Herpesvirus genus. EBV readily infects human B cells in culture transforming them into permanent cell lines in which every cell carries multiple episomal copies of the viral genome (reviewed by Dambaugh et al., 1986). Only the so-called latent viral genes are constitutively expressed in transformed cells and these include the EBV nuclear antigens (EBNAs) and the latent membrane protein (LMP).

EBNA is invariably associated with EBV-transformed cells as well as Burkitt's lymphoma and nasopharyngeal carcinoma both of which are human tumours thought to be caused by EBV (Pope et al., 1969; Reedman and Klein, 1973). Initially EBNA was found to be a high MW basic protein of 170-230kDa composed of a 48kDa subunit in an oligomeric complex with a 53kDa polypeptide of probable cellular origin (Luka et al., 1978, 1980). It was later shown that the 48kDa protein is the degradation product of a larger component of EBNA (Luka et al., 1983) and it was demonstrated by immunoblotting studies that the major component of EBNA has a MW of 65kDa to 70kDa depending on the strain of the resident virus genome in the cell type under investigation (Strnad et al., 1981). This form of EBNA, termed EBNA1, is important in episomal maintainence of the viral genome in transformed cells (Reisman et al., 1985). A second type of EBNA termed EBNA2 with a MW of 82kDa has been identified (Strnad et al., 1981) and there is now direct evidence from the comparison of natural EBV isolates with differing EBNA2 allelles, that this protein plays a continuing role in the expression of the transformed phenotype (Rickinson et al., 1987).

LMP is expressed in latently infected proliferating lymphocytes and can convert Rat-1 cells (which are immortalized cells) to a tumourigenic phenotype (Wang et al., 1985). Thus, EBV posesses more than one transforming function, in the same way as SV40, polyoma virus and adenovirus.

(d) Summary: From the above account it can be seen that SV40, polyoma virus, adenovirus and EBV all encode more than one transforming function. The viral sequences encoding these 37functions are retained by the transformed cell, which suggests that their continued expression is important for maintenance of the transformed phenotype. No single transforming function appears to be sufficient for oncogenic transformation of primary cells in culture.

1.2.3 Oncogenic herpesviruses

Marek's disease virus (MDV), herpesvirus saimiri (HVS) and EBV all produce tumours in animals and all are members of the gammaherpesvirus sub-family. MDV induces malignant lymphomas in chickens (Kato and Hirai, 1985) and HVS isolated from squirrel monkeys induces lymphomas on inoculation of marmosets (Meléndez et al., 1970). EBV also induces lymphomas in marmosets and is believed to be involved in the aetiology of Burkitt's lymphoma and nasopharyngeal carcinoma in man (Dambaugh et al., 1986). Herpesviruses which may be causative agents of cancer of the cervix include the alphaherpesvirus, HSV and the betaherpesvirus, human cytomegalovirus (HCMV).

A potential role for HCMV in the aetiology of cervical cancer is suggested by the fact it is a sexually transmissible cause of cervicitis and urethritis (Evans, 1976), and has oncogenic potential in vitro (Fletcher et al., 1986).

Seroepidemiological studies of the association between HCMV infection and cervical neoplasia have produced conflicting results but HCMV DNA, RNA and antigens have all been detected in HCMV transformed cells and in tumours (reviewed by Macnab, 1987). Strong evidence for the retention of integrated HCMV sequences in cervical neoplasia has been obtained by Fletcher et al., (1986), who detected sequences hybridizing to HCMV DNA in 2/43 biopsies taken from patients with cervical intra-epithelial neoplasia (CIN). In one of these biopsies the DNA sequences detected contained four restriction sites colinear with those of the prototype strain AD169 (Fletcher et al., 1986). Subsequent identification of HCMV DNA sequences in clones of an EMBL3 library constructed from the CIN DNA further confirmed the association (Macnab and Fletcher, unpublished results). Interestingly, HCMV sequences detected represented the major enhancer and the region responsible for the initiation of morphological transformation (reviewed by Macnab, 1987).

1.2.4 The role of HSV in the aetiology of cervical cancer

Cervical cancer is the most common malignant disease of the female reproductive tract, second only to carcinoma of the breast as a cause of death from neoplasia for women and it is also a disease of relatively young women with a peak incidence in the 45 to 49 year age group (Parker, 1969).

Much clinical and laboratory data exists to link both HSV and human papillomavirus (HPV) with the causation of cervical cancer (reviewed by: Baird, 1985; Macnab, 1987) and it has been suggested that HSV and HPV may have a synergistic effect in oncogenesis (zur Hausen, 1982). The evidence that HPV is a causative agent of human genital cancer has been strengthened

by detection of specific types (HPV-16 and HPV-18) in a high percentage of genital cancer biopsies (Dürst et al., 1983; Scholl et al., 1985; Macnab et al., 1986; Murdoch et al., 1988). It has been demonstrated recently that an enhancer in the upstream regulatory region of HPV-16 shows specificity for cervical carcinoma cells (HeLa cells) as compared with MCF-7 cells (Gloss et al., 1987). The significance of this interesting finding is not yet clear, but it may relate to HPV16 tissue tropism which may have an important bearing on the role of HPV16 in the aetiology of cervical cancer.

Early evidence of a causative role for HSV in cervical cancer came from the demonstration of an increased incidence of abnormal cervical smears showing premalignant change in women with genital HSV-2 infection (Naib et al., 1966). Subsequent seroepidemiological studies added to the evidence of an association between HSV-2 infection and cervical cancer, as will now be described.

(a) Seroepidemiological studies: A major problem in demonstrating a cause-effect relationship between HSV-2 infection and cervical cancer is that there is an association between genital HSV-2 infection, a range of other venereal infections, promiscuity and smoking. Many of these factors may be important in the aetiology of cervical cancer. If an epidemiological study is to be meaningful therefore, control groups must be carefully matched with test groups for many factors including age, sexual behaviour and smoking habits (Baird, 1985). While there are some studies which suggest that HSV-2 infection may be an aetiological agent of cervical cancer (Nahmias et al., 1974; Aurelian et al., 1975), not all studies agree with this conclusion (Vonka et al., 1984a,

1984b).

(b) Evidence of retained HSV information: RNA homologous to HSV DNA has been demonstrated in pre-malignant cervical cells but not in matched control biopsies and it was shown using specific cloned fragments of the HSV genome that R_S regions and a region with map units 0.07-0.4, hybridize preferentially (McDougall et al., 1980; Eglin et al., 1981). As mentioned in section 1.2.4, R_S regions of the HSV genome show considerable homology to regions of human DNA. In addition, cellular DNA sequences in tumour cells with homology to the plasmid vector pBR322, have been found to undergo amplification in tumour cells (Park, 1983; Macnab et al., 1984; Cameron et al., 1985). Thus, the detection of RNA homologous to HSV DNA in cervical neoplasia does not necessarily indicate the persistence of viral sequences in the cell (Macnab, 1987).

HSV antigens have been demonstrated in exfoliated cervical cells from 60-90% of cases of cervical dysplasia, CIN and invasive cervical cancer (Royston and Aurelian, 1970). In addition the major HSV DNA binding protein has been detected by monospecific antisera in over 30% of cervical tumor specimens and CIN biopsies (Dreesman et al., 1980).

HSV-2 DNA sequences have been detected in biopsies of cervical carcinoma in situ (CIN) and invasive cervical cancer and almost all studies reveal the presence of BglIIn fragment sequences in a small proportion of cases (Galloway and McDougall, 1983; Park et al., 1983; Macnab et al., 1985b, 1986; Prakash et al., 1985). Overall, approximately 10% of cervical cancer or CIN biopsies show detectable HSV sequences. The authenticity of these results was reinforced by the

demonstration of five restriction sites characteristic of the BglIIn fragment of HSV-2, in a restriction fragment of cervical cancer cell DNA which had been detected using the BglIIn fragment to probe Southern blots (Park et al., 1983). The detection of HSV DNA in cervical cancer specimens is circumstantial evidence that HSV has an aetiological role. Significance is added however, by the fact that the retained HSV DNA fragment most frequently detected is from one of the two regions of the HSV-2 genome with in vitro transforming activity (see below).

An animal model for HSV-induced cervical cancer has been developed, which adds to the evidence for a causative role of HSV in the human disease (Wentz et al., 1981). In one study 24/40 mice to which UV inactivated HSV-2 was administered intra-vaginally, developed invasive carcinoma of the cervix as compared with 0/25 of a control group exposed to uninoculated culture medium (Wentz et al., 1981). Using a similar system it has been shown that the Skinner vaccine (Skinner et al., 1987) protects mice against development of carcinoma of the cervix following exposure to HSV-2, reducing the incidence to less than half (Chen et al., 1986).

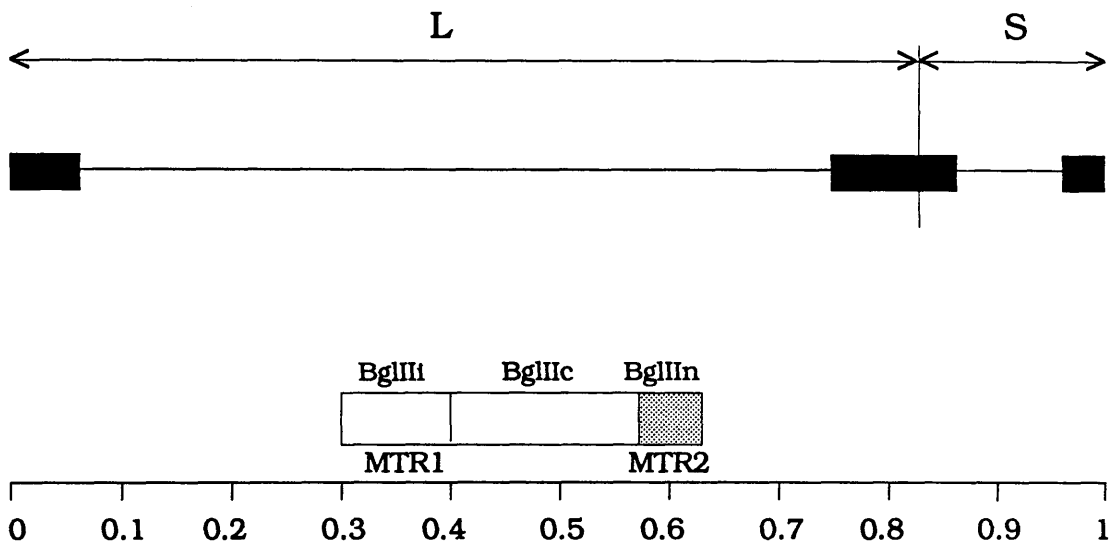
1.2.5 Morphological transformation of cultured cells by HSV

(a) Morphological transformation regions of HSV: While the link between HSV infection and cervical cancer remains controversial, it is now well established that abortive infections with defective HSV can transform normal rodent embryo cells with a finite lifespan and give rise to tumorigenic transformed cell lines (Galloway and McDougall, 1983; Cameron et al., 1985; Jariwalla et al., 1986). Three

regions of the HSV genome are associated with morphological transformation of cultured cells, one in HSV-1 and two in HSV-2 (figure 1.7). The morphological transformation region (MTR) of HSV-1 (MTRI) maps in BglIIi (Camacho and Spear, 1978; Reyes et al., 1979). In HSV-2, one morphological transforming region MTRII maps in BglIIn (Reyes et al., 1979; Macnab and McDougall, 1980; Galloway and McDougall, 1981; Cameron et al., 1985), while the other, MTRIII maps in BglIIc (Jariwalla et al., 1983). The transforming activity mapping in BglIIn was subsequently further localized to a 737 b.p. subfragment of the left half region of BglIIn (Galloway et al., 1984).

(b) The "hit and run" hypothesis: By analogy with other DNA tumour viruses like SV40, polyomavirus and adenovirus, one might expect that HSV DNA fragments from the MTRs would be integrated into the cell chromosome and encode transforming viral proteins. However, in HSV transformed cell lines the quantity of viral DNA present is always less than one copy per cell and varies with passage in culture (Galloway et al., 1980; Cameron et al., 1985). Southern blotting analysis on a range of HSV-transformed rat cell lines, showed that no HSV sequences of 1 k.b. or more were detectable even if present at only 0.1 copies per cell (Cameron et al., 1985). Thus, it is very unlikely that sufficient HSV DNA could be retained in most HSV-transformed cell lines, to encode a protein responsible for maintenance of the transformed phenotype. This does not exclude the possibility that active recombination of HSV sequences into the host cell chromosome might be important for HSV oncogenesis (Galloway and McDougall, 1983). Short HSV DNA sequences might be difficult to detect by Southern blotting analysis because they are

Figure 1.7; Transforming regions of HSV. Diagram to show the location of genomic regions associated with transformation by HSV-1 (**MTR-1**) and HSV-2 (**MTR-2** and **BglIIc**) as described in section 1.2.5.


L
S
BglIIi
BglIIc
BglIIn
MTR1
MTR2
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1

rearranged after integration so that they are not in a homologous position in all cells. Alternatively, they may act in a "hit and run" manner, activating an oncogene perhaps and then being rapidly excised from the host cell chromosome (Galloway and McDougall, 1983).

A system favouring stable integration of viral sequences was produced by co-transfection of NIH 3T3 cells with the HSV-2 BglIIn fragment and the pAG60 plasmid which confers resistance to the antibiotic G418 (Saavedra and Kessous-Elbaz, 1985). Co-transfection with the complete BglIIn fragment gave colonies of both normal and transformed morphology, but an overall reduction in efficiency of colony formation in G418 as compared with pAG60 alone. This suggests that the BglIIn fragment has an inhibitory effect on cell proliferation, perhaps due to a lethal mutagenic action. The inhibitory effect may account for rapid excision of the BglIIn fragment from the genome of transformed cells. Sub-fragments corresponding to each end of BglIIn will transform NIH 3T3 cells when co-transfected with pAG60 and no inhibitory effect on colony formation is seen (Saavedra and Kessous-Elbaz, 1985). Thus, BglIIn appears to be required in its entirety for the inhibitory effect, which could result from the combined actions of the three proteins totally encoded by BglIIn which have MWs of 38kDa, 58kDa and 61kDa (Galloway et al., 1982).

The failure of HSV-transformed cells in tissue culture to retain viral sequences contrasts with the presence of HSV sequences in a small but significant proportion of cervical cancer and CIN biopsies. The differences observed are not understood, but may result from the fact that cervical cancers are generally of epithelial origin while the rat cells

examined in tissue culture are predominantly fibroblastic (Macnab, 1987).

1.2.6 Possible mechanisms of HSV oncogenesis

(a) Trans-activation of cellular genes: Evidence for the trans-activation of cellular genes by HSV is presented in section 1.1.11. No HSV genes have as yet been shown to function as oncogenes in the same way as the adenovirus E1A gene or the SV40 gene for large T antigen. This may be because HSV-coded transcriptional activators are too vigorous in their activity and cause lethal alterations in the cell (Nevins, 1986). The pseudorabies IE gene for example is very toxic to cells and when co-transfected with a selectable marker produces a marked reduction in the number of surviving colonies. Attenuated strains of HSV with mutant IE genes might therefore have immortalizing/transforming activity (Nevins, 1986). This possibility seems more likely in the light of reports that natural isolates of HSV-1 frequently have ts mutations in the IE gene encoding Vmw175 (Knipe et al., 1981; Post et al., 1981).

(b) Activation of cellular genes by reduced methylation: Experiments with mouse retroviruses have provided strong evidence that transcription is increased by demethylation of certain DNA sequences (Jähner et al., 1982). In addition, a number of examples are known in which tumour cells show reduced DNA methylation when compared with normal control cells (Adams and Burdon, 1983).

Rat embryo cells show a marked reduction in the methylation of cellular DNA synthesised following infection

with HSV-2, while in HSV transformed cells, DNA shows reduced levels of methylation compared with control rat embryo cells (Dr J. Macnab, personal communication). It has been postulated that transformed cells resulting after an abortive HSV infection, may have been selected as a result of HSV-induced undermethylation and possible consequent expression of a cellular protooncogene.

(c) Mutagenesis and amplification of cellular genes: Chromosomal aberrations, including both random damage and specific gaps have been reported in HSV infected cells suggesting a mutagenic effect of the virus (Stich et al., 1964). The virus was subsequently demonstrated to have a mutagenic effect on a specific cellular gene. Treatment of permissive cells with UV inactivated HSV-1 and infection of non-permissive cells with wild-type HSV-2 have both been found to increase the mutation frequency in the cellular hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene (Schlehofer and zur Hausen, 1982; Pilon et al., 1985). In cells infected with HSV-2, the transcription rate for the HGPRT gene and the size of transcripts are similar to uninfected cells, suggesting that the mutagenic effect is due to point mutations rather than gene rearrangements (Pilon et al., 1986). Such mutations could have oncogenic effects if they activated oncogenes like c-ras or inactivated antioncogenes for which an individual is heterozygous.

Expression of the BamHIg fragment of the HSV-1 MTRI region in bacterial cells, using expression vectors puC7, 8 and 9 with the viral DNA fragment inserted in both orientations, has allowed assessment of the mutagenic effects of the encoded peptide (Shillitoe et al., 1986). Expression

of the fragment in E.coli C resulted in the increased reversion rate of a bacterial frameshift mutation for only one of the constructs. This suggests that the BamHIg fragment encodes a mutagenic peptide which may perhaps be involved in cell transformation. The peptide is thought to consist of 37 amino acids, 6 of which are encoded by the vector (Shillitoe et al., 1986).

Evidence also exists for gene amplification by HSV. A wide range of chemical and physical carcinogens have been shown to selectively amplify integrated SV40 sequences in an SV40-transformed Chinese hamster cell line (Lavi, 1981) and both HSV-1 and HSV-2 have been found to amplify integrated SV40 genes in the same way (Schlehofer et al., 1983). Further studies indicated that HSV DNA polymerase is important in amplification. DNA polymerase negative mutants of HSV-1 e.g. tsH, do not induce amplification (Matz et al., 1984) and inhibitors of DNA polymerase alpha e.g. aphidicolin, inhibit the gene amplification effect of HSV (Heilbronn et al., 1985). As described in section 1.2.1, amplified and mutated cellular oncogenes have been found in many tumours. This suggests that the mutagenic and gene amplifying properties of HSV, may be at least partly responsible for its oncogenic actions.

(d) Activation of cellular genes by promoter or enhancer insertion: The insertion of an HSV promoter into the cellular genome is one mechanism by which HSV could induce a cellular gene. Sequences as little as 100 b.p. long can have promoter function (McKnight and Kingsbury, 1982; Everett, 1983). Such sequences would escape detection by the Southern blot analysis of Cameron et al., (1985).

Homology between viral and cellular DNA sequences allows

for viral integration into cellular DNA by homologous recombination. R_S and R_L regions of the HSV-1 and HSV-2 genomes and the centre of U_L for HSV-2 only, show considerable homology to regions of both human and mouse cell DNA (Peden et al., 1982; Gomez-Marquez et al., 1985; Spector et al., 1987).

Part of the 737 b.p. transforming fragment within MTRII is an IS-like sequence element and does not correspond to a similar sequence in HSV-1 (Galloway et al., 1984). This sequence element may be important in transformation perhaps functioning as an enhancer or as a mutagen and activating a cellular oncogene (Galloway et al., 1984). IS-like sequence elements have also been found in MTRIII but not in the corresponding HSV-1 region (Jones et al., 1986). The significance of IS elements is less clear however, following the discovery that these sequences are common and widely dispersed in the HSV genome (reviewed by Macnab, 1987).

(e) Activation of endogenous viruses: HSV infection has been shown to activate endogenous C-type virus (MuX) from a feline cell line (F81) transformed by the Moloney strain of murine sarcoma virus (Hampar et al., 1976; Boyd et al., 1978). This activation of MuX may be the result of mutagenesis or of integration of an HSV promoter or enhancer element near to the integrated endogenous virus (Macnab, 1987). HSV-1 sequences responsible for the induction of MuX map in the MTR1 and have been found to be inactivated by digestion with endonucleases cutting in the thymidine kinase (TK) gene (Boyd et al., 1980). However, other investigators have found the inducing sequences in MTR1 to be distinct from the TK gene and also distinct from the mutagenic BamHIg fragment and the DNA polymerase gene

(Shillitoe et al., 1986).

(f) Expression of virus-encoded transforming proteins: The expression of virus-encoded enzymes involved in DNA synthesis or degradation would be likely to interfere with cellular DNA replication and repair mechanisms. It has been suggested that by creating an imbalance in cellular deoxyribonucleotide pools, HSV might enhance mutagenesis (Huszar and Bacchetti, 1983).

i. Ribonucleotide reductase: The HSV encoded ribonucleotide reductase (RR) is of interest because the smaller of the two subunits composing this enzyme is encoded by the BglIIn fragment of the HSV-2 genome and the larger subunit is mostly encoded by the adjacent BglIIc fragment. The RR subunits are encoded by two transcripts with colinear 3' ends that map within the BglIIn fragment of HSV-2 (McLauchlan and Clements, 1983). Both BglIIn and BglIIc fragments have their own transforming activity.

The RR small subunit of 38kDa MW is termed Vmw38 (Bacchetti et al., 1984; Frame et al., 1985) and the large subunit of 140kDa MW is termed Vmw136 (also known as ICP6 for HSV-1 and ICP10 for HSV-2). There is evidence suggesting that RR enzyme activity depends on formation of a complex containing both small and large subunits (Frame et al., 1985; Dutia et al., 1986; Bacchetti et al., 1986) and this suggests that viral RR activity does not account for the oncogenic properties of the BglIIn fragment of HSV-2. Further, the 737 b.p. transforming sub-fragment of BglIIn does not lie within the RR coding sequences (Galloway et al., 1984). In addition, the 481 b.p. fragment of BglIIc which transforms established

Rat-2 cells does not encode the carboxy terminal of the large subunit of RR which is the region to which the enzyme's catalytic function has been mapped (Jariwalla et al., 1986).

Cells transformed by the BglIIn fragment of HSV-2 have not been found to show any increase in RR activity (Cameron et al., 1985). However, it is possible that transient expression of the RR small subunit may occur after transfection with the BglIIn fragment and that this could interfere with cellular nucleotide metabolism and initiate oncogenic transformation (reviewed by Macnab, 1987).

ii. Protein kinase: DNA sequence analysis of the US3 genes of HSV-1 and HSV-2 and the corresponding gene of varicella zoster virus indicated that they encode proteins which are homologous with members of the protein kinase family of eukaryotes and the v-src oncogene (McGeoch and Davison, 1986). These observations were interesting since a number of oncogenes including v-src, v-yes, v-abl and v-fos are known to encode protein kinases (Hunter 1984).

A protein kinase has recently been identified as the product of the HSV-1 US3 gene (Purves et al., 1987; Frame et al., 1987). The novel protein kinase differs from that in uninfected cells with respect to substrate specificity and chromatographic properties and was absent from cells infected with a virus mutant from which a major portion of the US3 open reading frame was deleted (Purves et al., 1987). In addition, a monospecific antiserum raised against a synthetic oligopeptide corresponding to the carboxy-terminal eight amino acids encoded by US3, reacts on immunoblotting with a 68kDa polypeptide in HSV-1 infected cell extracts and in a purified preparation of HSV-1 protein kinase (Frame et al., 1987).

The conserved nature of the US3 gene indicates that its product must have an important function *in vivo*, despite the fact that mutant studies show it is not essential for virus growth in cell culture (Purves *et al.*, 1987). It is well established that phosphorylation is a major mechanism for the regulation of activity of cellular enzymes (Krebs, 1985) and the phosphorylation of cellular or viral enzymes by the viral protein kinase may facilitate viral multiplication in certain cell types.

(g) Expression of virion-associated host shutoff (vhs) functions: It has recently been shown that an HSV-1 virion-associated host shutoff function maps within a region which corresponds to BglIIn sequences of HSV-2 which have transforming activity (Kwong *et al.*, 1988). The transforming sequences within the BglIIn fragment have been shown to reside in an open reading frame encoding a 61kDa protein (Galloway *et al.*, 1984) and this is homologous to the HSV-1 open reading frame to which the vhs protein has been mapped (Kwong *et al.*, 1988). The significance of the co-mapping of transforming and host shutoff functions is not yet known, but it is possible that the cellular stress of protein synthesis inhibition may lead to amplification and other rearrangements of oncogenes and cell cycle regulatory genes, which may in turn lead to transformation (Kwong *et al.*, 1988).

(h) General considerations: Oncogenic DNA viruses appear in general to require more than one gene to establish and maintain neoplastic transformation, which seems likely to be a two step process at least, of which the first step is immortalization (Glaichenhaus *et al.*, 1985). Consistent with

this is the report that neoplastic transformation of Syrian hamster embryo cells by the BglIIc HSV-2 fragment involves at least two steps (Jariwalla et al., 1983). The left 64% of the BglIIc fragment is sufficient to induce immortalization but DNA sequences from the whole of the BglIIc fragment are required for tumorigenic transformation. It is interesting to find that functions important for immortalization and transformation are closely linked on the HSV genome just as they are for adenovirus, polyomavirus and SV40 genomes. From sections 1.2.1 and 1.2.2 it is seen that these appear to be distinct functions; immortalization occurring due to transcriptional changes in the nucleus and transformation occurring due to changes in the cytoplasm or cell membrane. The reason these functions are closely linked on the viral genome is not clear, but it would seem unlikely to be a chance association. Neither is it clear how a virus would benefit from causing a fatal neoplastic disease in its host. One explanation is that the immortalizing and transforming activities of viral genes are functions important in latency, which will, under certain unusual circumstances, produce oncogenic transformation (Livingston and Bradley, 1987). One such circumstance might be the occasional emergence of a mutant virus with lethal oncogenic effects. Although the relationship between a wild-type virus and its natural host may be the product of millions of years of evolution towards a state of balanced pathogenicity (Mims, 1977), mutant viruses will not necessarily display the appropriate behaviour.

As described in section 1.1, complex virus-host cell interactions occur in HSV infection so that HSV oncogenesis in nature may well involve a highly complex interplay between numerous viral and host cell factors. Adding to the

complexity, the large size of the HSV genome suggests that it may encode a much greater number of potentially oncogenic functions than small DNA viruses like SV40 and polyoma virus, as the HSV genome is approximately 30 times larger than the genomes of either of these viruses.

1.2.7 Evidence for virus-induced cellular polypeptides in HSV transformed cells

Although Cameron et al.,(1985) showed by Southern blotting analysis on HSV-transformed rat embryo (RE) cells, that no HSV sequences of 1 k.b. or more were detectable, even if present at levels of only 0.1 copies per cell, other evidence indicated that virus-induced antigens continued to be expressed in HSV transformed cells. Stable post-crisis cultures of transformed cells showed cytoplasmic and perinuclear immunofluorescence with sera raised against HSV infected cells (Macnab, 1974; Macnab et al., 1980). To identify these virus-induced antigens, direct and indirect immunofluorescence tests were carried out using a battery of monoclonals directed against specific HSV-coded polypeptides. However, no HSV coded polypeptides could be consistently detected in the transformed cells. The possibility was therefore considered that virus induced cell-coded polypeptides might account for the immunofluorescence results (Macnab et al., 1985a).

Immunoprecipitation (i.p.) assays were used to define the virus-induced polypeptide species. Analysis of immunoprecipitates by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) showed that antiserum raised against HSV infected cells recognizes a group of HSV transformed cell polypeptides that are not detected in untransformed control primary or secondary

rat embryo (RE) cells. Comigrating polypeptides of 40kDa and 90kDa molecular weight (MW) can be i.p. with the serum of tumour bearing rats (TBS), from transformed but not untransformed RE cells (Macnab et al., 1985a).

A number of monoclonal antibodies were tested in i.p. assays, for reactivity against the 40kDa and 90kDa HSV transformed cell polypeptides in i.p. assays in an effort to identify the molecules. A positive result was obtained with the TG7A monoclonal (generated by Dr N.B. LaThangue, University College, London), an antibody raised against affinity purified DNA binding proteins from HSV-2 infected cells (Macnab et al., 1985a). Western blotting studies showed that TG7A recognizes polypeptides of 40kDa and 90kDa in uninfected baby hamster kidney (BHK) cells. HSV infection resulted in the induction of either 40kDa or 90kDa polypeptides depending on the serotype of virus used (LaThangue and Latchman, 1988). Screening of a panel of HSV strains suggested that as a general rule HSV-2 infection causes the 90kDa polypeptide accumulation, while HSV-1 infection causes 40kDa polypeptide accumulation. One-dimensional V8 protease peptide maps of polypeptides i.p. by TG7A appear to show that the virus-induced polypeptides are related to those constitutively synthesised in uninfected cells (LaThangue and Latchman, 1988).

TG7A was shown to recognize polypeptides of 40kDa and 90kDa present in HSV transformed but not control primary RE cells cultures. From one-dimensional peptide mapping studies using V8 protease, the 40kDa and 90kDa polypeptides i.p. by TG7A appear to be highly similar to those i.p. by TBS (Macnab et al., 1985a). TG7A also recognized polypeptides of 40 and 90kDa in all transformed and immortalized rat cells tested but

not in primary or secondary RE cell cultures (Macnab et al., 1985a). Thus, the TG7A monoclonal antibody recognizes polypeptides which are a feature characteristic of immortalized cells as well as polypeptides of the same or similar MW which are induced on HSV infection. The relationship between these polypeptides is of great interest. It has been suggested that HSV may initiate oncogenic transformation by increasing the expression of immortalizing cellular polypeptides (Macnab et al., 1985a). It is therefore most important to identify these polypeptides and the genes which encode them.

1.3 Tumour antigens

Tumour antigens can be defined as immunological determinants present in tumour tissue, which elicit an antibody response in a tumour bearing animal (Maltzman et al., 1981). They may represent macromolecules with a causative role in oncogenesis, or may simply be a reflection of differences between the metabolism of normal and tumour cells.

1.3.1 Tumour progression and tumour antigens

Each tumour cell exists in a competitive microenvironment surrounded by other tumour cells as well as normal cells which infiltrate the tumour (Nicolson, 1987). During the progression of tumours, neoplastic cells are thought to accumulate genetic alterations generated by random somatic mutational events (Nowell, 1986). Cytogenetic studies show that chromosome abnormalities become progressively more pronounced as tumours advance to more malignant phenotypes, which is evidence of the decreased genetic stability of

cancers (Nowell, 1986; Wolman, 1983). Tumour evolution occurs rapidly, with host selection pressures acting to enrich those tumour cell subpopulations which are most adept at survival and growth (Nowell, 1986; Nicolson, 1987).

Virtually any characteristic of neoplastic cells should be subject to independent variation, selection and evolution leading to tumours of increasing autonomy from host regulation and increasing resistance to host immune surveillance mechanisms (Nowell, 1976, 1986; Fidler and Hart, 1982). The period for which a tumour cell would continue to express a tumour antigen *in vivo* would therefore be expected to depend on the survival advantage it conferred.

1.3.2 Explanations for tumour antigen expression

(a) Increased levels of a normal cellular protein; i. The cellular tumour antigen, p53: This is an example of a normal cellular protein expressed at increased levels in many tumours. As mentioned in section 1.2.1, p53 has been shown to be the product of an immortalizing oncogene (Jenkins *et al.*, 1984; Eliyahu *et al.*, 1984; Parada *et al.*, 1984) and has been shown to compete with cellular DNA polymerase alpha for binding to T antigen (Gannon and Lane, 1987) and to inhibit SV40 DNA replication (Braithwaite *et al.*, 1987). This suggests p53 may have a physiological role in cellular DNA replication. p53 was first observed in SV40 transformed cells, forming a stable complex with the viral large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). In immunoprecipitation assays, monospecific antibodies prepared against the viral large T antigen co-immunoprecipitated the cellular p53 protein with the viral antigen in a constant

precipitation ratio (Lane and Crawford, 1979).

Increased synthesis of p53 occurs in a wide range of transformed and tumour cells, of rodent and human origin (Crawford et al., 1981; Dippold et al., 1981; Maltzman et al., 1981). In addition to an increased rate of synthesis, the amount of steady-state p53 evaluated by radioimmunoassay, is higher in tumour cells than normal cells (Benchimol et al., 1982). Cells transformed by DNA and RNA viruses as well as chemical carcinogens, all appear to contain the same p53 molecule (DeLeo et al., 1979; Linzer and Levine, 1979; Rotter et al., 1981). This suggests that the increased synthesis of p53 is a common secondary event following a primary signal that induces malignant transformation.

p53 has been shown to be expressed phase-specifically in mouse prenatal development, being present in cultures from 12-14 day embryos, but not 16 day embryos (Mora et al.,1980; Chandrasekaran et al., 1981). Thus, p53 is a candidate oncofoetal antigen (see section 1.3.2).

It has been suggested that p53 is stabilized by complex formation with SV40 large T antigen, as the half-life of p53 is much longer in mouse 3T3 cells after transformation with SV40 (Oren et al., 1981). p53 also forms a complex with the viral tumour antigen E1B-p58, in adenovirus transformed cells (Sarnow et al., 1982) and with the mammalian HSP70 (Pinhasi-Kimbi et al., 1986). However, p53 does not appear to form complexes in a number of transformed cell types. In chemically transformed cells for example, p53 has only been demonstrated in unbound form (DeLeo et al., 1979). The accumulation of p53 in these cells is probably mediated by some mechanism other than stabilization through the formation of protein:protein complexes.

ii. Reasons for autoantibody production: By definition, a tumour cell protein must elicit an immune response in the tumour bearing animal to be termed a tumour antigen. In some circumstances at least, p53 satisfies this criterion. Mice bearing syngeneic SV40-induced and Abelson-MuLV-induced tumours have been shown to produce anti-p53 antibodies (Lane and Crawford, 1979; Rotter et al., 1980). In addition, a survey of human serum has shown that a proportion of cancer patients have anti-p53 antibodies unlike healthy controls (Crawford et al., 1982).

There are several ways in which antibodies might be induced to a normal cellular component. The production of antibody against many antigens is dependent on cooperation between T and B lymphocytes. If autoreactive T lymphocytes are tolerized and thereby unable to cooperate with B lymphocytes in generating autoantibodies, the provision of new carrier determinants could enable non-tolerized T lymphocytes to cooperate in the process of antibody production (Roitt, 1984). A carrier-hapten-like association between a foreign protein and the normal cell protein could have this effect. Thus complex formation between SV40 large T antigen and p53 may explain the production of antibodies against p53 by mice bearing SV40-induced tumours (Lane and Crawford, 1979).

Another way immunological tolerance can be bypassed is when a foreign antigen, bearing a similar epitope (hapten) to a self antigen but with a different carrier moiety, induces the synthesis of a cross-reacting antibody (Roitt, 1984). Thus rabbits bearing Rous sarcoma virus (RSV) induced tumours, have been shown to produce antibodies to the viral $pp60^{v\text{-}src}$ which cross-react with the rabbit pp60c-src (Collett et al., 1978).

(b) **Re-expression of antigens normally restricted to pre-natal life:** Transformed cells may re-express genes normally only expressed pre-natally in order to gain competence for survival in the tumour environment (Gerhards & Mehnert, 1986). Antigens which are normally expressed either exclusively or predominantly during foetal development, may be recognized as foreign by the mature immune system and therefore constitute tumour antigens.

There are two clinically important examples found in man. One is alpha foetoprotein (AFP), a protein found in human malignant hepatomas. Detection of AFP in the blood of a patient strongly suggests this condition is present (MacLeod, 1978). Another is carcinoembryonic antigen (CEA), which is found in some human colonic carcinomas (Neville and Laurence, 1974). Serial estimations of CEA levels may allow the earlier detection of recurrence after operative removal of colonic cancers (MacLeod, 1978).

Oncofoetal antigens (OFA) have been defined as "antigenic substances expressed exclusively as phase-specific autoantigens in the developing embryo or foetal tissues, of metazoans and their tumours" (Coggin, 1986a). Using this precise definition it is incorrect to classify AFP and CEA as OFAs, as they have been discovered in some diseased non-tumour tissue and show some limited expression in normal adult tissues. They would be more correctly classified as differentiation antigens (Coggin, 1986b).

Polypeptide antigens of 44kDa and 200kDa which appear to fulfill all the requirements of the above definition, have been detected in a range of cell lysates using a monoclonal antibody immunoaffinity column (Payne and Coggin, 1985). These are expressed in mouse, hamster and human foetal tissues

as stage-dependent antigens, being detected in mouse embryo fibroblast (MEF) cultures prepared from embryos of 12 to 13 days gestation but not embryos of 19 to 21 days. These are present in a range of neoplasms (including mouse sarcomas, and human colonic, rectal and ovarian carcinomas), but have not been detected in any normal tissue examined to date. They are immunogenic in mouse, hamster and man, both in pregnancy (where the pregnant animal is immunized by foetal antigens) and in neoplastic disease.

Similar polypeptides of 35kDa and 40kDa have been identified in MEF cell cultures prepared from embryos of 16 days, but not 12 or 18days gestation (Gerhards and Mehnert 1986). These are referred to as stage-dependent embryonic antigens (SDEAs). A rabbit antiserum raised against the detergent solubilized fraction of an SV40- transformed MEF cell line was used to i.p. these SDEAs. Comigrating polypeptides on SDS-PAGE were also i.p. from SV40 transformed cells of hamster, rat, monkey and human origin, from polyoma virus transformed cells of rat and mouse origin and also from cell lines derived from human tumours (HeLa and rhabdomyosarcoma cell lines). No comigrating polypeptides were i.p. from primary mouse kidney cell cultures of 12 day old mice, or from nontransformed cell lines of hamster, monkey or human origin (Gerhards and Mehnert 1986). As yet, no evidence has been presented suggesting that these polypeptides are autoantigenic. It is therefore not yet possible to classify them as oncofoetal antigens using the definition of Coggin, (1986a).

There have been many other reports of embryonic antigens that are expressed in tumours, including a rat embryonic antigen maximally expressed at 14-16 days gestation, which has

been isolated from chemically induced hepatomas and corresponds to a 65-70kDa polypeptide (Price, 1974). A further human example is a sarcoma associated 102kDa polypeptide present in melanoma, osteogenic sarcoma, colorectal cancer and 1st trimester foetal membranes (Brown, 1983). The numerous examples of antigens expressed in both embryogenesis and neoplasia, suggest that the re-expression of embryonic genes may be advantageous for tumour cell survival.

(c) Antigenic changes due to mutations and rearrangements of cellular genes: This can occur following retrovirus infection, evidence of recombination between viral and cellular genes to produce novel proteins being seen in the case of Abelson murine leukaemia virus (A-MuLV). The defective genome of A-MuLV encodes the genetic information for a 120-160 kDa protein (p120) which consists of the N-terminal portion of the viral gag protein linked to a gene segment of the host genome (Reynolds et al., 1978). Some deletion mutants in p120 fail to transform cells which suggests an important role in the transformation process for this protein (Rosenberg and Witte, 1980).

It is likely but as yet unproven, that tumour antigens expressed by some carcinogen-induced tumours result from mutations and rearrangements in cellular genes. Each individual tumour induced by a chemical carcinogen expresses its own unique tumour antigen or tumour-specific transplantation antigen (TSTAs), so named because expression of TSTAs by the cells of transplanted tumours results in transplant rejection (Levine, 1982). The TSTAs induced by chemical carcinogens (eg methylcholanthrene) are highly polymorphic and are specific to the tumour rather than the

transforming agent. Thus, animals immunized with the TSTA prepared from a tumour induced by methylcholanthrene are protected against transplants of the same tumour, but not against transplants of other tumours also induced by methylcholanthrene (Baldwin and Price, 1982; Law et al., 1980).

The antigenic diversity of the TSTAs of chemically induced tumours may arise because of pre-existing antigenic diversity of normal cells (Burnet, 1964). According to this view, transformation leads to clonal expansion of cell populations that were originally marked by distinct antigens. Equally possible, is the alternative that unique TSTAs arise as a consequence of mutational events induced by chemical carcinogens (Srivastava et al., 1987). Whether the antigenic diversity originates pre- or post- transformation, there is likely to be some mechanism for its generation. The mechanism may be similar to that for generation of antibody diversity, which involves somatic mutational events and rearrangements of the cellular genome (Honjo, 1983; Roitt, 1984).

The TSTAs of two methylcholanthrene-induced tumours, Meth A and CI-4, have been purified (DuBois et al., 1982; Dubois and Law, 1986) and have been shown to be similar to eachother and to a TSTA purified from the SV40-induced mKSA sarcoma (DuBois, 1984). All are acidic proteins of 75-86kDa, and are predominantly cytosolic, although they are present at the cell surface (DuBois, 1984). They are not glycoproteins, viral antigens or histocompatibility antigens (DeLeo et al., 1977; Appella et al., 1978; DuBois et al., 1982, 1984). Despite their similarities, not one of these TSTAs offers cross-protection in in vivo tumour rejection assays (DuBois et al., 1982, 1984; DuBois and Law, 1986).

The Meth A TSTA consists of two phosphorylated polypeptide isoforms with similar MW (84kDa and 86kDa) and pI (Ullrich et al., 1986). Mouse HSP90 also exists as two isoforms and on two-dimensional gel electrophoresis these comigrate exactly with the two TSTA isoforms. The two isoforms of HSP90 are referred to as HSP84 and HSP86 (Moore et al., 1987). Both HSP84 and HSP86 are i.p. from normal and heat-shocked cells by antisera to Meth A TSTA. This evidence suggests that the Meth A TSTA is either related or identical to the mouse HSP90 (Ullrich et al., 1986). The authors state that they have determined the nucleotide sequence of a mouse HSP84 cDNA (Moore et al., 1987), yet from the preceding publication it appears that Meth A TSTA amino acid sequence data, not HSP90 data, was used to prepare the oligonucleotide probes for cDNA library screening (Ullrich et al., 1986). Although they may be closely related or perhaps even identical, a distinction between HSP90 and Meth A TSTA should be maintained in view of the subtle but important differences between different TSTAs.

(d) Antigenic changes due to differences in tumour cell physiology: Tumour-associated carbohydrate antigens are frequently altered in malignancy as a result of differences in tumour cell physiology. They include: (a) carbohydrates bound to ceramides inserted in the lipid bilayer (glycolipids and glycosphingolipids); and (b) carbohydrates bound to cell surface proteins (glycoproteins) like fibronectin (reviewed by Hakomori, 1984).

Glycolipid tumour antigens include blood group A-like antigen in human cancer (gastric, colonic and hepatic adenocarcinoma) of blood group O individuals. Blood group

antigens are present on blood cells and various epithelial cells and since the majority of human cancers are derived from epithelial cells, changes in blood group antigens are an important topic in human tumour immunology (Hakomori, 1984). The Forssman antigen is another glycolipid antigen which is expressed in a variety of human cancers (gastrointestinal, lung and breast). The enzyme activity for synthesis of the Forssman glycolipid has been found to be greatly increased for the majority of squamous carcinomas of the lung (Taniguchi et al., 1981).

The monoclonal antibody Ca1 which distinguishes between various malignant and non malignant human cells, is directed against a glycoprotein tumour antigen (Bramwell et al., 1983). The carbohydrate chains of this glycoprotein show no novel structure and it has been suggested that recognition by Ca1 occurs because the carbohydrate groups attached to the protein moiety are densely spaced (Bramwell et al., 1983).

In general, the dominant factors affecting the antigenicity and immunogenicity of tumour-associated carbohydrates are: (a) uniqueness of carbohydrate structures; (b) the density of distribution of carbohydrate chains; and (c) the degree of exposure of carbohydrate structures to the immune system (reviewed by Hakomori, 1984).

(e) Virus-induced antigens; i. RNA tumour viruses: The transforming gene of Rous sarcoma virus (RSV) encodes a 60kDa polypeptide designated $pp60^{v\text{-}src}$ (Brugge and Erikson, 1977; Purchio et al., 1978), which has protein kinase activity specific for tyrosine residues (Collett and Erikson, 1978). $pp60^{v\text{-}src}$ is detectable in RSV-transformed chicken cells and RSV-induced hamster tumour cells by immunoprecipitation of

radiolabelled cell extracts with serum from rabbits bearing RSV-induced tumours (Brugge and Erikson, 1977). $pp60^{v-src}$ forms a complex with two co-precipitating cellular proteins. One is a 50kDa phosphotyrosine containing protein which is a potential substrate for $pp60^{v-src}$ (Brugge and Darrow, 1982) and the other is the HSP90 (Opperman et al., 1981; Schuh et al., 1985; Sanchez et al., 1985). The purpose of complex formation with HSP90 is not known, but it may be involved in the regulation of activity, processing or transport of the $pp60^{v-src}$ (Schuh et al., 1985). Studies on RSV strains encoding mutant src gene products have shown that they induce a partially transformed phenotype, which suggests that $pp60^{v-src}$ must interact with multiple cellular targets to elicit a fully transformed phenotype (review, Sefton and Hunter, 1984).

Abelson murine leukaemia virus (A-MuLV) will transform bone marrow-derived lymphoid cells and the transformed cells express three different tumour antigens. These include a cellular developmental antigen, the p53 tumour antigen which is not associated with any other proteins (Rotter et al., 1981) and the virus encoded p120 protein described above (Levine, 1982). The cellular developmental antigen is also expressed in normal cells of the pre-B lymphocyte sub-class, which are the target cells of A-MuLV (Rosenberg and Baltimore, 1976). A-MuLV may only transform cells expressing this developmental antigen, perhaps because this plays an important role in oncogenesis.

MuLV infection is widespread in mice, as are antibodies to surface MuLV antigens (DeLeo et al., 1977; Nowinski and Kaehler, 1974). In one study on a series of BALB/c and C57BL/6 cell lines, 7/17 different cell lines expressed cell

surface antigens associated with MuLV (DeLeo et al., 1977). With the widespread occurrence of MuLV in the mouse the possible contribution of MuLV antigens must always be considered in interpreting results of immunological analysis of mouse cells whether normal or malignant (DeLeo et al., 1977). RE cells derived from some breeds of rat eg. Sprague Dawley, have high levels of endogenous C-type viruses readily detected by reverse transcriptase assays (Rasheed et al., 1976). The colony of Hooded Lister rats used to provide RE cells for the author's experiments, was previously tested for endogenous C-type virus infection and no evidence of infection was found. Cells were assayed for reverse transcriptase after both infection and transformation by HSV and Sprague Dawley RE cells were used as a positive control (A. Bunce, J.C.M. Macnab, and I. Pragnell, unpublished results). Hooded Lister RE cells were also examined by electron microscopy and no C-type virus particles were found (Macnab, unpublished results).

(ii) DNA tumour viruses: Viruses like SV40, adenovirus and EBV express immunogenic viral proteins in the cells they transform (see section 1.2.2).

1.3.3 Tumorigenicity, immunogenicity and resistance to natural killer cell lysis

Transformed cells do not need to be resistant to either cellular or humoral immune responses when growing in tissue culture, but they do require such resistance if they are to survive and produce tumours following injection into an animal. Perhaps as a consequence, many transformed cell lines fail to produce tumours in vivo, despite showing the

characteristic transformation-related properties in vitro (Lewis and Cook, 1985).

The oncogenicity of transformed rat cell lines induced by different adenoviruses has been correlated with loss of expression of a 45kDa cellular polypeptide, identified as the class I major histocompatibility complex (MHC) heavy chain (Schrier et al., 1983). Tumour cell lysis by cytotoxic T cells is MHC-restricted (Zinkernagel and Doherty, 1979) and therefore tumour cells expressing viral antigens unaccompanied by class I MHC antigens, will escape immune surveillance by cytotoxic T cells (Schrier et al., 1983). Cells transformed by the highly oncogenic adenovirus 12 do not express the 45kDa polypeptide which may be an effect of the virus, or may be due to viral transformation of only those cells lacking the class I MHC (Schrier et al., 1983).

There is evidence that the tumorigenicity of transformed cells depends on their resistance to lysis by natural killer (NK) cells. A direct correlation has been shown between resistance of transformed cell lines to lysis by NK cells and their ability to induce tumours in immunocompetent animals (Cook, 1982; Raska and Gallimore, 1982). By the use of hybrids between SV40-transformed and adenovirus type 2-transformed cells, it has been possible to show that adenovirus early gene expression governs the cytolytic susceptibility of the hybrid cells (Cook et al., 1983). It has been suggested that one of the functions of the early gene products of DNA tumour viruses might be to regulate the level of susceptibility of virus-induced neoplasms to the host's immune defences (Lewis and Cook, 1985). The expression of immunogenic viral tumour antigens may be a viral mechanism for protecting the host, in case oncogenesis should occur

"accidentally" following infection. Thus, certain viruses may actively participate in the destruction of any tumour cells which they induce, by the expression of tumour antigens which can be recognized by the host's immune surveillance system.

1.4 Stress proteins

Stress proteins will be defined here as cell proteins produced in response to a variety of stressful stimuli including exposure to raised temperature (heat-shock proteins) and glucose deprivation (glucose regulated proteins). Raised levels of some stress proteins have been detected in virus infected cells and in transformed and tumour cells, suggesting that they may have a role in viral oncogenesis.

1.4.1 The heat-shock response

The response of cells to raised temperature is termed the heat-shock response. Heat-shock proteins (HSPs) expressed in this response are highly conserved indicating an essential function. They have been observed in most organisms and cell types examined to date including bacteria, protozoans, yeasts, higher plants, Drosophila and mammalian cells (reviewed by Schlesinger, 1982a). The major species of HSP in mammalian cells are in three size classes of 80-90kDa, 70-73kDa and 27-28kDa (Hickey et al., 1986). Much attention has been focussed on the 70-73kDa proteins which are generally the most abundant. In Drosophila and yeast cells a multigene family related to HSP70 has been identified (Ignolia and Craig, 1982; Ignolia et al., 1982). The HSP70-related genes in this family are referred to as cognate genes. In most mammalian cells there appear to be two forms of HSP70; a constitutive 73kDa

form present at appreciable levels under normal conditions and an induced 72kDa form which is only prominent after exposure to physiological stress. Peptide mapping and immunological studies show that 72kDa and 73kDa forms are similar but not identical (Welch and Feramisco, 1984).

(a) Regulation of the heat-shock response: Expression of HSP70 is controlled at both transcriptional and translational levels (Lindquist, 1981; Schlesinger et al., 1982a). Deletion analysis of the Drosophila HSP70 gene established that sequences between -10 and -66 nucleotides upstream from the RNA start site are essential and sufficient (in these assay systems) for heat-shock induction (Pelham, 1982; Mirault et al., 1982). This region contains a TATA box and a short conserved sequence element found in a similar location in most Drosophila heat-shock genes (Pelham, 1982). The consensus sequence is protected in footprinting experiments by a transcription factor which shows increased activity in heat-shocked cells (Parker and Topol, 1984).

HSP70 mRNA is efficiently translated during heat-shock, while most pre-existing cellular mRNAs are poorly translated. This preferential translation of HSP mRNA is at least in part due to features of the 5' noncoding region of the mRNA (Bonner et al., 1984; McGarry and Lindquist, 1985). In addition, the stability of HSP70 mRNA in heat-shocked cells appears to be greater than in control cells (Theodorakis and Morimoto, 1987).

(b) Developmental activation of HSP genes: In addition, to transient induction by various stresses, HSPs and related proteins are expressed at certain stages of development. Two

HSP70 related proteins are induced during the sporulation of yeast (Kurtz and Lindquist, 1984). Several HSPs are induced during oogenesis in Drosophila, including HSP26 and HSP84 (Zimmerman et al., 1983) and an HSP70-like protein is one of the first proteins synthesised in mouse embryogenesis (Bensaude et al., 1983). HSPs and related cognate proteins may therefore have important functions in development as well as in the response to stress.

(c) HSP induction by viruses and oncogenes: HSP70 is induced in eukaryotic cells by SV40, polyomavirus infection (Khandjian and Türler, 1982), adenovirus (Nevins, 1982) and HSV infection (Notarianni and Preston, 1982). Expression of the adenovirus E1A gene induces HSP70 at the transcriptional level (Nevins, 1982; Kao and Nevins, 1983). HSV infection also induces HSP90 (Notarianni and Preston, 1982).

Several of the E.coli HSPs induced on Bacteriophage lambda infection, are used by the virus for its own replication (Friedman et al., 1984). In the same way, eukaryotic HSPs induced by viruses may be used in viral replication, or their induction may just reflect the stress of infection. Induction of HSP70 by the adenovirus E1A gene product, argues for a positive role of HSP70 in the viral life-cycle (Schlesinger, 1986). In 293 cells, which are an adenovirus type 5 transformed human cell line, there is a very high level of expression of HSP70 (Nevins, 1982; Kao and Nevins, 1983). The expression of HSP70 and E1A genes fluctuate during the cell cycle, with peaks of E1A expression just prior to peaks of HSP70 expression. This implies that in 293 cells, the E1A gene product may regulate the HSP70 gene in a cell cycle-specific manner (Nevins, 1986). The expression

of the myc oncogene may also activate the HSP70 gene (Kingston et al., 1984).

1.4.2 Functions of major HSPs

Heat-shock proteins (HSPs) are produced when cells are stressed by exposure to raised temperature and a wide range of seemingly unrelated stimuli like amino acid analogues, arsenite and hydrogen peroxide. Most inducing stimuli are either known or thought to cause denaturation of cellular proteins, as indicated in table 1.3 (review, Ananthan et al., 1986). The physiological function of the heat-shock response is still unclear, but it has been shown for a variety of cell types that brief exposure to elevated temperatures renders them more resistant to further thermal insult (Landry et al., 1982; Tanguay, 1983).

(a) HSP70 functions: A number of HSP70 functions have been identified including: (i) binding to nucleolar proteins following heat induced damage (Lewis and Pelham, 1985), (ii) binding to a number of proteins including the p53 tumour antigen (Pinhasi-Kimhi et al., 1986), and (iii) disruption of the clathrin coat of coated vesicles (Ungewickell, 1985).

The binding activities are ATP-reversible and disruption of the clathrin coat of vesicles is ATP-dependent. A general model formed on the basis of these findings is that HSP70 binds to hydrophobic regions of proteins which are either naturally exposed or revealed because of faulty synthesis or denaturation. HSP70 binding prevents or disrupts inappropriate protein-protein interactions and reversal of binding requires the hydrolysis of ATP (Lewis and Pelham, 1985; Pelham, 1986). Hence, HSP70 may have a "chaperoning"

Inducing agent/treatment	**Proposed effects**
heat-shock	increased unfolding of proteins
amino acid analogs (eg.puromycin)	abnomal proteins
arsenite, iodoacetamide	binding to sulfhydryl groups, conformational changes in proteins
ethanol	translation errors
return from anoxia, hydrogen peroxide, superoxide ions	oxygen toxicity, free radical fragmentation of proteins
ammonium chloride	inhibition of proteolysis
hydroxylamine	cleavage of asparagine-glycine bonds in proteins

Table 1.3; Agents or treatments that activate HSP genes, and their proposed effects. (Ananthan et al., 1986).

role, binding to incorrectly or incompletely folded proteins and preventing their aggregation (Pelham, 1986).

Newly synthesised secretory and organellar proteins need to be maintained in an unfolded state for translocation across membranes (Eilers and Schatz, 1986). Post- translational transport of some mitochondrial and secretory proteins requires both cytosolic factor(s) and ATP hydrolysis, which perhaps indicates the need for an ATP-dependent "unfoldase" to create and maintain the loosely folded configuration of the precursor proteins (Eilers and Schatz, 1986; Rothman and Kornberg, 1986).

The ATP-reversible protein binding properties of HSP70 suggest that it might be such a protein. Recent studies indicate that HSP70 is a cytosolic factor involved in translocation of proteins across membranes. In one study on translocation, an *in vitro* assay was used which involved the uptake of a yeast protein (prepro-alpha-factor) into microsomal vesicles (Chirico *et al.*, 1988). An activity was purified from yeast cytosol which stimulates protein translocation across the microsomal membranes and was found to consist of two constitutively expressed members of the HSP70 family (Chirico *et al.*, 1988). Substrate which had been denatured with urea was translocated 10X faster in this assay, indicating that conformation influences translocation (Chirico *et al.*, 1988).

The role of HSP70 *in vivo* has been studied using nonviable strains of yeast with mutated HSP70 genes, rescued by plasmids expressing the HSP70 gene placed under the control of the GAL1 promoter (Deshaies *et al.*, 1988). In this system the HSP70 gene is repressed when glucose is added to the culture. Effects of HSP70 depletion that were detected

include accumulation of prepro-alpha factor and of a mitochondrial F_1ATPase subunit. This implies that HSP70 has a physiological role in the import of proteins into endoplasmic reticulum and mitochondria (Deshaies et al., 1988). However, the exact function of HSP70 and whether it interacts directly with the translocated protein has not yet been shown.

(b) Interactions of HSPs with oncogene products: Complex formation has been detected between p53 and the constitutive 73kDa form of HSP70 in a cell line derived by co-transformation of RE cells with murine p53 and activated Ha-ras, but similar complexes were not detected in normal cells (Pinhasi-Kimhi et al., 1986; Hinds et al., 1987). It has been suggested that HSP70 may stabilize p53 in the same way as the SV40 large T antigen (Pinhasi-Kimhi et al., 1986). A mutant p53 cDNA clone which yields up to thirty five fold more transformed cell foci when co-transfected with Ha-ras, encodes a product that binds the constitutive HSP70 more effectively than non-mutant p53 (Hinds et al., 1987). This suggests that formation of this complex could have functional significance in transformation. HSP70 might therefore be involved in cell proliferation, which is in keeping with the apparent cell cycle dependance of the expression of the human HSP70 gene (Kao et al., 1985).

The RSV transforming oncogene $pp60^{v\text{-}src}$ forms a complex with two co-precipitating cellular proteins in RSV transformed cells. One is a 50kDa phosphotyrosine containing cellular protein which is a potential substrate for $pp60^{v\text{-}src}$ (Brugge and Darrow, 1982) and the other is the HSP90 (Opperman et al., 1981; Schuh et al., 1985; Sánchez et al., 1985). HSP90 and the 50kDa polypeptide also co-precipitate in i.p.s of v-fps,

v-yes, v-ros and v-fes oncogene products which all have tyrosine protein kinase activity (Brugge et al., 1983). In chick cells transformed by nondefective strains of RSV the complex between $pp60^{v\text{-}src}$, HSP90 and the 50kDa polypeptide, has a half life of 9 to 15 min. (Brugge et al., 1983). However, when cells were infected with a src gene ts mutant of RSV and grown at NPT, the half-life of the complex was increased to well over 3 h. Thus, the transforming activity of $pp60^{v\text{-}src}$ is reduced by a mutation in the v-src oncogene, a mutation which also increases the stability of complexes between $pp60^{v\text{-}src}$ and two cellular polypeptides (Brugge et al., 1983). The purpose of $pp60^{v\text{-}src}$-HSP90 complex formation is not known, but may be involved in transport or processing of the oncogene product or in the regulation of its activity (Schuh et al., 1985).

(c) Interaction of HSP90 with steroid receptors:

Non-transformed (non-activated) steroid receptors have a sedimentation coefficient of 8 Svedberg units (S) and a MW of 250-300kDa (Gorski et al., 1968; Baulieu et al., 1971). When in the 8S form, chick oviduct steroid hormone receptors (for progesterone, oestrogen, androgen and glucocorticosteroid) all contain a 90kDa non-hormone binding protein (Joab et al., 1984; Groyer et al., 1985). Transformation (i.e. activation) of the progesterone receptor includes acquisition of DNA binding properties, enhanced hormone binding and conversion to the 4S form of the receptor. This implies separation of the 90kDa protein from the steroid binding component (Wolfson et al., 1980). In chick oviduct cells the 90kDa component of the untransformed progesterone receptor has been shown to be the HSP90 (Catelli et al., 1985). In addition, the untransformed

glucocorticoid receptor of L-929 mouse fibroblasts appears to contain the HSP90 (Sanchez et al., 1985).

As in the case of the $pp60^{v\text{-}src}$, steroid receptor complexing with HSP90 may be important in receptor transport or processing, or may regulate activity by keeping the steroid receptor in its inactive form (Catelli et al., 1985; Schuh et al., 1985).

(d) HSP90 and TSTAs: Murine HSP90 appears very similar if not identical to a TSTA of a methylcholanthrene-induced sarcoma (Ullrich et al., 1986). Thus, another function of members of the HSP90 family may be to act as a TSTA (see section 1.3.2.

1.4.3 Ubiquitin

Ubiquitin is an 8kDa heat-shock inducible polypeptide (Bond and Schlesinger, 1985) which appears to play a role in chromatin structure and in ATP-dependent proteolytic degradation of unstable cellular proteins (Busch and Goldknopf, 1981; Hershko, 1983).

The amino acid sequence of ubiquitin is very highly conserved in eukaryotic cells indicating an important function (Ozkaynak et al., 1984). It has been found as part of an unusual Y-shaped protein species in which the C-terminal glycine residue of ubiquitin is joined to the epsilon-amino group of an internal lysine residue of histone H2A (Busch and Goldknopf, 1981; Levinger and Varshavsky, 1982). Another unusual feature of ubiquitin is that it is translated as a polyprotein precursor which is cleaved proteolytically to generate the mature protein. The number of ubiquitin coding units varies from six in yeast (Ozkaynak et al., 1984) to nine

in man (Wiborg et al., 1985).

There are two distinct types of ubiquitin-protein conjugate detected from studies with reticulocyte extracts: : (i) ubiquitin joined at the epsilon amino group of internal lysine residues to give Y-shaped molecules (Busch and Goldknopf, 1981; Hershko and Ciechanover, 1982), and (ii) ubiquitin joined at the N-terminal amino group of the substrate protein (Hershko et al., 1984). Currently available indirect evidence is consistent with N-terminal ubiquitination being both necessary and sufficient for ubiquitin-dependent selective degradation of protein substrates (Hershko et al., 1984). N-terminal acetylation appears to block ubiquitination both in vivo and in vitro and may therefore have a physiological role in protecting polypeptides from proteolysis (Hershko et al., 1984).

The mouse mutant cell line ts85 is defective with respect to ubiquitination and studies using ts85 suggest that the turnover of most short-lived proteins in higher eukaryotes, proceeds through a ubiquitin-dependent pathway (Ciechanover et al., 1984).

1.4.4 A TG7A-recognized bacterial HSP is a proteolytic enzyme

The monoclonal antibody TG7A recognizes the 94kDa Lon gene product of E.coli, La protease (Chung & Goldberg., 1981), which is induced by heat-shock and bacteriophage T7 infection (Latchman et al., 1987). TG7A also recognizes mammalian cell polypeptides of approximately 90kDa which are induced by heat-shock and HSV infection (Latchman et al., 1987) and polypeptides of similar MW, present at raised levels in immortalized cells (Macnab et al., 1985a). The significance of these cross-reacting epitopes is heightened by the fact

that the bacterial and eukaryotic polypeptides recognized by TG7A are both induced by heat-shock treatment and viral infection (Latchman et al., 1987). This suggests that the TG7A-recognized epitope is highly conserved and may be functionally important.

TG7A recognition of an abundant polypeptide of immortalized eukaryotic cells (Macnab et al., 1985) is particularly interesting in the light of evidence suggesting that La protease is a regulator of bacterial cell division (Mizusawa & Gottesman, 1983). La protease proteolytically degrades the sulA protein which is an inhibitor of septation (Charette et al., 1981., Chung & Goldberg., 1981). La protease action is ATP-dependent and as ATP concentration increases in the cell cycle towards the time of cell division, it has been suggested that the degradation of a division inhibitor by La protease may increase simultaneously (Charette et al., 1981). Hence, La protease may regulate cell division by controlling the stability of the sulA protein (Mizusawa and Gottesman, 1983).

It is interesting to note that ubiquitin, a eukaryotic heat-shock protein, functions in proteolysis as does La protease (Hershko et al., 1984). Further, it has recently been shown that the HSV-1 IE protein Vmw175, specifically induces transcription of the human ubiquitin B gene (Kemp and Latchman, 1988). These findings suggest that the protein recognized by TG7A in heat-shocked and HSV-infected mammalian cells may be either ubiquitin, a ubiquitinated protein or a protein related to ubiquitin.

Heat-shock genes of E. coli are under the control of the htpR gene product, sigma^{32} which is a short half-life RNA polymerase sigma factor (Grossman et al., 1984; Goff et al.,

1984; Goff and Goldberg, 1985). The production of abnormal proteins in E. coli following treatment of cells with canavanine or puromycin stimulates the transcription of Lon and other heat-shock genes (Goff and Goldberg, 1985). It has been suggested that abnormal proteins may compete with a positive regulator of heat-shock genes (e.g. sigma^{32}) for degradation by proteolytic enzymes (Goff and Goldberg, 1985). By increasing the level of the positive regulator, heat-shock gene expression would be increased. A similar model for regulation of eukaryotic heat-shock gene transcription has been proposed (Ananthan et al., 1986). In support of this model the mouse cell line, ts85, which is ts in both ubiquitin-protein conjugation and the degradation of abnormal proteins, produces HSPs at elevated rates on temperature-shift up to the NPT (Finley et al., 1984). Further, HSP gene activation occurs in the absence of protein synthesis (Ashburner and Bonner, 1979) and genes are positively regulated by binding of a heat-shock gene-specific factor to defined promoter sequences in HSP genes (Wu, 1984).

Proteolytic mechanisms involving heat-shock proteins may therefore be important in regulation of prokaryotic and eukaryotic cell division. The sigma^{32} factor of E. coli and p53 of mammalian cells, are both metabolically unstable proteins and both have been implicated in growth control. The stabilization of p53 against selective degradation, probably involving the ubiquitin system, has been associated with the neoplastic transformation of eukaryotic cells (Croy and Pardee, 1983; Oren and Levine, 1983).

1.4.5 Glucose regulated proteins

Glucose regulated proteins (GRPs) are a class of stress

proteins similar to HSPs. The GRPs are cellular proteins synthesised constitutively at detectable levels under normal tissue culture conditions (Hightower and White, 1982). The two major GRPs observed in mammalian and avian species have MWs of 78-80kDa and 94-100kDa (Shiu et al., 1977; Pouyssegur et al., 1977; Lee, 1981). Their level is markedly increased when cells are starved of glucose or exposed to glycosylation inhibitors such as 2-deoxyglucose, glucosamine or tunicamycin (Pouyssegur et al., 1977; Lee, 1981). In addition, treatment of chicken and rat cells with the calcium ionophore A23187 has been reported to increase the level of the two major GRPs (Wu et al., 1981; Welch et al., 1983).

Chick embryo fibroblasts (CEF) transformed by various strains of Rous sarcoma virus (RSV) show a significant increase in two membrane associated proteins of 78 and 95kDa in MW (Stone et al., 1974). These proteins were originally thought to be transformation- specific, but were later shown to be induced by low glucose concentration, due to rapid glucose utilization by transformed cells (Shiu et al., 1977; Peluso et al., 1978). Due to the influence of glucose on the level of these proteins, they have become known as the glucose regulated proteins (GRP) and are designated GRP78 and GRP95 according to MW (Shiu et al., 1977).

GRP78 is related to HSP70 and is found within the endoplasmic reticulum. GRP78, also called BiP, associates in an ATP-reversible manner with immunoglobulin heavy chains (Pelham, 1986). GRP78 only binds to the test substrate yeast invertase if the enzyme has not been glycosylated, which suggests that GRP78 interacts preferentially with incorrectly processed proteins, in a similar manner to the related HSP70 (Kassenbrock et al., 1988).

While HSPs and GRPs are distinct protein species, they share some homologies and are both induced by a variety of stresses. Under some conditions the induction and suppression of these two sets of proteins bear a reciprocal relationship. For example, while prolonged oxygen deprivation triggers the synthesis of GRPs, recovery from oxygen deprivation triggers synthesis of HSPs (Sciandra et al., 1984). Similarly, low extracellular pH induces GRPs, while high extracellular pH induces HSPs (Whelan and Hightower, 1985). These observations imply that cells respond to different adverse conditions by synthesising different sets of stress proteins.

2 MATERIALS

2.1 Animals

Hooded Lister rats from a closed colony maintained at the Institute of Virology for over 20 years, were used to prepare rat embryo (RE) fibroblast cultures and tumour bearing serum (TBS).

2.2 Cells

RE fibroblast cultures were prepared from 16-20 day sibling embryos. Primary or secondary RE cultures were used. Bn5 cells are RE cells transformed by transfection with the BglIIn clone of the HSV-2 (HG52 strain) genome, which encodes the small subunit of the ribonucleotide reductase. The tumour cell line Bn5T was cultured from tumours induced by Bn5 cells (Cameron et al.,1985). The Hood cell line was one of several obtained by continuous passage of RE cells in culture (Macnab, 1979). These cell lines were provided by Dr Macnab.

Rat-1 and Rat-2 are continuous contact inhibited rat cell lines and LA24 cells are Rat-1 cells transformed by a temperature-sensitive (ts) mutant of RSV. These cell lines were provided by Dr J. Wyke (Department of Oncology, St. Bartholomew's Hospital, London), LA90 cells are Rous sarcoma virus (RSV) transformed mouse 3T3 cells and were supplied by Dr J. Brugge (Department of Microbiology, Stony Brook, New York, USA).

The embryonal carcinoma cell line PC1315 and in vitro differentiated cell lines EB28/5, EB28/10n, EB26/1A and EB37/19D (Morgan et al.,1983) were provided by Dr M.L.Hooper (Department of Pathology, University Medical School, Edinburgh).

Baby hamster kidney (BHK) cells, established by Macpherson and Stoker (1962) and maintained in this Institute,

were used in preparation and titration of virus stocks.

2.3 Virus

Virus stocks were supplied by Mrs M. Murphy (Institute of Virology, Glasgow). Wild-type viruses used included HSV-2 strain HG52 (Timbury, 1971), and HSV-1 strain 17 syn^+ (Brown et al., 1973). The ts mutant of HSV-1 strain 17, tsK, was initially isolated in the syncytial (syn) plaque morphology by Crombie (Marsden et al., 1976). The tsK syn^+ form was produced by Dr V.G.Preston (Institute of Virology, Glasgow).

2.4 Tissue culture media and solutions

Cells were grown in Glasgow modified Eagle's medium, supplemented with 100 units/ml penicillin and 100ug/ml streptomycin all supplied by Gibco Ltd., Paisley, Scotland, Mycostatin (Squibb & Sons, Houndslow, Middlesex, England) was added to a concentration of 10U/ml. The following media and solutions were used for tissue culture:

(a) EFX: Eagle's medium containing X% foetal calf serum and 1%(v/v) non-essential amino acids, both from Gibco Ltd.

(b) EF2-met: Eagle's minus methionine, prepared at the Institute of Virology, Glasgow.

(c) EF2-Pi: Eagle's minus phosphate, prepared at the Institute of Virology, Glasgow.

(d) ETCX: Eagle's medium containing 10%(v/v) tryptose phosphate broth (Difco Laboratories, West Molesley, England) and X% calf serum, prepared at the Institute of Virology using blood obtained from the abattoir in Dumfries, Scotland.

(e) EHuX: Eagle's medium containing X% pooled human serum prepared at the Institute of Virology using pathogen-free blood obtained from the Blood Transfusion Service, Glasgow.

(f) Versene: 0.6mM disodium ethylenediaminetetraacetate (EDTA) dissolved in phosphate buffered saline (PBS) containing 0.002%(w/v) phenol red.

(g) Trypsin: Trypsin (Difco Laboratories, West Molesley, England), was used as a 0.25%(w/v) solution in tris-saline.

2.5 Chemicals

Analytical reagent grade chemicals were supplied by the following companies:

(a) Aldrich Chemical Company, Gillingham, Dorset, England: N,N'- diallyltartardiamide (DATD)

(b) BCL, Boehringer Mannheim, Lewes, E. Sussex, England: calf thymus transfer RNA (tRNA)

(c) BDH Chemicals Ltd., Poole, Dorset, England: ammonium bicarbonate, bromophenol blue, disodium ethylenediamine-tetraacetate (EDTA), disodium hydrogen phosphate (Na_2HPO_4), glycine, magnesium chloride ($MgCl_2$), 2-mercaptoethanol, potassium chloride (KCl), sodium chloride (NaCl), sodium deoxycholate, sodium dihydrogen phosphate (NaH_2PO_4), sodium dodecyl sulphate (SDS), tris(hydroxymethyl)aminomethane (Tris)

(d) Bethesda Research Laboratories (UK) Ltd., Cambridge, England: sucrose (ultra-pure)

(e) Biorad Laboratories, Richmond, California, USA: ammonium persulphate, Coomassie brilliant blue R-250, N,N,N',N'-tetra -methylethylenediamine (TEMED)

(f) Fluka Chemie Ag., CH-9470, Buchs, Switzerland: guanidinium isothiocyanate

(g) James Burroughs Ltd., London, England: ethanol

(h) Koch-Light Laboratories, Haverhill, Suffolk, England: acrylamide, cyanogen bromide, dimethylsulphoxide (DMSO), L-methionine, trichloracetic acid (TCA)

(i) May & Baker Ltd., Dagenham, Essex, England: acetic acid (glacial), chloroform, hydrochloric acid, methanol

(j) Pharmacia Ltd., Milton-Keynes, England: Ficoll (type 400)

(k) Pierce and Warriner, Chester, England: trifluoracetic anhydride (TFA)

(l) Rathburn Chemicals Ltd., Walkerburn, Scotland: acetonitrile (HPLC grade)

(m) Sigma Chemical Company Ltd, London, England: agarose (type 1), antifoam A, benzamidine, cycloheximide, diethylpyrocarbonate (DEPC), heparin, methionine, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), phenanthroline, phenylmethylsulphonyl fluoride (PMSF), potassium thiocyanate, protein-A sepharose CL-4B, Sarkosyl, triton X-100

2.6 Standard buffer solutions

(a) General use:

phosphate buffered saline (PBS)	0.17M NaCl, 3.4mM KCl, 1mM Na_2HPO_4, 2mM KH_2PO_4 pH7.2

(b) Total cell RNA preparation:

lysis buffer	4M guanidinium isothiocyanate, 100mM 2-mercaptoethanol, 0.5%(w/v) Sarkosyl, 0.33%(v/v) antifoam A, 2mM EDTA, 50mM

	tris-Cl pH 7.5
extraction buffer	0.5%(w/v) SDS, 5mM EDTA, 10mM tris-Cl pH7.4

(c) Cytoplasmic RNA preparation:

lysis buffer	0.14M NaCl, 2mM $MgCl_2$, 0.5% NP40, 10ug/ml cycloheximide, 0.2M tris-Cl pH8.5
TSE	0.5% SDS, 5mM EDTA, 10mM tris-Cl pH8.5

(d) Oligo(dT)-cellulose chromatography:

binding buffer	0.5M NaCl, 1mM EDTA, 10mM tris-Cl pH7.5
elution buffer	1mM EDTA, 10mM tris-Cl pH7.5

(e) Polysome preparation and immunoselection:

buffer A^-	25mM NaCl, 5mM $MgCl_2$, 1ug/ml cycloheximide, 0.2mg/ml heparin, 25mM tris-Cl pH7.5
resuspension buffer	0.15M NaCl, 5mM $MgCl^2$, 1ug/ml cycloheximide, 0.2mg/ml heparin, 10mM HEPES pH7.6
elution buffer	20mM EDTA, 0.2mg/ml heparin, 20ug/ml calf thymus tRNA, 25mM tris-Cl pH7.4

(f) DNA studies:

Hind III restriction enzyme buffer	50mM NaCl, 10mM $MgCl^2$, 50mM tris-Cl pH8.0
TBE buffer (10X)	8.9mM boric acid, 0.3mM EDTA, 8.9mM tris-Cl pH8.3
Minigel sample buffer	5X TBE buffer containing 10%(w/v) Ficoll, 0.2%(w/v) bromophenol blue, 100mM EDTA
Alkaline electrophoresis buffer	30mM NaOH, 1mM EDTA
Alkaline loading buffer	50mM NaOH, 1mM EDTA, 2.5% Ficoll, 0.025% bromophenol blue

(g) Immunoprecipitation assays:

RIPA	0.1%(w/v) SDS, 1%(w/v) sodium deoxy-cholate, 1%(v/v) triton X-100, 1mM

	$MgCl_2$, 150mM NaCl, 10mM tris-Cl pH7.4
tris-saline	140mM NaCl, 30mM KCl, 280mM Na_2HPO_4, 1mg/ml glucose, 25mM tris-Cl pH7.4

(h) Peptide mapping:

rehydration buffer	0.1%(w/v) SDS, 1mM EDTA, 0.125M tris-Cl pH6.8

(i) SDS-Polyacrylamide gel electrophoresis (SDS-PAGE):

electrophoresis tank buffer	0.1%(w/v) SDS, 53mM glycine, 53mM tris
stacking gel buffer (final concentration)	0.1%(w/v) SDS, 0.125M tris-Cl pH6.7
separating gel buffer (final concentration)	0.1%(w/v) SDS, 0.375M tris-Cl pH8.9
electrophoresis sample buffer	2%(w/v) SDS, 10% glycerol, 700mM 2-mercaptoethanol, 50mM tris-Cl pH6.7, 0.001%(w/v) bromophenol blue

(j) Electrophoretic transfer of polypeptides to immobilon membranes:

transfer buffer	0.05%(w/v) SDS, 10mM phosphate buffer pH6.8

2.7 Radiochemicals

The following radiolabelled compounds were obtained from Amersham International plc., Amersham, Buckinghamshire, England:

alpha-^{32}P-deoxyadenosine triphosphate (dATP)	> 3,000Ci/mmol
2,6 ^{3}H-mannose	30-60Ci/mmol
^{35}S-methionine	> 800Ci/mmol
^{131}I	200mCi/ml
^{32}P-orthophosphate	10mCi/ml

2.8 Enzymes

Hind III and Klenow fragment from Bethesda Research Laboratories (UK) Ltd, Cambridge, England.

RNase A from Sigma Chemical Company Ltd., London.

V8 protease and chymotrypsin were from Miles Scientific, Naperville, Illinois, USA.

Trypsin from Worthington Biochemicals Corporation, New Jersey, USA.

2.9 Immunological reagents

Protein-A sepharose from Sigma Chemical Company Ltd., London.

Rabbit anti-mouse immunoglobulin from Sera-lab, Crawley Down, W.Sussex, England.

TG7A monoclonal antibody was supplied by Dr N.B. La Thangue (NIMR, Mill Hill, London). TG7A hybridoma was prepared using mice immunized with DNA-binding proteins from HSV-2 infected cells (Macnab et al., 1985a).

AC88 monoclonal antibody was supplied by Dr D.O.Toft (Department of Biochemistry, Mayo Clinic, Rochester, Minnesota, USA). AC88 monoclonal is directed against an 88kDa polypeptide from the aquatic fungus Achyla ambisexualis. This 88kDa polypeptide complexes with steroid receptors as does the mammalian 90kDa heat-shock protein which is also recognized by AC88 (Riehl et al., 1985).

2.10 Miscellaneous materials

Eppendorf reaction vials: Starstedt Ltd., Leicester, England.

Falcon plastic centrifuge tubes, 15ml and 50ml; Falcon rotating plastic 850 cm^2 culture bottles: Becton Dickinson Labware, New Jersey, USA.

Plastic tissue culture flasks: Nunclon Ltd., Roskilde, Denmark.

Plastic petri dishes, 50mm: Sterilin Ltd., Feltham, Middlesex, England.

Oligo(dT)-cellulose: Collaborative Research Inc., Lexington, Massachusetts, USA.

In vitro translation kit; Canine pancreatic microsomal membranes; Human placental ribonuclease inhibitor (HPRI): Amersham International plc., Amersham, Bucks, England.

Protein molecular weight standards: Bethesda Research Laboratories (UK) Ltd., Cambridge, England.

Silver staining kit: Bio-Rad Laboratories Ltd., Watford, Herts, England.

PD-10 columns containing Sephadex G-25 M: Pharmacia Fine Chemicals, Uppsala, Sweden.

Centricon microconcentrating tubes: Amicon Corp., Danvers, Massachusetts, USA.

Immobilon transfer membrane: Millipore Corp., Bedford, Massachusetts, USA.

Ecoscint scintillation fluid: National Diagnostics, Manville, New Jersey, USA

EN^{3}HANCE: New England Nuclear, Boston, Massachusetts, USA.

Photographic film: Kodak Ltd., London, England.

3 METHODS

3.1 Cell culture

3.1.1 RE cell cultures

Embryos were removed from a pregnant rat at 16-20 days gestation. They were eviscerated to remove blood forming organs, finely minced and washed in PBS. Fragments of tissue were trypsinized for 30 min. at 37°C, and remaining lumps of tissue trypsinized a further 30 min. Foetal calf serum was added to the suspension of dissociated cells to a concentration of 10%. Cells were pelleted by centrifugation at 1000 revolutions per min. (r.p.m.) in an MSE Coolspin, 10 min. at 4°C. They were resuspended in EF10 and counted in a haemocytometer. Cells were seeded in EF10 into rotating plastic 850cm^2 culture bottles at a density of 2X10^8 cells per bottle. Cells used for assays were harvested from culture bottles by washing monolayers twice with 20ml trypsin, resuspending the detached cells in EF5 and then pipetting up and down to dissociate cell aggregates. Cells were seeded in 50mm petri dishes at a density of 1.5X10^6 cells per dish and these secondary cultures used when subconfluent.

3.1.2 Cell lines

Cultures of Bn5T cells and other rat cell lines were grown at 37°C in rotating plastic 850cm^2 culture bottles, containing 150ml EF5. Culture bottles were split 3:1 when confluent. Cells were harvested as described above and if required for assays, seeded in 50mm petri dishes at a density of 2X10^6 cells per dish. For BHK cells ETC10 was the medium used and CO_2 added to culture bottles giving an atmospheric

concentration of 5%. Mouse embryonal carcinoma cell lines were harvested using a solution called TVP (0.025% trypsin, 1mM EDTA, 1% v/v chick serum) and were cultured in EF10.

3.1.3 Cell storage

Cells were harvested by trypsinization, pelleted by centrifugation and resuspended in EF15 containing 10%(v/v) DMSO, at a concentration of 10^7 cells/ml. Cells were aliquoted into black-cap vials, placed in an insulating polystyrene container for gradual cooling, stored overnight at -70°C and subsequently at -140°C.

For recovery cells were thawed rapidly and 5ml of EF10 added per ml of cell suspension to dilute the DMSO. Cells were grown in tissue culture flasks and culture medium changed the following day.

3.2 Production of virus stocks

Stocks of HSV-2 strain HG52 were prepared by infecting subconfluent BHK cells in 80oz bottles at a multiplicity of infection (m.o.i.) of approximately 0.003 plaque forming units (p.f.u.) per cell. Virus was absorbed for 1 h in 5ml ETC2 per culture bottle and cells incubated in 25ml ETC5 at 31°C for 3 days until extensive cytopathic effect (c.p.e.) had developed. Infected cells were harvested into the medium by shaking (glass beads added if necessary) and were pelleted by centrifugation at 1000 r.p.m. in an MSE Coolspin, 15 min. at 4°C. Cell associated virus was released by sonicating the cell pellet in 2 volumes of ETC5 and cell debris pelleted by a further low speed spin. The supernate was aliquoted in 1ml volumes and stored at -70°C. Sterility checks were performed routinely by streaking virus stocks on blood agar plates.

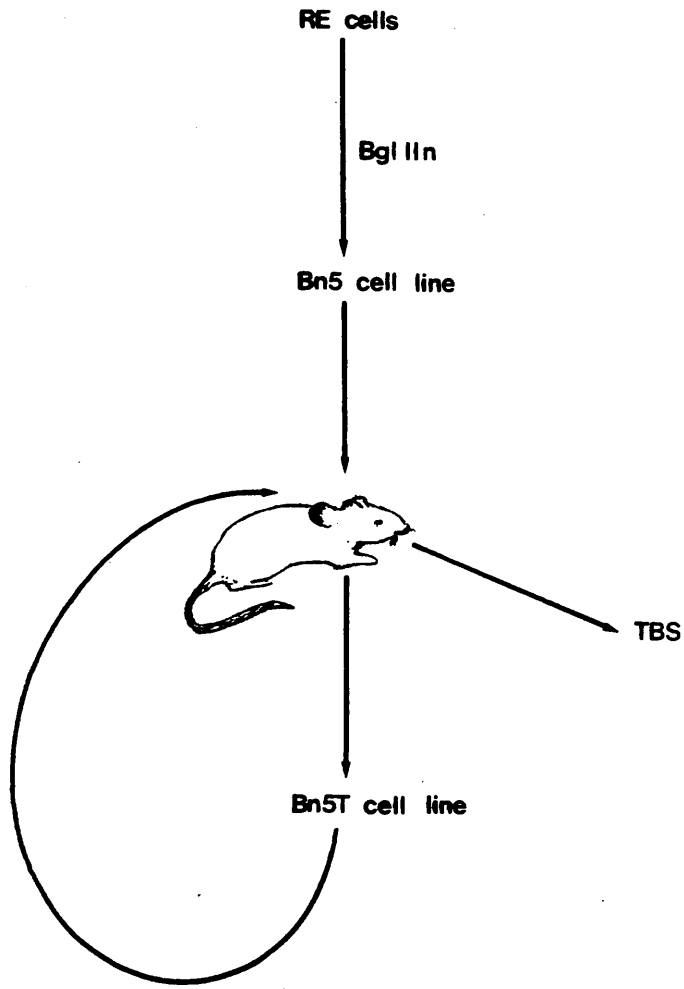
3.2.1 Titration of virus stocks

Virus was titrated on subconfluent monolayers of BHK cells in 50mm petri dishes. Serial ten-fold dilutions of virus were made in complete PBS containing 5% calf serum. For the different virus dilutions, inocula of 0.1ml were added to BHK monolayers from which growth medium had been removed. After adsorption of virus for 1 h at 37°C, cells were overlaid with EHu5 to neutralize unadsorbed virus and de novo synthesised virus to prevent formation of secondary plaques. Plates were incubated at 31°C for 3 days. Monolayers were then overlaid with Giemsa stain for 10 min., the stain washed off with water and virus plaques counted under a dissecting microscope.

3.3 Preparation of tumour bearing serum

To prepare tumour bearing serum (TBS), Hooded Lister rats were injected subcutaneously with Bn5T cells (fig 3.1). Adult rats (over 8 weeks) injected with $5X10^7$ cells developed palpable tumours within 1-2 months. Pre-weaned rats being immunologically immature, required fewer cells for tumour induction, but usually gave poorer antisera. Rats with palpable tumours were anaesthetized and bled by cardiac puncture. Blood was incubated in a 37°C waterbath for 1 h, which allowed clotting to occur. After incubation for 30 min. the clot was freed at the sides of the container with a 5ml pipette, to encourage its retraction. After incubation for 1 h the overlying liquid was transferred to a Falcon 15ml conical tube and centrifuged at 1000 r.p.m. in an MSE Coolspin, 5 min. at 4°C. Serum supernate was aliquoted and stored at -20°C.

Figure 3.1; The origins of Bn5T cells and tumour bearing serum (TBS). The Bn5 cell line was derived from RE cells by transformation with the BglIIn fragment of HSV-2 (Cameron et al., 1985). The Bn5T cell line was derived from a tumour induced in a rat by injection of Bn5 cells. TBS is obtained from rats bearing tumours induced by Bn5T cells.


RE cells
BglIIn
Bn5 cell line
TBS
Bn5T cell line

3.4 Preparation and analysis of RNA and polysomes

3.4.1 Preparation of total cellular RNA

A modification of the method of Chirgwin et al.(1979), was used in the preparation of total cellular RNA.

Monolayers of cells grown in rotating plastic 850cm^2 culture bottles, were washed 3X with cold PBS, then harvested by the addition of 5ml of lysis buffer to each culture bottle. One gram of caesium chloride was added to each 2.5ml of lysate and dissolved. The lysate was layered onto a 1.2ml cushion of 5.7M caesium chloride in a 12ml polyallomer tube and centrifuged at 35,000 r.p.m. on a Sorvall TST41 rotor, 18 h at 20°C. Following centrifugation the supernate was discarded and the RNA containing pellet dissolved in extraction buffer. This was extracted with an equal volume of a 4:1 mixture of chloroform and 1-butanol. The organic phase was extracted again with fresh extraction buffer, then the two aqueous phases were combined. Finally RNA in the aqueous phase was ethanol precipitated and then redissolved in DW for estimation of the RNA concentration.

(a) Ethanol precipitation: 0.1 volumes of 3M sodium acetate pH7.2 and 2.5 volumes of ethanol were added to the RNA containing solution and mixed thoroughly. After storage overnight at -20°C, RNA was pelleted by centrifugation at 10,000 r.p.m in a Sorvall SS34 rotor, 10 min. at 4°C. RNA was dissolved in distilled water and precipitated with sodium acetate and ethanol. The precipitating RNA was stored at -70°C until required. Before use, RNA was pelleted as before and the pellet lyophilized prior to dissolving in distilled water.

(b) **Estimation of RNA concentration:** The absorbance of an aqueous solution of RNA at 260nm was used to estimate concentration. An absorbance of 1.0 unit was taken to indicate an RNA concentration of 40ug/ml.

3.4.2 Preparation of cytoplasmic RNA

A method provided by Dr C. Preston was used to prepare cytoplasmic RNA. The steps described below, between cell lysis and the first phenol/chloroform extraction, were carried out very rapidly at 4°C. This minimizes RNA degradation by the cellular RNases which are released on cell lysis.

Burrlers of cells were washed 3X with cold PBS and scraped into 25ml PBS per burrler. Cells were pelleted in an MSE Coolspin at 1,000 r.p.m. for 5 min. at 4°C. Each pellet was resuspended in 5ml lysis buffer, pipetted up and down 4X through a wide bore 10 ml pipette and then centrifuged in an MSE Coolspin at 2,500 r.p.m. for 3 min. at 4°C. The supernate was then poured into a 100ml conical flask containing 5ml TSE, 10ml DW and 15ml of an equal mixture of phenol and chloroform at room temperature. The solutions were mixed rapidly and left for 5-10 min. with occasional mixing, after which they were transferred to a 50ml tube and centrifuged in an MSE Coolspin at 1,000 r.p.m. for 10 min. at 4°C. Subsequently the aqueous layer was transferred to another 100ml flask to which 10ml of phenol/chloroform was added. As before the solutions were mixed, left 10 min. and then spun to collect the aqueous phase. A third phenol/chloroform extraction was then carried out and the final aqueous phase was then extracted in the same way with 10ml of chloroform, but without phenol. RNA present in the final aqueous phase was ethanol precipitated as described in section 3.4.1.

3.4.3 Preparation of poly(A^+)mRNA

Poly(A^+)mRNA was prepared by oligo(dT)-cellulose column chromatography. Each column was washed with 10 bed volumes of binding buffer prior to use. An equal volume of 2X binding buffer was added to the sample solution which was then heated to 65°C and cooled to room temperature before loading. The flow-through was reloaded twice. The column was washed with 5 bed-volumes of binding buffer and poly(A^+)mRNA was then eluted with 2 bed-volumes of elution buffer. Poly(A^+)mRNA was ethanol precipitated and stored at -70°C until required.

3.4.4 Preparation of total cell polysomes

Polysomes were prepared by the magnesium precipitation technique of Palmiter (1974) with some modifications.

RNases are a major problem in studies involving polysomes. They are released on cell lysis and are difficult to remove completely from contaminated glasswear, and the following measures were therefore taken to minimize RNase contamination (Palacios et al., 1972; Shapiro and Young, 1981). All glassware was filled with 0.1% DEPC in water, incubated at 37°C overnight, then emptied and autoclaved. A stock of glasswear treated this way was reserved for polysome work. Sterile plastic tubes and pipettes were used whenever possible, and gloves worn at all times. Solutions were made with distilled water, and unless they contained heat-labile components (eg. heparin, sucrose and detergents), were autoclaved before use.

Monolayers of Bn5T cells grown in rotating plastic 850cm^2 culture bottles were used in preparation of polysomes. The culture medium was replaced with EF5 containing 20ug/ml cycloheximide 15 min. prior to harvesting, to reduce polysome

run-off. After 3 washes with cold PBS containing 20ug/ml cycloheximide, cells were scraped into a small volume of PBS, transferred into pre-weighed tubes and pelleted by centrifugation at 1000 r.p.m. in an MSE Coolspin, 5 min. at 4°C. After removal of the supernate the weight of the cell pellet was determined, and cells resuspended in 6ml/g buffer A^-.

The following steps were carried out within 4 min., to minimize polysome degradation by RNases released on cell lysis. Cells suspended in buffer A^- were transferred into a 30ml Dounce homogenizer and 10% Triton X-100 was added to a concentration of 2.5%. Homogenization was carried out with 10 strokes of the pestle. The resulting lysate was centrifuged at 16,000 r.p.m. on a Sorvall SS34 rotor, 30 seconds at 4°C, to spin out cellular debris. 1M $MgCl_2$ was then added to the supernatant to give a concentration of 100mM $MgCl_2$.

The cell lysate was incubated on ice for 1 h. During this time a cloudy polysome flocculate appeared. The suspension was layered onto a 10ml pad of 0.5M sucrose in buffer A^-, in a 36ml polyallomer centrifuge tube. Polysomes were pelleted through the sucrose pad by centrifugation at 13,000 r.p.m. on an AH627 rotor, 15 min. at 4°C. The polysome pellet was resuspended in 10mM HEPES pH7.6. 1ml of HEPES buffer was added per gram of cells used in the preparation. The suspension was transferred to a 1ml Dounce homogenizer and given 10 strokes with the pestle. Unless polysomes were to be *in vitro* translated, the following were added to the given concentration and mixed gently: 0.1mg/ml heparin, 1ug/ml cycloheximide, 5mM $MgCl_2$, 0.5%(v/v) triton X-100, 0.5%(w/v) sodium deoxycholate and 150mM sodium chloride. Polysome aggregates were then removed by centrifugation at 13,000

r.p.m. in an MSE Microcentaur microfuge, 5 min. at 4°C and the supernate stored at -70°C until required.

3.4.5 Polysome sedimentation profiles

The quality of a polysome preparation was assessed from its sedimentation profile (Gough and Adams, 1978; Shapiro and Young, 1981). Between 0.5 and 2.0 A_{260} units of polysomes in 0.5ml HEPES pH7.6 were layered on top of a continuous 0.5 - 1.5M sucrose gradient. The two sucrose solutions used to pour the gradient were 0.5M and 1.5M sucrose in buffer A^-. The gradients were poured in 12ml polyallomer tubes using a proportioning pump. When samples were loaded, tubes were centrifuged at 40,000 r.p.m. on a TST41 rotor, 90 min. at 4°C. The sedimentation profile was then determined by pumping gradient fractions through a spectrophotometer flow cell and recording the changes in A^{260} with a Cecil CE500 control record module.

3.4.6 Immunoselection of polysomes

Bn5T polysomes were immunoselected with TBS and TG7A by a modification of the method of Kraus and Rosenburg (1982).

Polysomes prepared from 1 gram of cells were diluted to a concentration of 15 A^{260} units/ml with resuspension buffer. Protein-A sepharose affinity column purified TBS or TG7A antibody (see section 5.1.2) was added to polysomes at a ratio of 1mg antibody per 160 A^{260} units. After 18 h incubation at 4°C, the polysome/antibody mixture was applied at a flow rate of 5ml/h to a protein-A sepharose column of 0.2ml bed volume which had been previously equilibriated with buffer A^-. The flow-through fraction was passed over the column once more,

then collected. The column was washed with 100 column volumes of buffer A^- (Benchimol et al., 1984). Specifically bound polysomes were then eluted with 5 column volumes of elution buffer. Each column volume of eluate was collected separately in an Eppendorf tube containing an equal volume of phenol/chloroform, then vortexed immediately for 2 seconds. After phenol/chloroform extraction poly(A^+)mRNA was prepared on a 0.2ml oligo(dT)-cellulose column followed by ethanol precipitation (see section 3.4.1). Flow-through fraction polysomes were adjusted to 20mM EDTA and 1.5% SDS and poly(A^+)mRNA prepared as before.

3.4.7 In vitro translation of RNA and polysomes

Reticulocyte lysate obtained from Amersham International plc., was used for in vitro translation (IVT) reactions. Reticulocyte lysate and ^{35}S-methionine used in this procedure were stored in liquid nitrogen.

For each small-scale IVT reaction 12ul lysate and 1.5ul ^{35}S-methionine were added to 1.5ul of a solution of RNA or polysomes, in a 1.5ml Eppendorf tube. 1ug - 4ug total cell/polysomal RNA or 0.05ug to 0.25ug poly(A^+)mRNA were added per tube and the contents vortexed 2 seconds then incubated in a 30°C waterbath for 1 h. The reaction was stopped by the addition of 15ul of a solution of 1%(w/v) methionine, 100ug/ml RNase A and 100mM EDTA, followed by a 15 min. incubation at 30°C. Samples were centrifuged at 13,000 r.p.m. in an MSE Microcentaur microfuge, 10 min. at 4°C. Supernates were collected and pellets discarded. For electrophoretic analysis of total in vitro translated polypeptides, 0.5 volumes of 3X electrophoresis sample buffer was added to supernates, which were then heated 3 min. in a boiling water bath prior to

counting. For i.p. assay, an equal volume of 2X RIPA buffer was added to supernates prior to counting. Samples were counted as described in section 3.6.6.

3.5 Analysis of cDNA prepared from Bn5T RNA

For the electrophoretic analysis of radiolabelled cDNA radiolabelled DNA size markers were prepared from Hind III restriction fragments of phage lambda DNA.

3.5.1 Preparation of agarose minigels

Minigels were prepared to estimate DNA concentration and to check that phage lambda DNA was properly digested by Hind III before radiolabelling. They were prepared using a 0.6% solution of agarose in TBE buffer and 2ul ethidium bromide was added to each 30ml aliquot of this solution to allow visualization of DNA under long wave UV illumination. Gels were run with TBE electrophoresis buffer at 10V/cm until the sample dye-front had migrated the appropriate distance.

3.5.2 Estimation of phage lambda DNA concentration

Approximate estimations of DNA concentration were obtained by visual comparison with a DNA standard of known concentration, after electrophoresis on ethidium stained agarose gels visualized under long wave UV illumination.

3.5.3 Preparation of end-labelled lambda Hind III DNA fragments

Lambda DNA was incubated with 10U/ug HindIII restriction in 20ul Hind III restriction enzyme buffer (REB) for 1 h at 37oC. The products of this reaction were analyzed on a minigel before proceeding further.

End-labelling of restriction fragments was achieved by incubation of 0.5ug of digested DNA with 1uCi dATP and 0.5U Klenow fragment in a total volume of 20ul Hind III REB for 10 min. at room temperature. Samples for electrophoresis were prepared by adding 1ul of reaction products to 20ul alkaline loading buffer.

3.5.4 Alkaline agarose gel electrophoresis

The sizes of first and second cDNA strands synthesised by reverse transcriptase were checked by alkaline agarose gel electrophoresis (McDonnell et al., 1977). A 1% agarose solution was prepared by adding 2g agarose to 200ml of a solution containing 50mM NaCl and 1mM EDTA and heating in a microwave oven until the agarose dissolved. The solution was cooled to 50oC and then poured to give a horizontal slab gel, which when set was mounted in an electrophoresis tank. Alkaline electrophoresis buffer was then added to the tank covering the gel to a depth of 5mm and buffer was allowed to soak into the gel for at least 30 min. before loading the samples of DNA.

Samples of cDNA corresponding to 20,000 c.p.m. were ethanol precipitated (see section 3.4.1) and then dissolved in 20ul of alkaline loading buffer for electrophoretic analysis. Before loading excess electrophoresis buffer was removed from above the surface of the gel leaving a 1mm layer of buffer covering the gel. Electrophoresis was carried out at 1.5V/cm for 18h, after which the gel was carefully placed on a piece of filter paper and dried under a vacuum. The gel was then autoradiographed using an intensifying screen.

3.6 Radiolabelling and immunoprecipitation of polypeptides

3.6.1 In vivo radiolabelling of polypeptides

Polypeptides were radiolabelled in vivo to allow for autoradiograph detection following SDS polyacrylamide gel electrophoresis (SDS-PAGE).

3.6.2 Radiolabelling of polypeptides with ^{35}S-methionine

Uninfected cell monolayers in 50mm petri dishes were overlaid with 4ml EF2-met and incubated at 37°C for 4 h to deplete cells of methionine. Culture medium was then replaced with 2ml EF2-met containing 100uCi/ml ^{35}S-methionine and cells incubated for the required time, usually 18h, before harvesting.

To radiolabel infected cell polypeptides, subconfluent monolayers were depleted of methionine as before. The culture medium was removed and the virus applied in 0.2ml PBS and absorbed for 1 h at the required temperature. The overlay was replaced by 2ml EF2-met containing 100uCi/ml ^{35}S-methionine and the infected cells incubated for the appropriate time period.

3.6.3 Pulse-chase labelling of polypeptides with ^{35}S-methionine

Uninfected cell monolayers were labelled with ^{35}S-methionine as before. After a 10 min. or 60 min. pulse, radiolabel was removed and the monolayer washed 3X with EF5. Cells were overlaid with 4ml of EF5 and incubated at 37°C. After varying chase periods, plates of cells were harvested.

3.6.4 Radiolabelling of polypeptides with ^{32}P-orthophosphate

Cells were depleted of phosphate by incubation in EF2-Pi for 4 h. They were labelled for 18 h in EF2-Pi containing 150uCi/ml ^{32}P-orthophosphate.

3.6.5 Harvesting radiolabelled cells

Monolayers of uninfected cells were washed 3X with PBS and stored at -70°C until required. Infected cells were scraped into the culture medium and pelleted by centrifugation at 1000 r.p.m. in an MSE Coolspin, 10 min. at 4°C. Pelleted cells were washed by resuspension in PBS, repelleting and removal of the supernate. Cell pellets were stored at -70°C.

For preparation of samples of total infected/uninfected cell polypeptides, cells were lysed in electrophoresis sample buffer (0.25ml/2X10^6 cells), followed by heating at 100°C for 3 min. Cell debris was removed by centrifugation at 13,000 r.p.m. in an MSE Microcentaur microfuge, 15 min. at 4°C, and the supernate aliquoted and stored at -70°C.

For preparation of lysate for immunoprecipitation (i.p.) assays, cells were suspended in RIPA buffer (0.5ml/2X10^6 cells) containing protease inhibitors (0.030mg/ml benzamidine, 0.100mg/ml phenanthroline, 0.034mg/ml PMSF). Protease inhibitors were stored as a stock solution in ethanol at -20°C, and were added to RIPA buffer immediately before use, to minimize inactivation by hydrolysis in aqueous solution. Crude lysate was sonicated for 3 min. to disrupt cell aggregates, incubated for 30 min. on ice, then centrifuged to remove cell debris and the supernate stored at -70°C.

3.6.6 Scintillation counting

To quantify incorporation of radioactivity into polypeptides, 5ul samples of lysate were placed on Whatman's filter discs which were washed 2X with 10% Trichloracetic acid and 2X with 5% Trichloracetic acid, thus precipitating polypeptides onto the filter discs and removing unincorporated radiolabel. Filter discs were then washed 2X with ethanol, dried under a heat lamp, placed in vials with 3ml scintillation fluid and counted in a scintillation counter.

For the electrophoretic analysis of total infected/uninfected cell polypeptides, samples were loaded in volumes corresponding to $70X10^3$ counts per minute (c.p.m.). For standard i.p. reactions a volume of lysate corresponding to $4X10^6$ c.p.m. was used. For peptide mapping analysis a volume corresponding to $4X10^7$ c.p.m. was used to get a stronger signal.

3.6.7 Immunoprecipitation of polypeptides

The i.p. procedure was based on a method described by Kessler (1975). For each reaction a volume of lysate corresponding to the appropriate number of radioactive counts (see section 3.6.6) was diluted with RIPA buffer to a volume of 100ul. Antibody was added and the mixture incubated for 1 h at 4^oC. For i.p.s with TBS, 5ul of antibody were added per 100ul of lysate, giving a dilution of approximately 1/20(v/v). For mouse monoclonals TG7A and AC88, 2.5 to 5ul antibody were added. For mouse monoclonals only, a second antibody (rabbit anti-mouse immunoglobulin) was added at the end of the first incubation and the antigen-antibody mixture incubated a further 1 h.

Immune complexes were precipitated by addition of 60ul

protein-A sepharose (12.5%(w/v) suspension in RIPA), and incubation for 1 h on a rotating mixer at 4°C. Next, i.p.s were washed 3X with RIPA, then 1X with tris-saline. In each wash cycle the pellet was resuspended in 0.5ml of buffer, vortexed 2 seconds, centrifuged at 13,000 r.p.m. in an MSE Microcentaur microfuge, 2 min. at 4°C and the supernate discarded. The final pellet was resuspended in 30ul of electrophoresis sample buffer and heated at 100°C for 3 min. to dissociate antigen, antibody and protein-A sepharose. The latter was removed by a further 2 min. microfuge spin and the supernate containing antigen and antibody, collected and stored at -70°C until required.

3.6.8 In vitro radiolabelling of polypeptides

The chloramine-T method was used to radiolabel polypeptides *in vitro* (Greenwood *et al.*, 1983). 10ul of a 1mg/ml solution of polypeptide was placed in an Eppendorf tube kept on ice. 10ul of 0.1M sodium phosphate pH7.5 was added followed by 200uCi of ^{131}I and 10ul of 1mg/ml chloramine-T. After 20 seconds incubation, 20ul of a 1mg/ml solution of sodium metabisulphite was added to stop the reaction. After a further 20 seconds incubation 1ml of PBS was added and the iodinated polypeptide separated from unreacted iodide by desalting on a Pharmacia PD-10 column (Sephadex G-25 M).

3.7 Electrophoretic analysis of polypeptides

3.7.1 SDS polyacrylamide gel electrophoresis

Polypeptides were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on either single concentration or gradient slab polyacrylamide gels (Laemmli, 1970). Single

concentration gels contained 7.5% or 9% acrylamide and gradient gels 6-15% acrylamide in resolving gel buffer. Acrylamide was cross-linked with 1 part in 40 (w/w) N-N'-methylenebisacrylamide. Gels were polymerized by the addition of ammonium persulphate to 0.04%(w/v) and TEMED to 0.04%(v/v). Gradients were poured with a proportioning pump and overlaid with a few drops of butan-2-ol to produce a level surface. After polymerization the butan-2-ol was removed by washing with deionized water. The upper surface of the gel was overlaid with 1X stacking gel buffer which prevented drying out and allowed gel storage for up to 2 weeks at 4°C.

The stacking gel was prepared on the day the gel was run. This contained 5% acrylamide in stacking gel buffer. The acrylamide was cross-linked with 1 part in 40(w/w) N-N'-Diallyl-tartar-diamide. Wells were formed using teflon combs.

Tank buffer was prepared from a 10X stock. Samples for analysis were loaded in electrophoresis sample buffer. Gels were run at 0.45mA/cm^2 for 3-4 h at room temperature until the bromophenol blue dye front reached the bottom of the separating gel.

3.7.2 Peptide mapping

One-dimensional peptide mapping was used as a test of polypeptide relatedness and a modified version of the method of Cleveland et al., (1977), was used. Polypeptides were labelled in vivo with ^{35}S-methionine and resolved by electrophoresis on 7.5% SDS-polyacrylamide gels. Gels were fixed and dried down onto Whatmans 3MM filter paper. Direct autoradiography was carried out overnight at room temperature, using flashed Kodak XS film. Marks made with radioactive dye

allowed accurate alignment of autoradiograph and gel. Polypeptide bands located by autoradiography were excised from the gel with a scalpel blade. Excised gel slices were rehydrated for one h in rehydration buffer. Polypeptides were digested without prior elution by placing gel slices in the sample wells of a second SDS-polyacrylamide gel (the peptide mapping gel), then overlaying each slice with proteolytic enzyme. Digestion proceeded in the stacking gel during subsequent electrophoresis.

In preparing the peptide mapping gel the separating gel was cast allowing for a long stacking gel (4cm in length from the bottom of the sample wells). The separating gel was single concentration 15% acrylamide gel, cross-linked by N-N'-methylenebisacrylamide present at a ratio of 1 part in 40 (w/w). Gel slices were pushed to the bottom of empty wells in the stacking gel with a toothpick, taking care to avoid trapping air bubbles underneath. The spaces around each slice were filled by overlaying with 10ul rehydration buffer containing 20% glycerol (v/v). The upper chamber of the gel kit was filled with tank buffer prior to loading the proteolytic enzyme. A stock solution of enzyme was diluted to the required concentration with rehydration buffer containing 10% glycerol (v/v) and bromophenol blue dye. Enzyme was then added to sample wells in 20ul aliquots.

Electrophoresis was performed in the usual manner at room temperature, with the exception that the current was turned off for 1 h when the dye-front reached a point 3-4mm from the bottom of the stacking gel. When electrophoresis was completed, gels were examined by fluorography as described in section 3.7.3. For peptide mapping gels films were exposed first for 3 weeks and then 2 months.

3.7.3 Autoradiography

Gels were fixed in a solution containing 50% methanol and 7% acetic acid for a minimum of 15 min., but were often stored in this overnight. Gels were next soaked in a 5% methanol, 7% acetic acid solution for a minimum of 15 min. or longer if necessary, until swollen to the original size, then washed in water for 30 min. Unless bands were to be excised for peptide mapping, they were then soaked in EN^{3}HANCE for 1 h and then washed for a further 30 min. in water. However, if bands were to be peptide mapped, all washes were kept to 15 min. and gels were not EN^{3}HANCED in order to minimize acid hydrolysis. Gels were dried under vacuum at 80^{o}C onto a sheet of Whatman 3MM filter paper and then autoradiographed with flashed Kodak X-Omat XS-1 film which was exposed at -70^{o}C if gels were EN^{3}HANCED or room temperature if not.

3.7.4 Coomassie blue staining

Gels were stained in a solution of 0.25% Coomassie blue in 50% methanol, 7% acetic acid. Standard slab gels of 1.5mm thickness were stained for at least 2 h, while gels 0.5mm thick (used for polypeptide blotting) required only 15 min.

Destaining was carried out in a shaking water bath at 37^{o}C. Destaining was faster if 50% methanol, 7% acetic acid was used for the first 3-4 washes of 15 min. each. Over the following 24 h gels were given 3-4 washes in 5% methanol, 7% acetic acid.

3.7.5 Silver staining

The Bio-Rad silver staining kit was used according to the protocol supplied by the manufacturers. Silver staining was carried out following Coomassie blue staining if increased

sensitivity was required.

3.8 Amino acid sequence analysis

Amino acid analysis and sequencing was carried out in Dr J. Walker's laboratory at the MRC Laboratory of Molecular Biology (L.M.B.), Hills Road, Cambridge, England.

At least 20pmol of a polypeptide are usually required for successful amino-acid sequencing. Results given in section 9.1.1 indicate that less than 0.5pmol of the 90kDa polypeptide to be sequenced in this study, are i.p. in a standard i.p. reaction. It was therefore decided to increase the scale of the reaction and make a number of other modifications.

3.8.1 Large-scale immunoprecipitation reactions

The author devised the following protocol, which proved to be simpler and more effective than a theoretically equivalent number of standard i.p. reactions.

1ml TBS and 2ml protein-A sepharose (12.5%(w/v) suspension) were added to 7ml RIPA buffer in a 50ml conical flask, and incubated at room temperature for 2 h. The protein-A sepharose was kept in suspension during incubations by mixing gently on a magnetic stirrer with a small magnet. 10ml of Bn5T cell lysate was added and the reaction mixture incubated at room temperature for a further 2 h. Two modifications were made to the standard technique of lysate preparation (section 3.6.5). Lysate concentration was increased 10X by lysis of $4X10^7$ cells/ml of RIPA buffer, and 10X more protease stock solution was added per ml of lysate.

The reactants were next introduced into a column consisting of a 5ml pipette plugged at the bottom with glass wool. During washing stages this retained the protein-A

sepharose with attached antibody and antigen. This simplified washing to the application of 3ml RIPA, followed by 3ml tris-saline at the top of the column while unbound material was run out at the bottom. No centrifugation step was required.

Bound molecules were eluted with 2.5ml 3M potassium isothiocyanate. The eluate was immediately desalted by passing over a Pharmacia PD-10 column containing Sephadex G-25M, previously equilibriated with 10mM ammonium bicarbonate. When all the sample had run into the column, polypeptides were eluted with 3.5ml 10mM ammonium bicarbonate. The sample was concentrated to about 300ul by freeze-drying followed by centrifugation in a Centricon microconcentrator (5,000 r.p.m. in a Sorvall SS34 rotor, up to 6 h at 4°C), which reduced the volume to approximately 50ul. Finally 0.5 volumes of 3X electrophoresis sample buffer were added to concentrated sample, which was stored at -70°C. Immediately prior to electrophoresis the sample was heated to 100°C for 3 min.

3.8.2 Transfer to Immobilon membrane

Following electrophoresis on a 0.5mm thick 7.5% SDS-polyacrylamide slab gel, polypeptides were electrophoretically transferred from gels onto Immobilon polyvinylidine difluoride (PVDF) transfer membranes. These are mechanically strong solid phase supports that bind proteins hydrophobically and do not need to be chemically activated (Matsudaira, 1987). The polyacrylamide gel was first soaked for 20 min. in transfer buffer. A piece of Immobilon membrane, cut to the same size as the gel, was immersed in methanol for 2 seconds, washed for 2 min. in de-ionized water and for a further 5 min. in

transfer buffer. The gel was placed on top of the membrane, then sandwiched with two pieces of Whatmans 3MM paper on either side. This sandwich was put into a Bio-Rad electroblotting kit, with the membrane on the +ve side of the gel. The kit was filled with transfer buffer and electro-transfer carried out at 300mA for 1 h.

On completion of transfer the membrane was washed for 5 min. in deionized water. The membrane was stained in a solution of 50% methanol and 0.1% Coomassie Blue, destained in 50% methanol and washed in water. The membrane was then allowed to dry. The stained band of interest was excised with a scalpel blade and stored at -20°C. For sequencing of intact polypeptide the excised strips of Immobilon membrane were placed directly into the reaction chamber of the automated sequencer. When the polypeptide was to be cleaved into peptide fragments the membrane was not stained with Coomassie blue as this dye interferes with the separation and purification of peptides on the type of gradient used. Instead the polypeptide band was located by autoradiography and then excised.

3.8.3 Polypeptide elution

This step was only necessary if the polypeptide was to be cleaved into peptides before sequencing.

Polypeptide was eluted from excised strips of Immobilon membrane by placing the strips in 1ml 70% formic acid in an Eppendorf tube and incubating for 1 h at room temperature while vortexing for 10 seconds every 5 min. The supernate was dried down to a pellet in a speedivac, then twice resuspended in 200ul of distilled water (DW) and dried to remove any trace of formic acid.

3.8.4 Cleavage with trypsin

The sample pellet was dissolved in 200ul 50mM ammonium bicarbonate and trypsin added in the ratio 1ug trypsin : 50ug sample polypeptide. Incubation for 4 h at 37^{o}C was followed by drying in a speedivac and the pellet was then twice resuspended in 200ul DW and dried down in a speedivac. The final pellet was dissolved in 15ul of 6M guanidine hydrochloride, 0.1% trifluoroacetic anhydride (TFA) immediately before HPLC.

3.8.5 Cleavage with cyanogen bromide

Polypeptides were dissolved in 70% formic acid and a 10-fold excess (by weight) of cyanogen bromide added. The digest was stirred for 4h at room temperature, in the absence of light. The reaction was terminated by dilution with 10 volumes of water and the solution was then freeze dried.

3.8.6 Peptide purification

Peptides were purified using an Applied Biosystems 130A separation system which separates by microbore HPLC. The system used for separation was a 0% - 80% acetonitrile gradient in 0.1% TFA. Peptides were detected by absorbance at 225nm as the column was automatically fractionated. Fractions were collected in Eppendorf tubes and were dried down in a speedivac, resuspended in 200ul D.W., then dried down again.

3.8.7 Amino acid sequence determination

Amino acid sequencing was carried out on an Applied Biosystems 470A gas phase sequencer, operated by Mr F.Northrop, (L.M.B.,Cambridge). Automated protein sequencers work on the Edman degradation principle (Edman, 1950), by

which the N-terminal amino acid is coupled with phenylisothiocyanate (PITC) and the derivatized amino acid is then cleaved from the polypeptide. Sequential removal of N-terminal amino acids occurs with repeated coupling/cleavage cycles. Amino acid derivatives are converted to the more stable phenylthiohydantoin (PTH) form.

PTH-amino acids were analyzed sequentially on an Applied Biosystems Model 120A PTH analyzer. This identifies PTH-amino acids according to their elution characteristics on high performance liquid chromatography (HPLC) using a sodium acetate/acetonitrile gradient. PTH-amino acids are detected by UV absorption monitoring at 270nm. By identification of PTH-amino acids derived in each successive cycle, the amino acid sequence is determined.

4 TUMOUR ANTIGENS IDENTIFIED BY IMMUNOPRECIPITATION ASSAYS

4.1 Background information

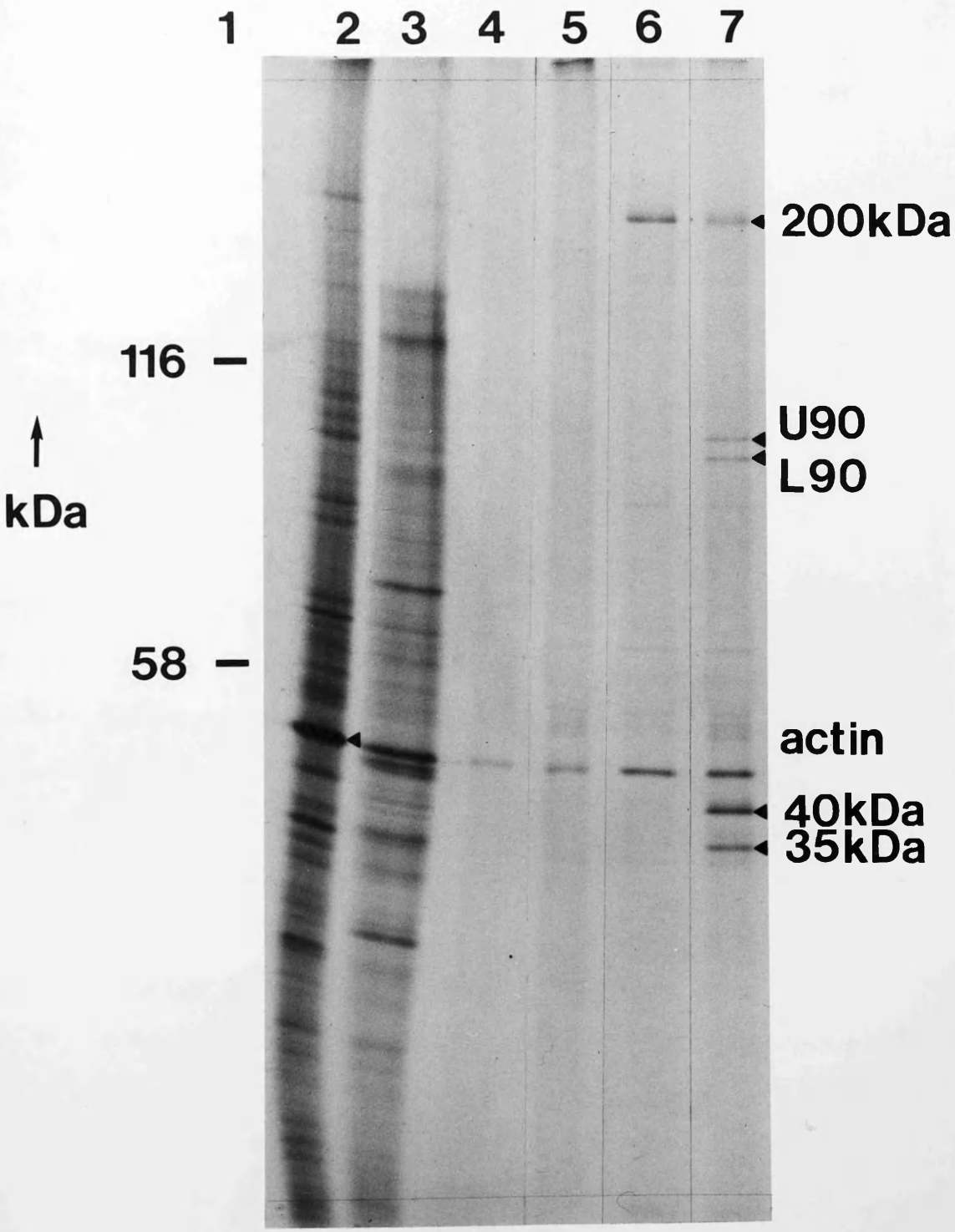
A set of cellular polypeptides is immunoprecipitated (i.p.) by the monoclonal antibody TG7A from HSV-transformed RE cells (Bn5T cells) but not from normal control RE cells (Macnab et al., 1985a); (figure 4.1). TG7A monoclonal was raised against DNA-binding proteins from HSV-2 infected cells and in Western blotting studies, it recognizes cellular polypeptides induced on HSV infection which are of similar MW to those i.p. from transformed cells (Dr N.B. LaThangue, personal communication). The serum taken from rats bearing tumours induced by Bn5T cells (tumour bearing serum/TBS) has been shown to i.p. polypeptides from Bn5T cell lysate which comigrate exactly with those i.p. by TG7A and which give closely similar or identical peptide maps. Like TG7A, TBS does not i.p. the polypeptide set from normal control RE cells. The same results were also obtained with rat antisera to HSV-2 infected cells (Macnab et al., 1985a). These findings suggest that certain cellular polypeptides induced by HSV-2 infection are related to polypeptides expressed at raised levels in HSV-2 transformed cells. On the basis of these and other observations the authors suggested that cellular polypeptides induced by HSV may be important in the initiation of transformation by the virus (Macnab et al., 1985a). A more detailed account of this work is given in section 1.2.7.

The results of further characterization studies on these polypeptides will be presented in this thesis together with the results of studies aimed at providing a probe to allow for screening of cDNA libraries to identify the encoding gene(s).

Figure 4.1; Electrophoretic analysis of RE and Bn5T cell polypeptides i.p. by TG7A. Autoradiograph of a 6-15% gradient polyacrylamide gel containing electrophoretically separated ^{35}S-methionine labelled polypeptides. MW markers are shown in track 1. Tracks 2 and 3 show a total Bn5T cell and HSV-2 infected (HG52 strain, 10 p.f.u./cell, harvested 8h. p.i.) RE cell polypeptide profiles respectively. Tracks 4 to 7 contain i.p.s of RE and Bn5T cells with control ascites and TG7A monoclonal arranged as follows: track 4, RE i.p. control ascites; track 5, RE i.p. TG7A; track 6, Bn5T i.p. control ascites; track 7, Bn5T i.p. TG7A.

The MW's of polypeptides i.p. by TG7A are indicated to the right of track 7. There is a 97kDa polypeptide band referred to as "U90", and a 93kDa polypeptide band referred to as "L90". For an explanation of this nomenclature see section 4.2.3.

On this particular gel a 200kDa polypeptide is seen in the track corresponding to a TG7A i.p. of RE cell lysate (track 6). This band is not always seen in such i.p.s, and is likely to represent myosin which tends to be precipitated non-specifically (Dr D. Lane, personal communication).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5
6
7
200kDa
116
U90
L90
kDa
58
actin
40kDa
35kDa

In this chapter, a detailed analysis of polypeptides expressed by normal control RE cells and by transformed Bn5T cells will by described. Much use was made of the immunoprecipitation technique throughout these studies, and the two antibody preparations most often employed were the monoclonal TG7A and the polyclonal TBS. TBS was often utilised as an alternative to TG7A, owing to the limited supply of the monoclonal antibody.

4.2 Analysis of Bn5T polypeptides immunoprecipitated by TBS

4.2.1 Techniques used

TBS is serum taken from rats bearing tumours induced by Bn5T cells and contains antibodies that i.p. a group of Bn5T polypeptides which are not detectable in control RE cells. These polypeptides can be demonstrated by *in vivo* radiolabelling of Bn5T cells with ^{35}S-methionine, i.p. with TBS, separation of i.p. polypeptides by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography of the resulting gel 4.1. This method of polypeptide labelling and detection was used throughout these studies, except where stated otherwise.

4.2.2 Estimates of molecular weight

Estimates of the molecular weight (MW) of polypeptides were made by comparison of their mobility on SDS-PAGE, with MW standards run on the same gels. MW estimates for Bn5T polypeptides i.p. by TBS are shown in Table 4.1. Values for each polypeptide are highly consistent and in general agree well with previously assigned MWs. However some important differences exist in the 32 - 36kDa region, and these are

Polypeptide number assignment	Mean of ten MW estimates	Original MW assignment	New MW assignment
1.	202.6 (SD=2.9)	200	200
2.	97.3 (SD=2.2)	90	97
3.	92.8 (SD=2.3)	90	93
4.	38.7 (SD=0.6)	40	40
5.	35.5 (SD=0.9)	32	35
6.	34.0 (SD=0.9)	32	34

Table 4.1; Molecular weight estimates in kDa for Bn5T polypeptides i.p. by TBS. Estimates were obtained from calibration curves constructed by plotting relative mobility on SDS-PAGE (6 - 15% acrylamide gradient gels) against Log_{10} MW for a number of BRL standard MW markers (myosin H chain - 200kDa, phosphorylase b - 97.4kDa, bovine serum albumin - 68kDa, ovalbumin - 43kDa, alphachymotrypsinogen - 25.7kDa). For every polypeptide ten estimates were made, each from a different gel. Mean values and standard deviations (SD) are given. Original MW assignments are taken from Macnab et al., (1985). New MW assignments are given, taking into account recent findings. On gels showing high resolution the 34kDa band is resolved into three components.

discussed in section 6.1.

4.2.3 Nomenclature

Each polypeptide is named according to: (1) Cell of origin, (2) antibody used to i.p., and (3) apparent MW (as determined by migration rate on SDS-PAGE). This information is given in the order 1:2:3. Thus, the 40kDa polypeptide i.p. by TBS from Bn5T cell lysate is named Bn5T:TBS:40. A slight variation is that the 93kDa polypeptide i.p. by TBS from these cells is named Bn5T:TBS:L90. This is because the 93kDa polypeptide migrates faster than the 97kDa polypeptide and thus produces the lowermost (hence "L90") of the two bands in the 90kDa region of the gel. The 97kDa polypeptide is named Bn5T:TBS:U90 for similar reasons. For polypeptides which have not been i.p. and are seen in total cell polypeptide profiles the abbreviation PP is inserted into the notation. Thus the polypeptide giving the major 40kDa band seen in total Bn5T polypeptide profiles is denoted Bn5T:PP:40. Polypeptides of 200, 97, 93, 40, 35 and 34kDa i.p. from Bn5T cell lysate by TBS will be referred to collectively as the Bn5T:TBS set.

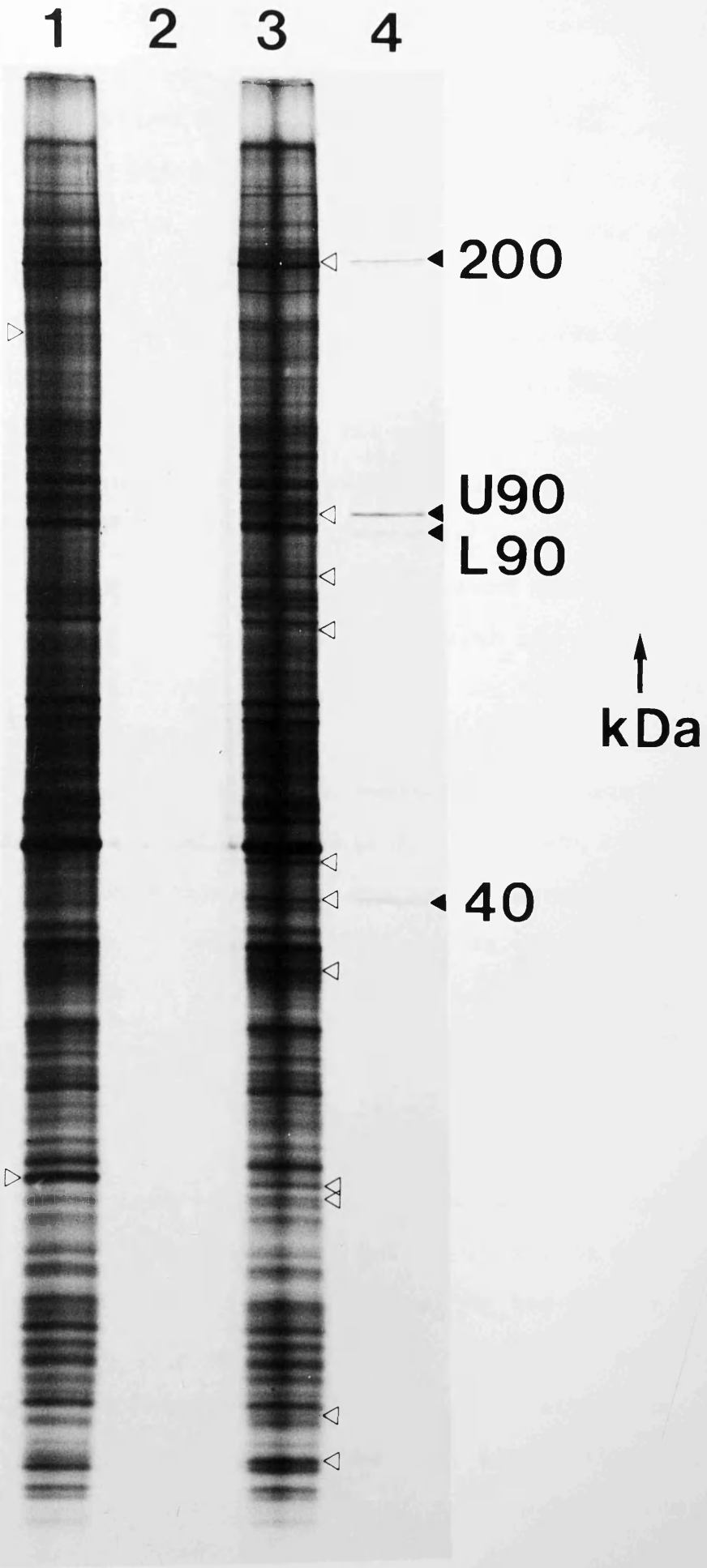
4.3 Analysis of RE and Bn5T total polypeptide profiles

Polypeptides radiolabelled *in vivo* with ^{35}S-methionine and separated by SDS-PAGE can be visualized by both autoradiography and Coomassie blue staining. A number of differences are seen between total polypeptide profiles of control RE (non-transformed) cells and Bn5T (transformed) cells (figure 4.2).

4.3.1 Autoradiography

Of the thirteen or more ^{35}S-methionine labelled

Figure 4.2; Analysis of transformed cell (Bn5T) and normal control cell (RE) polypeptides by SDS-PAGE. Autoradiograph of a 6-15% gradient polyacrylamide gel containing electrophoretically separated ^{35}S-methionine labelled polypeptides extracted from RE control cells (track 1) and transformed Bn5T cells (track 3). Polypeptide bands only present for one of these two cell types, are arrowed (tracks 1 and 3). Also compared are TBS i.p.s. of RE cell lysate (track 2) and Bn5T cell lysate (track 4). Polypeptides i.p. by TBS are indicated by solid arrows in track 4 and the designated MW values given on the right side of the gel.


1
2
3
4
200
U90
L90
kDa
40

polypeptides seen to be present at higher levels in Bn5T cells than in RE cells (figures 4.2 and 4.3), three appear to be i.p. by TBS (200, 97 and 40kDa). Use of TBS in i.p. assays therefore allows attention to be focussed on a proportion of these polypeptides which are not detectable in control RE cells and which may be responsible for some features of the oncogenic phenotype.

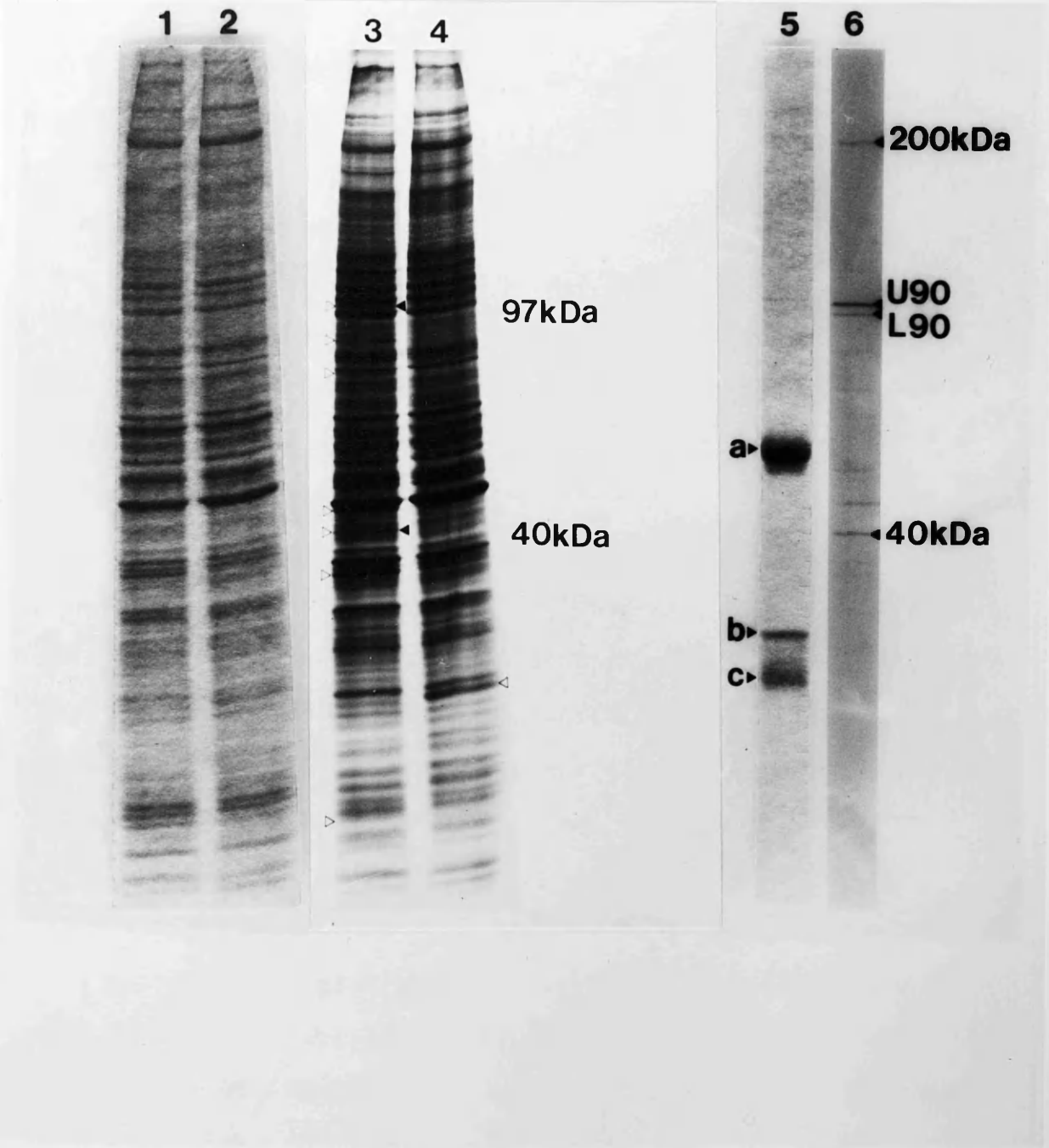
On comparison of total Bn5T cell polypeptides labelled with ^{35}S-methionine, with total control RE cell polypeptides labelled the same way, there are two prominent bands which are consistently observed for Bn5T cells and not observed for RE cells. These have apparent MWs of 84.8kDa (mean of seven estimates each from a different gel; standard deviation(SD)= 1.2) and 38.7kDa (observed to comigrate with the Bn5T:TBS:40 on more than ten different gels).

There are, in addition, at least four ^{35}S-methionine labelled polypeptides which are present at lower levels in Bn5T cells than in RE cells (figures 4.2 and 4.3). These may also be important determinants of the normal/transformed phenotype, but have not been investigated in this study.

4.3.2 Coomassie blue staining

Total cell polypeptides, radiolabelled with ^{35}S-methionine and separated by SDS-PAGE give some bands that are visible on autoradiography but not on Coomassie blue staining. Corresponding polypeptides may be too low in abundance to be detected by Coomassie blue staining and only detectable on autoradiography because of high methionine content or rapid turnover rate. Coomassie blue staining of polyacrylamide gels allows detection of 0.2ug polypeptide if

Figure 4.3; Electrophoretic analysis of RE and Bn5T polypeptides using both Coomassie blue and autoradiographic detection techniques. Examination of Coomassie blue stained and autoradiographed tracks from a single 6-15% gradient polyacrylamide gel containing electrophoretically separated ^{35}S-methionine labelled polypeptides. Tracks 1 and 2 show Coomassie blue stained tracks containing total Bn5T and RE cell polypeptides respectively. Tracks 3 and 4 show the corresponding autoradiograph on which visible differences between the two polypeptide profiles are indicated by the outlined arrows. Solid arrows indicate 97kDa and 40kDa Bn5T polypeptides which comigrate with U90 and 40kDa Bn5T polypeptides in TBS i.p.s. (track 6). Track 5 shows a Coomassie blue stained track containing four standard TBS i.p.s of Bn5T cell lysate. Track 6 shows the corresponding autoradiograph on which the i.p. polypeptides are arrowed. Comparison of tracks 5 and 6 shows that the U90 and L90 Bn5T polypeptides i.p. by TBS give faint bands on Coomassie blue staining. Non-specific bands labelled a, b and c represent serum components which bind to protein-A sepharose.


1
2
3
4
5
6
97kDa
40kDa
200kDa
U90
L90
a
b
c
40kDa

present in a sharp band (Hames, 1981).

In Coomassie blue stained gels of total Bn5T cell polypeptide profiles, no bands are seen that comigrate with the 35S-methionine labelled polypeptides i.p. by TBS. This suggests that Bn5T:TBS polypeptides are present at low abundance in the cell.

4.3.3 Bands comigrating with Bn5T polypeptides immunoprecipitated by TBS

Bn5T total cell polypeptide profiles show three bands on autoradiography which comigrate with bands in Bn5T:TBS i.p.s. These have MWs of 40kDa, 97kDa and 200kDa (figures 4.2 and 4.3). The fact that these bands are seen in total Bn5T cell polypeptide profiles but are not seen in total RE cell polypeptide profiles, suggests that they may be the polypeptides recognized by TBS.

(a) 40kDa and 97kDa bands: The 40kDa band is easily visible in ^{35}S-methionine labelled total Bn5T cell protein profiles, but the 97kDa band is more difficult to see. These bands are not detected by Coomassie blue staining and are not seen in total RE cell polypeptide profiles.

(b) 200kDa band: Analysis of this band is more complex. It can be seen from gels showing very fine resolution, that in ^{35}S-methionine labelled total Bn5T cell polypeptide profiles there are two 200kDa bands very close together (figure 4.2). The lower band is much stronger than the upper band. In total RE cell polypeptide profiles there is only one 200kDa band and it is of similar intensity to the strong Bn5T 200kDa band.

In Bn5T:TBS i.p.s there are two 200kDa bands which

comigrate with the two 200kDa bands in the total Bn5T cell polypeptide profile. However, in contrast to the total Bn5T cell polypeptide profile, it is the upper band in a Bn5T:TBS i.p. which is the strongest. This strong 200kDa band in Bn5T:TBS i.p.s comigrates with the weak 200kDa band which is present in total Bn5T cell, but not in total RE cell, polypeptide profiles. Thus, the major 200kDa polypeptide band in TBS i.p.s corresponds to the polypeptide specific to the transformed cell.

4.4 Summary

(1) The apparent MWs of Bn5T:TBS polypeptides were rigorously determined and a system of nomenclature defined.

(2) A detailed analysis of total Bn5T and RE cell polypeptide profiles was carried out.

5 POLYSOME IMMUNOSELECTION AND RNA STUDIES

The initial aim of this project was to use the polysome immunoselection technique to obtain a specific cDNA probe to study Bn5T cell gene(s) encoding polypeptides recognized by TBS and TG7A.

5.1 Polysome immunoselection

The author learned the details of polysome preparation and immunoselection techniques by reference to publications (Palmiter, 1974; Palacios et al., 1972; Gough and Adams, 1978; Shapiro and Young, 1981; Benchimol et al., 1984) and by communications with Dr M. Oren and Dr L. Crawford.

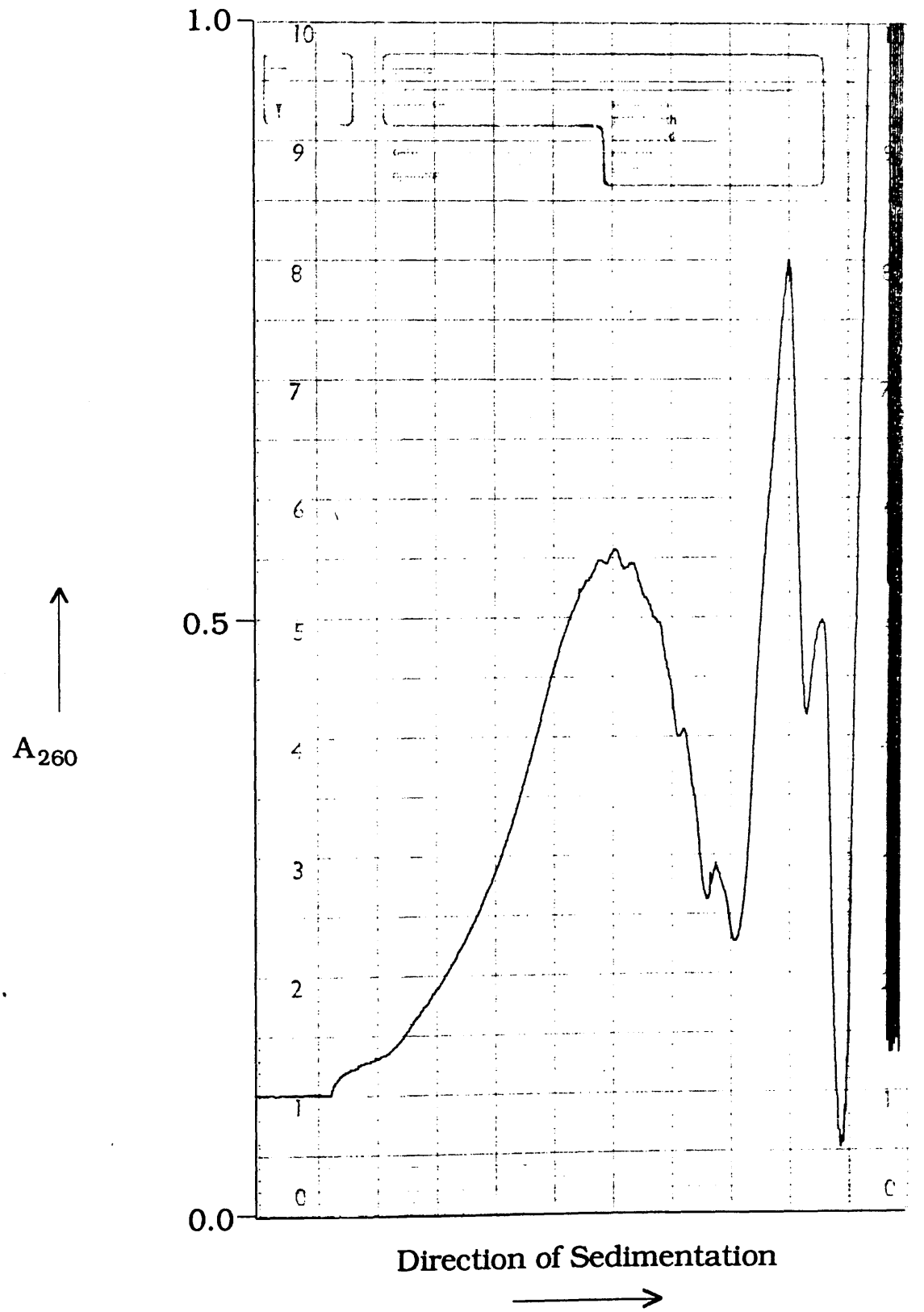
The polysome immunoselection technique has been used successfully for the selection of the low abundance mRNAs encoding p53 (Oren and Levine, 1983; Benchimol et al., 1984) and the precursor for ornithine transcarbamoylase (Kraus and Rosenberg, 1982). It was also used to select the message for the HSV-induced heat shock protein (HSP) of 57kDa recognized by the TI56 monoclonal (Patel et al., 1986). However, it was later learned that the same workers were unable to immunoselect polysomes from HSV-2 infected cells using the TG7A monoclonal (Dr N.B. LaThangue, personal communication).

5.1.1 Preparation of total cell polysomes

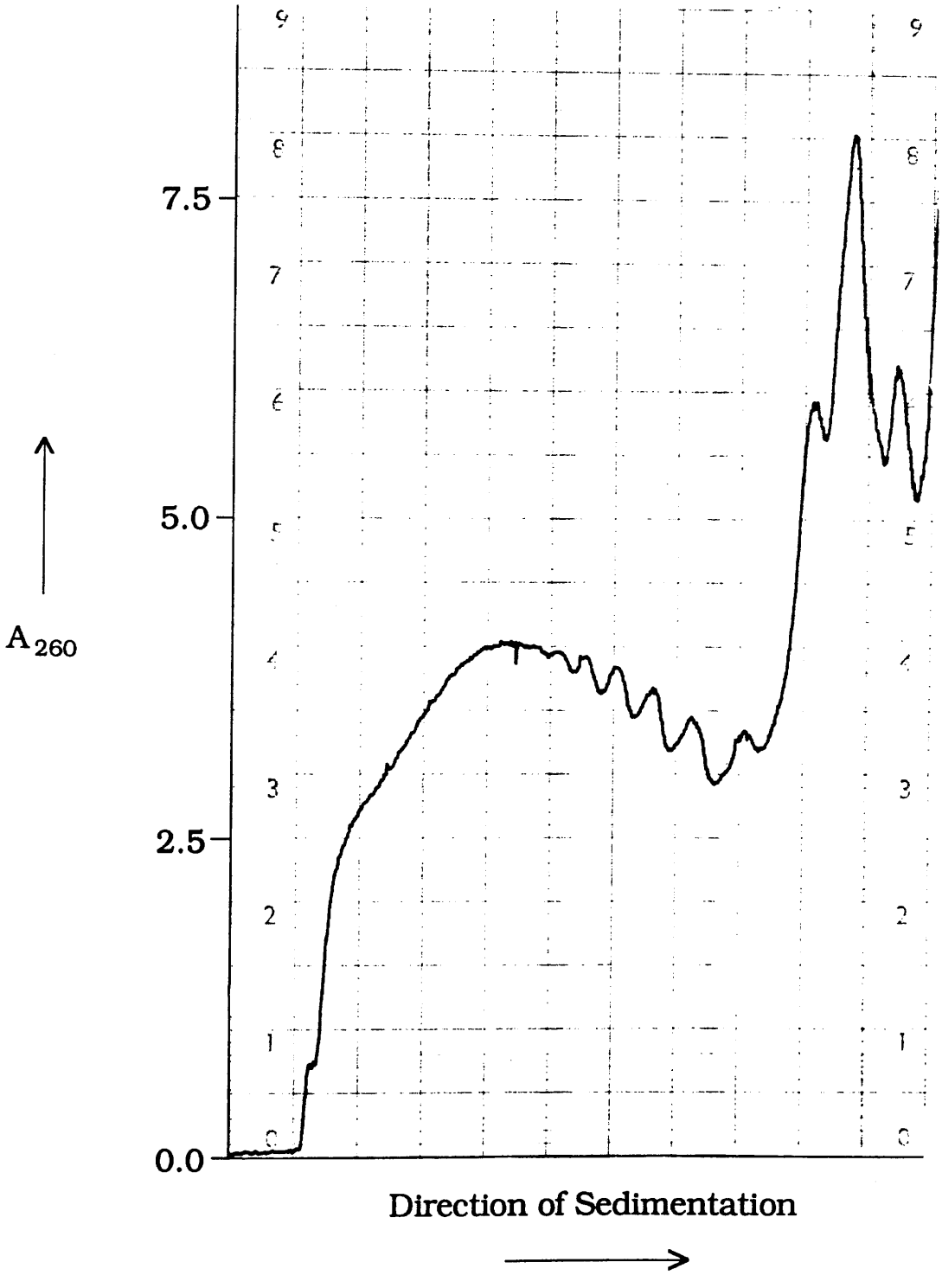
Polysomes were prepared as described in section 3.4.4 and the quality of preparations was assessed by sedimentation profile analysis, as described in section 3.4.5 (Palacios et al., 1972; Palmiter, 1974; Shapiro and Young, 1981). BHK cells were used to practise polysome preparation and a total of seven polysome preparations were made before a satisfactory polysome sedimentation profile was obtained (fig 5.1). An

Figure 5.1; Polysome profiles. Sedimentation profiles of polysomes following centrifugation through a 0.5M - 1.5M sucrose gradient (see section 3.4.5). (**A**) BHK cell polysomes; (**B**) Bn5T cell polysomes.

A

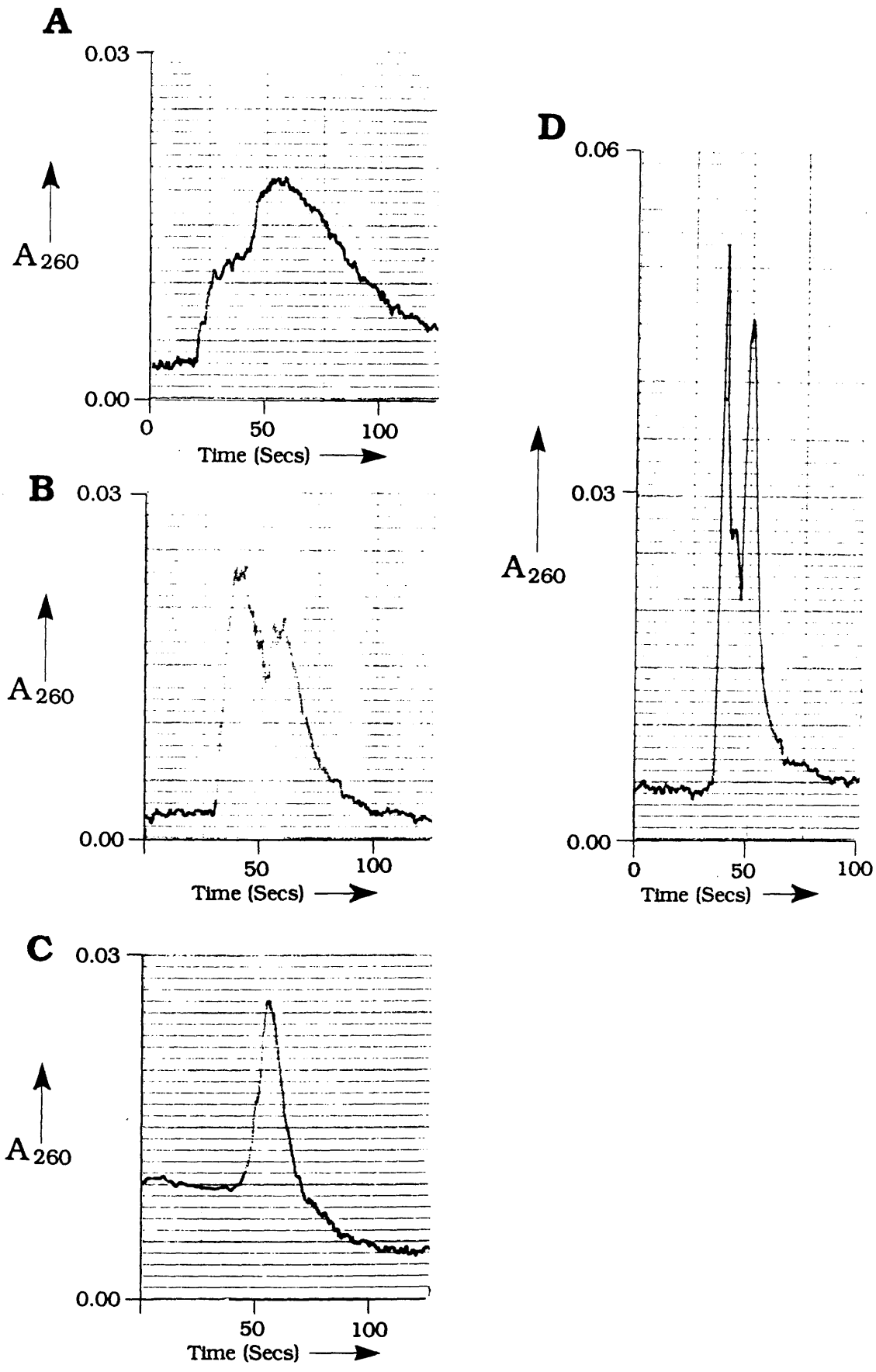


B



important factor contributing to the improvement in polysome preparations was the addition of cycloheximide (20ug/ml) to the PBS used to wash cell monolayers and harvest cells. This reduces the polysome "run-off" that occurs when cold PBS is applied (Dr C. Darnborough, personal communication; Dr C. Schneider, personal communication). Other alterations included: (a) changing the cell culture medium to EF5 containing cycloheximide (20ug/ml), 15 min. before harvesting, (b) reducing the time taken to homogenize cells, pellet nuclei and add 1M $MgCl_2$, to less than 4 min. (delays at this stage are very detrimental to polysome preparations; Dr M. Oren, personal communication), (c) preparation of sucrose gradients using highly pure BRL sucrose, to obviate the need to autoclave sucrose solutions (some batches of autoclaved sucrose solution had been previously found to raise the background absorbance of UV at 260nm and consequently obscure the polysome peaks), and (d) technical improvements in the method of sucrose gradient sedimentation profile analysis. With regard to the latter, experiments were carried out to optimize the resolving power of the pump, tubing and spectrophotometer system. The effects of alterations on resolution were assessed by pumping two 20ul boluses of RNA solution, separated by 200ul water, through the system while continuously monitoring the A_{260} using a Cecil CE500 control record module. Resolution of the two A_{260} peaks was increased by: (i) finding the optimum pump speed (a flow rate of 4ml per min. gave the best resolution; figure 5.2), and (ii) reducing the length of tubing through which gradient fractions were pumped before reaching the spectrophotometer flow cell. A major length reduction was achieved by placing the gradient next to the flow cell inside the spectrophotometer and using a

Figure 5.2; Optimizing conditions for recording polysome sedimentation profiles. Graphs **A**, **B** and **C** show the effect of different pump speeds on the resolution of A_{260} peaks corresponding to two 20ul boluses of an RNA solution, separated by 200ul distilled water (DW), which were pumped through a spectrophotometer flow cell. Graph **D** shows the effect of reducing the path length between sucrose gradient and flow cell from 40cm (as in A, B and C), down to 10cm.


A
0.03
0.00
A260
0
50
100
Time (Secs)
B
0.03
0.00
A260
50
100
Time (Secs)
C
0.03
0.00
A260
50
100
Time (Secs)
D
0.06
0.03
0.00
A260
0
50
100
Time (Secs)

pump placed outside to "pull" fractions through the flow cell (figure 5.2).

5.1.2 Immunoselection of polysomes

The technique of Kraus and Rosenberg (1982) was used to immunoselect Bn5T polysomes (see section 3.4.6). Polysome immunoselection was attempted twice with TBS and once with TG7A.

The first approach used to purify antibody for immunoselection and remove RNases, was purification by protein-A sepharose CL4B chromatography. 1ml TBS was applied to a 1ml protein-A sepharose column in 4ml 0.1M sodium phosphate pH7.4, at a flow rate of 5ml/h for 18h. After washing with 5 column volumes of the phosphate buffer, antibody was eluted with 0.1M glycine-Cl pH2.5. Eluate fractions were immediately adjusted to neutral pH by the addition of 1M tris-Cl pH9.0 (75ul/ml eluate). Antibody containing fractions were identified by approximate determination of their protein concentrations by measuring absorbance directly. An A_{280} reading of 1.0 was taken to indicate 1mg/ml of protein (Cantor and Schimmel, 1980). It was estimated that approximately 2.5mg antibody was purified from 1ml TBS. To reduce RNase activity further, human placental ribonuclease inhibitor (HPRI) was added to the purified antibody to a concentration 1U/ul. HPRI is a 50kDa protein which forms a 1:1 complex with bovine pancreatic RNase A and is a non-competitive inhibitor of the pancreatic enzyme with a K_i of $3X10^{-10}M$ (Blackburn et al., 1977).

On the first occasion polysome immunoselection was tried, no immunoselected message could be in vitro translated (IVT). The problem was unlikely to be due to RNase degradation of

polysomes as poly-A^+ RNA prepared from breakthrough fraction polysomes translated well *in vitro* and polypeptides of up to 200kDa were synthesised. The possible explanations considered were:

(a) No Bn5T polysomal epitopes recognized by TBS: The epitope recognized by TBS might depend on the conformation adopted by the polypeptide chain of the native protein. Alternatively, it might result from post-translational processing of the polypeptide.

(b) Inactivation of antibody during purification: The antibody might have been denatured by exposure to low pH glycine buffer. The antibody should have been tested in an immunoprecipitation assay before use in immunoselection to examine this possibility.

(c) Antibody and/or immune complexes removed by excessive washing: If the antibody showed low affinity binding to protein-A sepharose excessive washing could remove it. The use of TBS-protein-A sepharose immunoaffinity columns to purify Bn5T:TBS polypeptides had given very poor yields (Dr J. Macnab, personal communication), which would be consistent with low affinity binding. These columns, which were prepared by Dr J. Macnab and Mr A. Orr, were washed extensively before use of dimethyl pimelimidate to covalently bind the antibody to protein-A sepharose (Schneider *et al.*, 1982).

(d) RNase degradation of the immunoselected message: A very low concentration of immunoselected message leading to RNase susceptibility and inefficient ethanol precipitation.

On the second occasion that polysome immunoselection was attempted, both TBS and TG7A (ascites) were used for immunoselection. The following modifications were made to the protocol, taking into account the possible explanations listed above for the initial failure of polysome immunoselection.

Antibody was reacted with the protein-A sepharose before the addition of polysomes, so that the antibody could be washed without the need for elution and re-binding. Antibody bound to protein-A sepharose was washed with 20 bed volumes of buffer. Following this 10 bed volumes of buffer containing 1U/ul HPRI were added, the column sealed and then incubated on a rotating mixer for 2h. Buffer was then run through the column, polysomes added and the column sealed again and then incubated as before. Columns were then unsealed and washed with only 4 column volumes of buffer A^-.

Duplicate columns were eluted with 4 column volumes of polysome elution buffer containing HPRI (1U/ul), either with or without 2ug of calf thymus tRNA. The tRNA was to act as carrier RNA to protect the small amount of immunoselected message from RNases, and to aid ethanol precipitation. Eluted fractions were collected in pre-cooled Eppendorf tubes containing appropriate volumes of ethanol and sodium acetate, then vortexed and ethanol precipitated at -70^oC overnight. Breakthrough fraction polysomes were treated as before, that is, phenol extracted and then poly-A^+ selected.

IVT of immunoselected mRNA preparations did not produce any detectable polypeptide bands that were not also seen for the minus RNA control. Thus polysome immunoselection by TBS or TG7A was unsuccessful, either with or without the use of carrier RNA. IVT of TBS breakthrough fraction mRNA produced ill-defined polypeptide bands above 90kDa, suggesting that

RNases may have been a problem. In contrast however, IVT of TG7A breakthrough fraction mRNA gave a protein profile indistinguishable from IVT poly-A^+ RNA prepared from total polysomes (figure 5.3). This indicated that for TG7A immunoselection at least, the RNase problem had been overcome.

Following this negative result, it was decided to test whether or not the epitopes recognized by TBS and TG7A, are expressed on nascent polypeptide chains during translation on the polysomes. An approach suggested by Dr C. Preston, was to test whether polypeptides synthesised by IVT of Bn5T RNA could be i.p. with these antibodies. This led to the interesting finding described below, that the Bn5T:TBS set of polypeptides could not be detected in the IVT products of Bn5T RNA or polysomes.

5.2 Experiments to detect Bn5T:TBS polypeptides synthesised in vitro

5.2.1 Analysis of total polypeptides produced by IVT

(a) Total cytoplasmic RNA: ^{35}S-methionine labelled polypeptides resulting from *in vitro* translation (IVT) of total Bn5T cell RNA with rabbit reticulocyte lysate, were analysed by SDS-PAGE. Three different preparations of Bn5T cell cytoplasmic RNA were prepared by the author for IVT. The polypeptide products of six separate IVT reactions, were analyzed by SDS-PAGE, some of them more than once. Altogether ten different gels were examined and results were entirely consistent. As illustrated in figure 5.4, many Bn5T polypeptides were successfully synthesised *in vitro*, but in

Figure 5.3; Results of Bn5T polysome immunoselection with TG7A and TBS. Autoradiograph of a 6-15% gradient polyacrylamide gel containing electrophoretically separated ^{35}S-methionine labelled polypeptides. This shows the polypeptides IVT in rabbit reticulocyte lysate from poly-A$^+$ RNA prepared from total Bn5T cell polysomes (tracks 1 and 7) and breakthrough polysomes after TG7A and TBS immunoselection (tracks 2 and 8 respectively). Tracks 3 and 4 contain IVT products of TG7A immunoselected RNA and tracks 9 and 10 contain IVT products of TBS immunoselected RNA. RNA prepared from immunoselected polysomes was eluted from protein-A sepharose in the absence (tracks 3 and 9) or presence (tracks 4 and 10) of calf thymus carrier tRNA. The products of minus RNA control IVT reactions are seen on tracks 5 and 11. Total Bn5T cell polypeptides are shown in track 6.

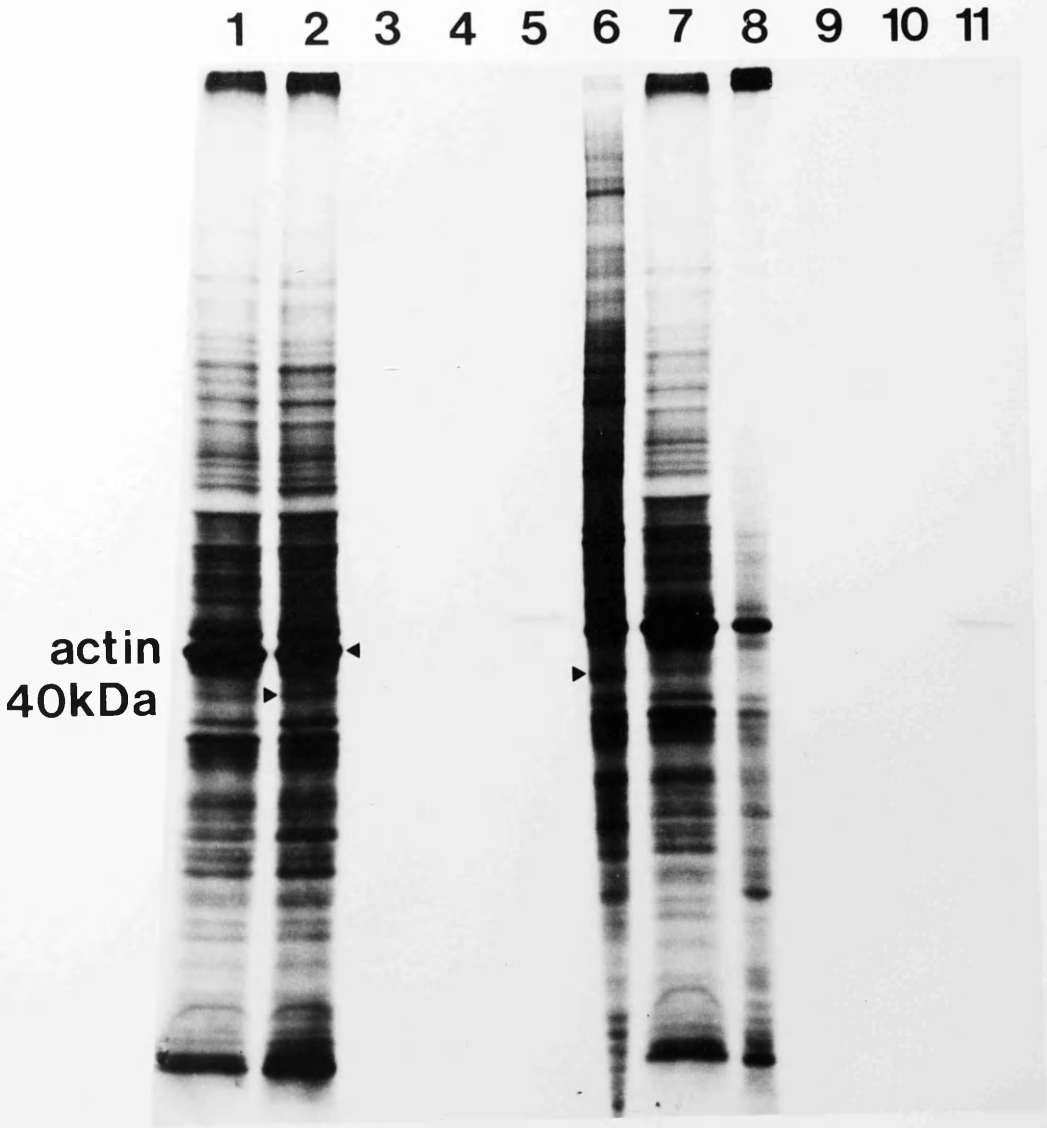
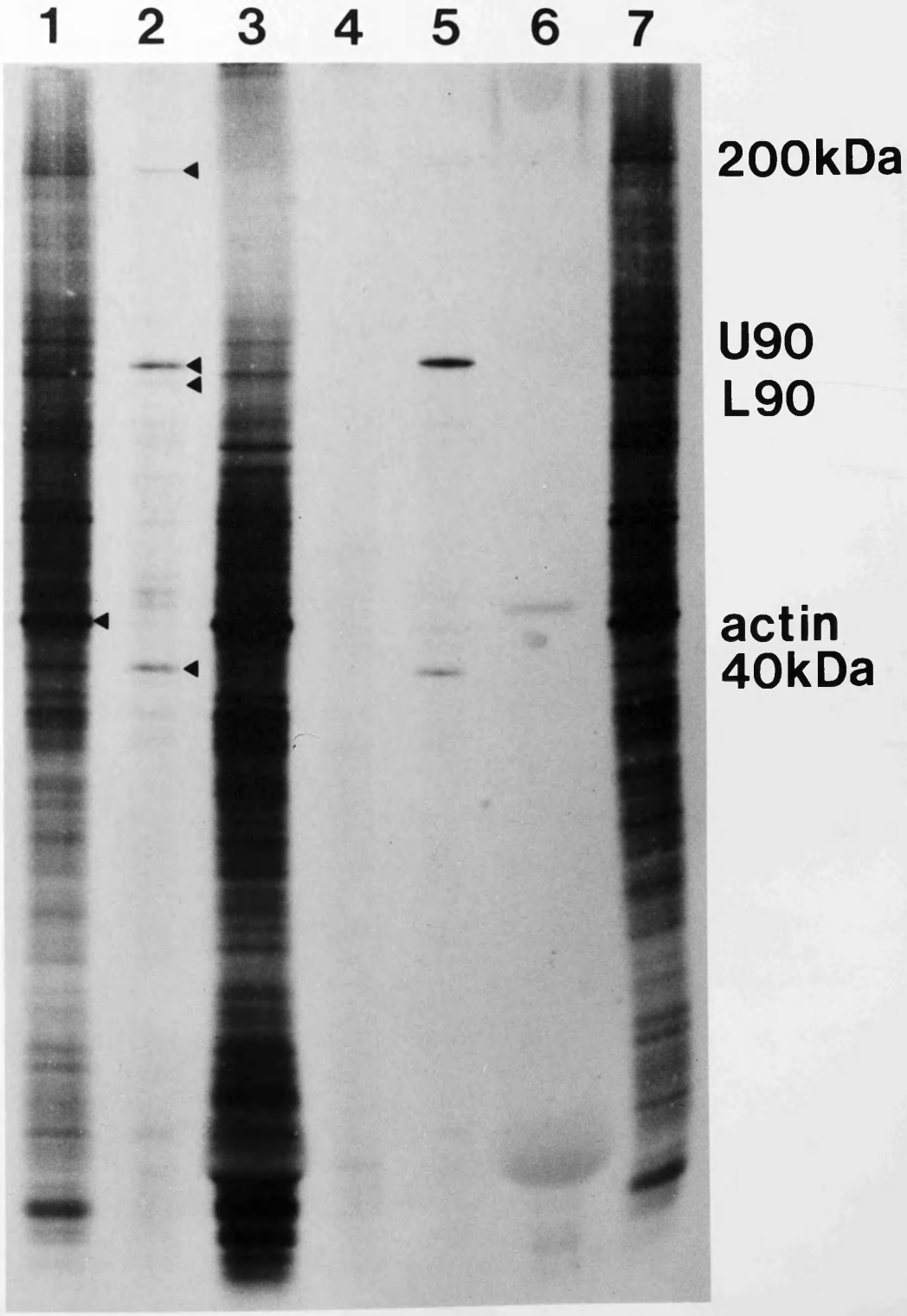

1 2 3 4 5 6 7 8 9 10 11
actin
40kDa

Figure 5.4; Electrophoretic analysis of Bn5T polypeptides synthesised <u>in vitro</u>. Autoradiograph of a 6-15% gradient polyacrylamide gel containing electrophoretically separated ^{35}S-methionine labelled polypeptides. Total Bn5T cell polypeptide profiles are seen in tracks 1 and 7 and track 2 shows a standard TBS i.p. of Bn5T cell lysate. Total polypeptides resulting from IVT of Bn5T cell cytoplasmic RNA are seen in track 3 and a TBS i.p. of these polypeptides is seen in track 4. Track 5 shows a TBS i.p. of IVT polypeptides mixed with total cell polypeptides and track 6 shows the products of a minus RNA control IVT reaction.


1
2
3
4
5
6
7
200kDa
U90
L90
actin
40kDa

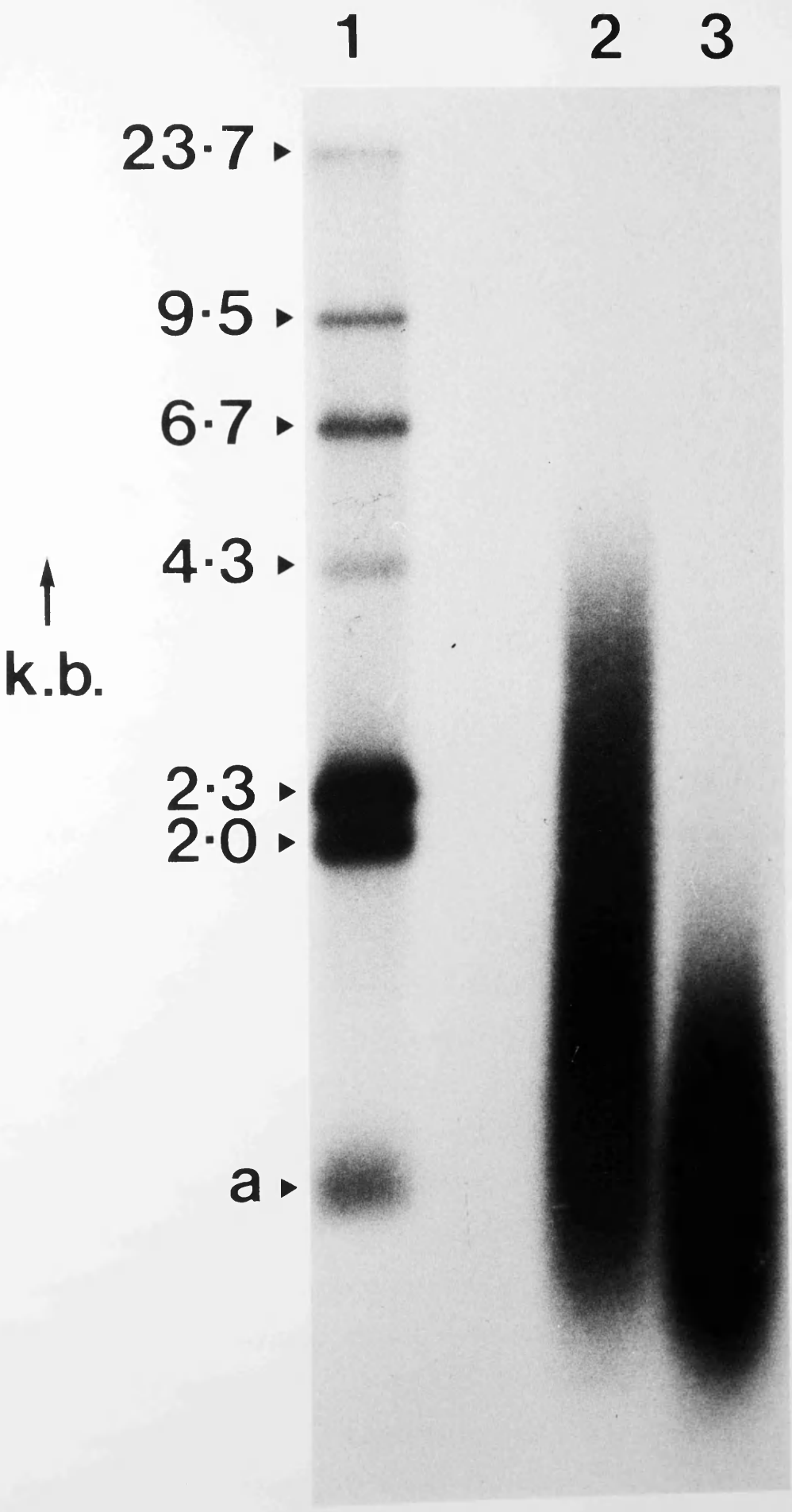
contrast to profiles of total Bn5T cell polypeptides, no 40kDa polypeptide band was seen.

(b) Poly-A^+ RNA: A total of five different preparations of Bn5T cell poly-A^+ RNA were prepared for IVT, three by Mr G. Patterson and two by the author. Poly-A^+ RNA was selected from total Bn5T RNA prepared by the guanidinium/caesium chloride method (see section 3.4.1) by either oligo-dT cellulose column chromatography or the use of Hybond messenger-activated paper selection, using the protocol provided by the suppliers. The latter technique was used by Mr G. Patterson for two of his three poly-A^+ RNA preparations. Poly-A^+ Bn5T RNA prepared by the author using an oligo-dT cellulose column was used for the preparation of a cDNA library by Dr J. Macnab. The author analysed the resulting 1st cDNA strand by alkaline denaturing gel electrophoresis (McDonnell et al., 1977). The majority of the cDNA was in the 1k.b. to 2k.b. range (fig 5.5) indicating that the poly-A^+ Bn5T RNA synthesised was of an acceptable quality.

IVT products of the five different preparations of poly-A^+ Bn5T RNA were analysed separately by SDS-PAGE on a total of six different gels. In every case, exactly the same result was obtained as for preparations of cytoplasmic RNA. No evidence of the 40kDa polypeptide band was seen on any of the polypeptide profiles.

(c) Total cell polysomes: Four different Bn5T cell polysome preparations were IVT and translation products analyzed by SDS-PAGE on ten different gels. The result was the same as for IVT of cytoplasmic RNA and poly-A^+, with one exception. On one occasion, polysomes were IVT for time periods ranging

Figure 5.5; Analysis of cDNAs by agarose gel electrophoresis. Autoradiograph of a alkaline denaturing (1%) agarose gel containing the electrophoretically separated first cDNA strands, synthesised from Bn5T RNA (track 2) and HSV-2 infected RE cell RNA (track 3). End-labelled lambda Hind III restriction fragments are seen in track 1 and their sizes in k.b. are indicated on the left side of the gel. The identity of band labelled "a" was not determined.


1
2
3
23·7
9·5
6·7
4·3
k.b.
2·3
2·0
a

from 2.5 min. to the standard period of 60 min. On SDS-PAGE analysis of volumes of IVT polypeptides corresponding to 7X104 c.p.m., it was found that a 40kDa band which is clearly visible at 2.5 min., becomes progressively less prominent with increasing times of incubation, such that at 60 min. it was not visible above background (data not shown). Shortage of time prevented repetition of this experiment.

(d) Poly-A^+ RNA prepared from total cell polysomes: Poly-A^+ RNA prepared from Bn5T polysomes, was IVT on two different occasions as part of the polysome immunoselection procedure. The IVT polypeptide products were analyzed by SDS-PAGE on a total of three gels. On one of these gels only, a weak 40kDa polypeptide band was seen (figure 5.3).

(e) Conclusions: A detectable 40kDa Bn5T polypeptide was produced by IVT, in some special circumstances, which comigrated with the Bn5T:TBS:40. However, the band produced on autoradiography was always weaker than that of the total cellular polypeptide profile. It is important to note that the 40kDa band of polypeptide profiles of Bn5T cell lysate, gives a very similar V8 protease peptide map to Bn5T:TBS:40 and Bn5T:TG7A:40 (see section 6.2.2). This implies that the Bn5T:TBS:40 is only synthesised to a limited extent, (at most), by IVT. Two possible explanations why the 40kDa polypeptide produced by IVT of polysomes was only detectable after short incubation periods are that: (i) the mRNA encoding the 40kDa polypeptide is unstable in reticulocyte lysate, and (ii) initiation of translation in reticulocyte lysate is inefficient and the 40kDa polypeptide synthesised *in vitro* results mainly from polysome "run-off". It is important

to note however, that the 40kDa polypeptide produced by IVT may not be related to the Bn5T:TBS:40 polypeptide. The only available evidence of identity is comigration on SDS-PAGE.

No conclusions can be reached from the analysis of IVT polypeptides about the *in vitro* synthesis of 97kDa and 200kDa members of the Bn5T:TBS set. The bands in total cell polypeptide profiles, thought to correspond to these i.p. polypeptides, are very faint and only visible on gels showing particularly good resolution. The bands seen on SDS-PAGE analysis of IVT polypeptides were never as well resolved as the bands seen for cell lysates.

5.2.2 Immunoprecipitation assays on polypeptides produced by IVT

(a) Total cytoplasmic RNA: IVT cytoplasmic RNA was i.p. by TBS on three different occasions by the author. It was also i.p. once with TBS and once with TG7A by Mr A. Orr. On SDS-PAGE analysis of TBS i.p.s, no polypeptide bands were seen, on any occasion, in the vicinity of polypeptide bands of the Bn5T:TBS set (figure 5.4). One positive control used in this experiment was a TBS i.p. of the following mixture: Radiolabelled Bn5T cell lysate added to reticulocyte lysate which had been IVT in the absence of RNA. The amount of radiolabelled cell lysate required for a single standard i.p. ($4X10^6$ c.p.m.) was used for this positive control i.p., and the volume:volume ratio of Bn5T cell lysate to reticulocyte lysate, never exceeded 1:20. In every case the positive control i.p. gave exactly the same result as a standard Bn5T:TBS i.p., indicating that no reticulocyte lysate component was interfering significantly with the i.p.

reaction. Ideally, another positive control should have been included, using an antibody directed against a normal cellular polypeptide to i.p. IVT Bn5T polypeptides and in repeat experiments this would need to be tested. The total polypeptide profile of IVT products of Bn5T RNA was a further positive control used in this experiment, and this showed that polypeptides over a MW range from 200kDa to less than 30kDa were being synthesised. The faint and poorly defined bands of high MW polypeptides indicate that they did not translate well. However, results were entirely satisfactory for polypeptides with MWs below 110kDa.

For the TG7A i.p. of <u>in vitro</u> translated Bn5T polypeptides very weak bands were seen comigrating with 200kDa, L90 and 40kDa Bn5T:TBS polypeptides. The significance of this is doubtful however, as there was a high background in this i.p. and many bands of greater prominence were visible (data not shown). Unfortunately, the limited supply of TG7A monoclonal made it impossible to repeat this experiment.

(b) Total cell polysomes: Polysomes from three different preparations were IVT and i.p. with TBS by the author. Mr G. Patterson also made a polysome preparation and assayed by i.p. with TBS. The i.p.s were separately analyzed by SDS-PAGE on five different gels. No evidence was seen on any of these gels of bands comigrating with those of the Bn5T:TBS set. In addition, two different preparations of polysomes were IVT and i.p. with TG7A, but no bands were seen on SDS-PAGE analysis of these i.p.s.

(c) Conclusions: Two possible reasons for the absence of detectable Bn5T:TBS polypeptides after IVT of Bn5T RNA and

polysomes are: (i) the levels of Bn5T:TBS polypeptides produced by IVT are too low for detection, and (ii) the polypeptides are not post-translationally processed in the IVT system, to forms recognizable by TBS or TG7A. Experiments were therefore carried out to investigate the possible post-translational processing of Bn5T:TBS polypeptides.

5.3 Experiments to detect post-translational processing

5.3.1 Analysis of polypeptides produced by IVT in the presence of canine pancreatic microsomal membranes

Microsomal membranes are used to study two main types of post-translational or co-translational processing. One is the removal of signal peptides (Dorner and Kemper, 1978) and the other is glycosylation of pre-secretory or membrane proteins (Rothman and Lodish, 1977). As a positive control human placental lactogen mRNA, which encodes a polypeptide with a signal peptide, was IVT in the presence or absence of microsomal membranes. As was expected, a polypeptide of approximately 30kDa was IVT without the microsomal membranes and on addition of the membranes a lower MW polypeptide band (approximately 20kDa) appeared. Thus, the microsomal membranes were working effectively.

Two different preparations of polysomes were IVT in the presence of canine pancreatic microsomal membranes on three different occasions, twice by the author and once by Mr G. Patterson. No evidence of a 40kDa polypeptide band was seen in the total polypeptide profile of polysomes IVT in the presence or absence of microsomal membranes. Nor was it possible to i.p. polypeptides migrating at a similar rate to

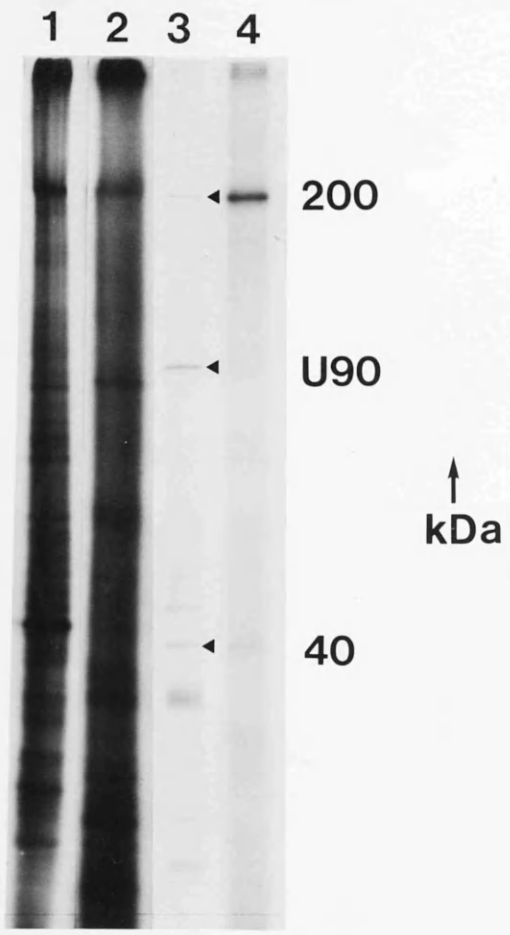


Figure 5.5; Analysis of transformed cell (Bn5T) polypeptides by SDS-PAGE. Autoradiograph of a 6-15% gradient polyacrylamide gel containing electrophoretically separated ^{35}S-methionine labelled (tracks 1 and 3), and ^{32}P-orthophosphate labelled polypeptides (tracks 2 and 4). Total cell polypeptides are seen in tracks 1 and 2, and TBS i.p. polypeptides in tracks 3 and 4. Polypeptides i.p. by TBS are indicated by solid arrows in track 3 and the designated MW values given on the right side of the gel.

Bn5T:TBS bands, despite three attempts using TBS and one attempt using TG7A (data not shown).

5.3.2 Metabolic labelling with ^{32}P-orthophosphate

Lysate from Bn5T cells radiolabelled *in vivo* with ^{32}P-orthophosphate was i.p. with TBS and then subjected to SDS-PAGE. Autoradiography showed a ^{32}P-labelled band comigrating with the ^{35}S-labelled Bn5T:TBS:200 and :40 of a standard Bn5T:TBS i.p. run in an adjacent track (figure 5.5). No ^{32}P-labelled bands were seen in the 90 - 100kDa region, a finding confirmed by Dr D.O. Toft (personal communication), nor were any seen in the 30-36kDa region. This suggests that the Bn5T:TBS:200 and :40 are phosphoproteins, unlike the Bn5T:TBS:U90, L90, :35 and :34.

5.3.3 Metabolic labelling with ^{3}H-mannose

It was not possible to demonstrate any glycosylated Bn5T:TBS i.p. polypeptides by radiolabelling with ^{3}H-mannose (data not shown). Treatment of Bn5T cells with tunicamycin resulted in the loss of the low MW component of the 34kDa band (see Table 4.1) indicating that it may be a glycoprotein, but no other changes were seen (data not shown).

5.4 Conclusions

The only direct evidence found here of post-translational processing of major Bn5T:TBS polypeptides, is for the phosphorylation of 40kDa and 200kDa polypeptides. No direct evidence was found of post-translational modifications common to all polypeptides of the Bn5T:TBS set, which might account for our inability to i.p. any one of these polypeptides from the products of IVT Bn5T RNA.

It is possible that Bn5T:TBS polypeptides require post-translational changes to occur such as complex formation with other cellular components, in order to be i.p. by TBS and TG7A. If this is true then TBS and TG7A are unlikely to recognize epitopes expressed by polysomes on which Bn5T:TBS polypeptides are synthesised. Another possible explanation for our inability to i.p. Bn5T:TBS polypeptides translated *in vitro*, is that the reticulocyte lysate system is unsuitable for the efficient IVT of mRNAs encoding Bn5T:TBS polypeptides. Whatever the correct explanation, our inability to detect IVT Bn5T:TBS polypeptides suggests that it would also be impossible to IVT mRNA immunoselected by TBS or TG7A. This presents a serious problem, as in order to demonstrate that a specific mRNA has been purified by the polysome immunoselection technique, it is important to be able to analyze that mRNA by IVT and SDS-PAGE. Thus, our inability to IVT polypeptides recognized by TBS or TG7A, suggests that polysome immunoselection is a technique ill-suited for the Bn5T:TBS system. Its use was therefore discontinued.

6 COMPARING TUMOUR ANTIGENS RECOGNISED BY TBS AND TG7A

The TG7A monoclonal antibody was previously found to recognize polypeptides of 90kDa, 40kDa and 32kDa in Bn5T cells (Macnab et al., 1985a). Bn5T polypeptides i.p. by TG7A and TBS, were analysed and compared by one-dimensional peptide mapping using Staphylococcus V8 protease at a single enzyme concentration. The 90kDa polypeptide band (U90 and L90 combined) gave indistinguishable results on peptide mapping, whether from Bn5T:TG7A or Bn5T:TBS i.p.s. The 40kDa polypeptides were found to be similar but not identical and the same was found for 32kDa polypeptides. Results are presented in this chapter of a further and detailed investigation into the relationship between Bn5T polypeptides recognized by TBS and TG7A.

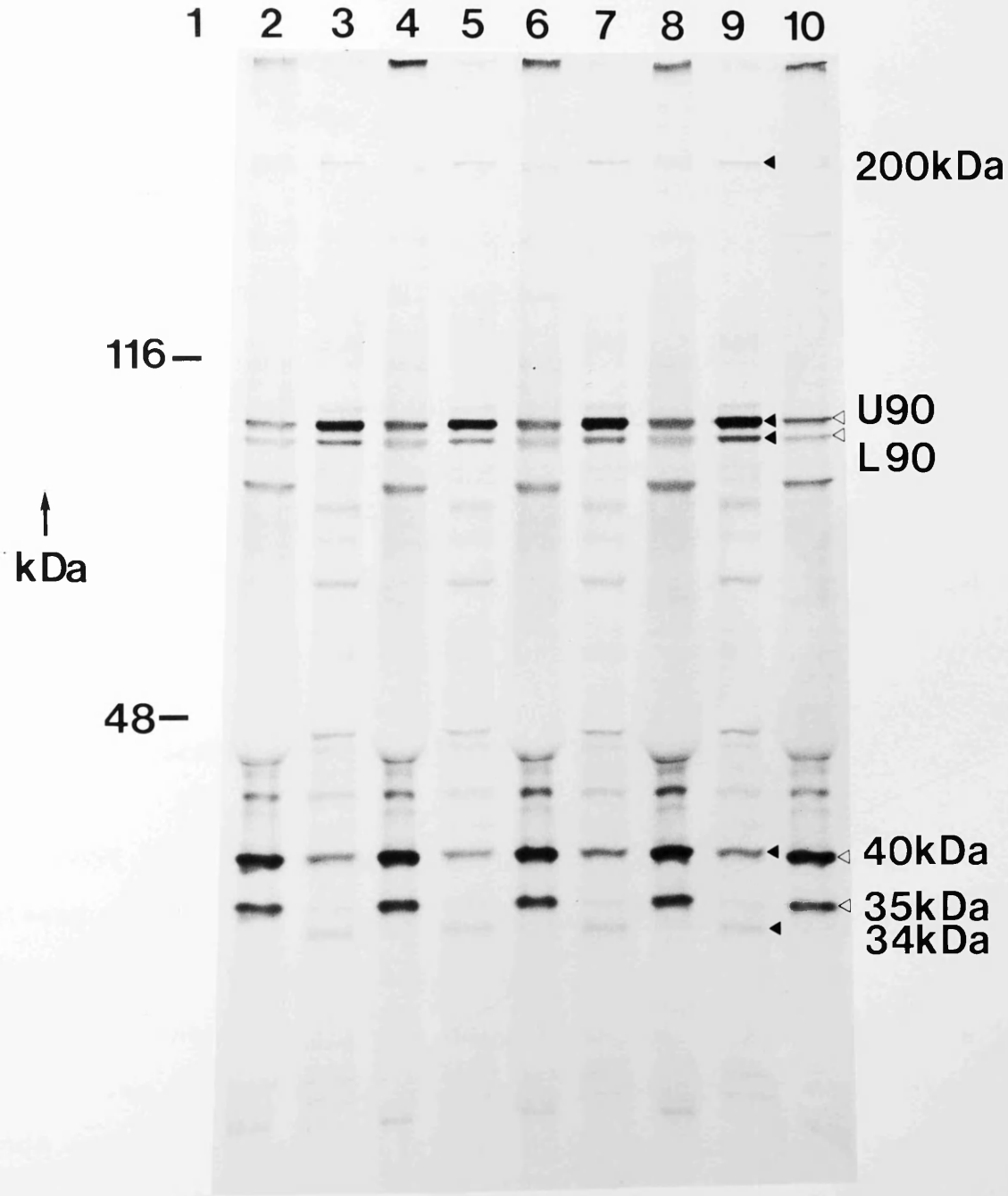
6.1 Analysis of immunoprecipitates by SDS-PAGE

In all cases where Bn5T:TBS:U90, L90 and 40kDa polypeptides could be i.p. by TBS from a cell lysate, these polypeptides were always i.p. in addition by the TG7A monoclonal. These polypeptides were never found to be i.p. by only one of these two antibody preparations.

(a) Comigrating polypeptides: Analysis by SDS-PAGE shows that for each polypeptide i.p. by TBS from Bn5T lysate, there is a comigrating polypeptide i.p. by TG7A (Figure 6.1). Thus, in each case there are polypeptides of 200, 97, 93, 40, 35, and 34kDa.

(b) Different precipitation ratios: The intensity of bands seen on autoradiography indicates that the precipitation ratio for TBS and TG7A polypeptides is quite different (figure

Figure 6.1; Comparison of Bn5T:TBS and Bn5T:TG7A polypeptides by SDS-PAGE. Autoradiograph of a 6-15% gradient polyacrylamide gel containing electrophoretically separated ^{35}S-methionine labelled polypeptides i.p. from Bn5T cell lysate by TG7A (tracks 2, 4, 6, 8 and 10) and TBS (tracks 3, 5, 7 and 9). Bands of interest are indicated by solid arrows for the TBS i.p. (track 9) and outlined arrows for the TG7A i.p. (track 10). The designated MW or notation for each of these bands is given on the right side of the gel. MW markers are shown in track 1.


1 2 3 4 5 6 7 8 9 10
200kDa
116—
U90
L90
kDa
48—
40kDa
35kDa
34kDa

6.2). For TBS the U90 is the major band, usually followed by the L90, 200kDa, 40kDa, 34kDa and 35kDa in order of intensity. The TBS 35kDa is often too faint to see. For TG7A in contrast, the strongest bands are of 40 and 35kDa, followed by the U90 and L90. The TG7A 200 and 34kDa bands are often too faint to see.

(c) Polypeptides of 34kDa and 35kDa: In Bn5T:TBS i.p.s the major band below 40kDa is of slightly lower apparent MW than that of TG7A i.p.s (figure 6.1). For TBS i.p.s the 34kDa polypeptide gives a major band, while for TG7A it is a 35kDa polypeptide which gives a major band.

6.2 One-dimensional peptide mapping analysis

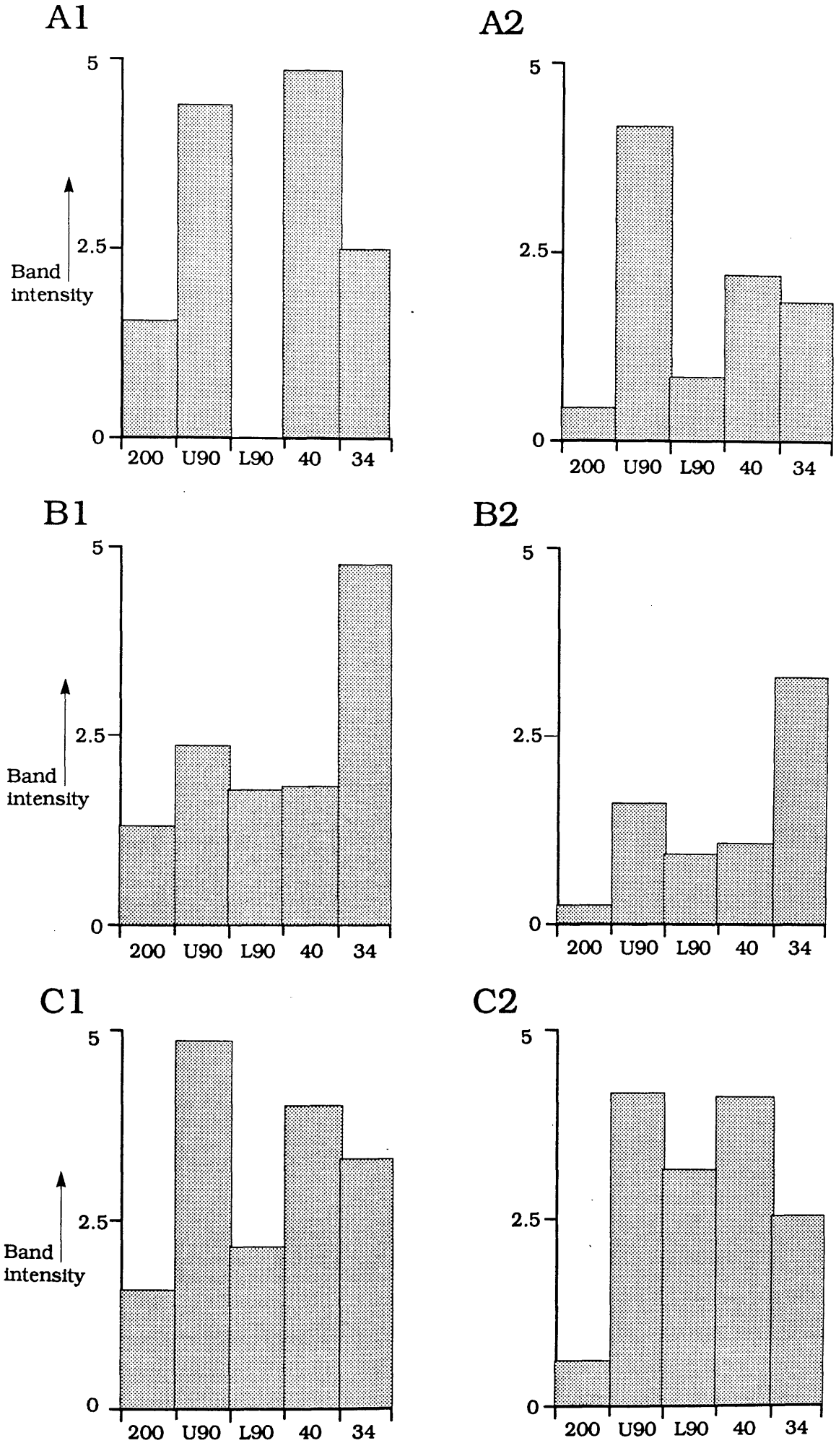
Comparisons were made by one-dimensional peptide mapping analysis using the Cleveland digest technique (Cleveland et al., 1977), between comigrating polypeptides i.p. by TBS and TG7A.

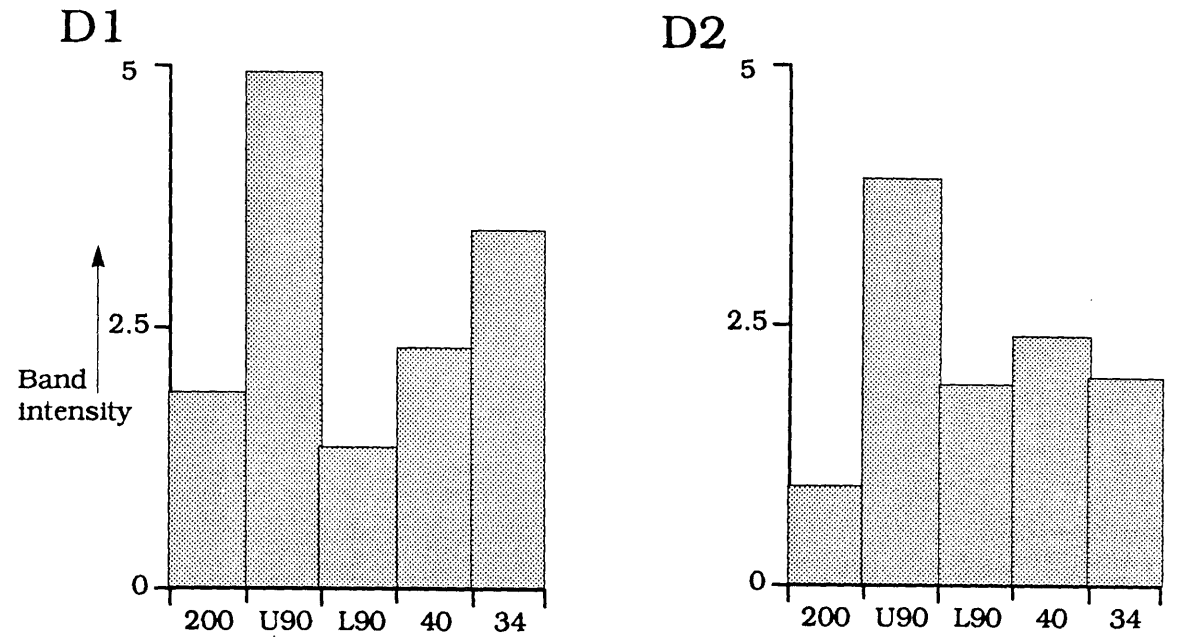
6.2.1 Comigrating polypeptides of 90 - 100kDa

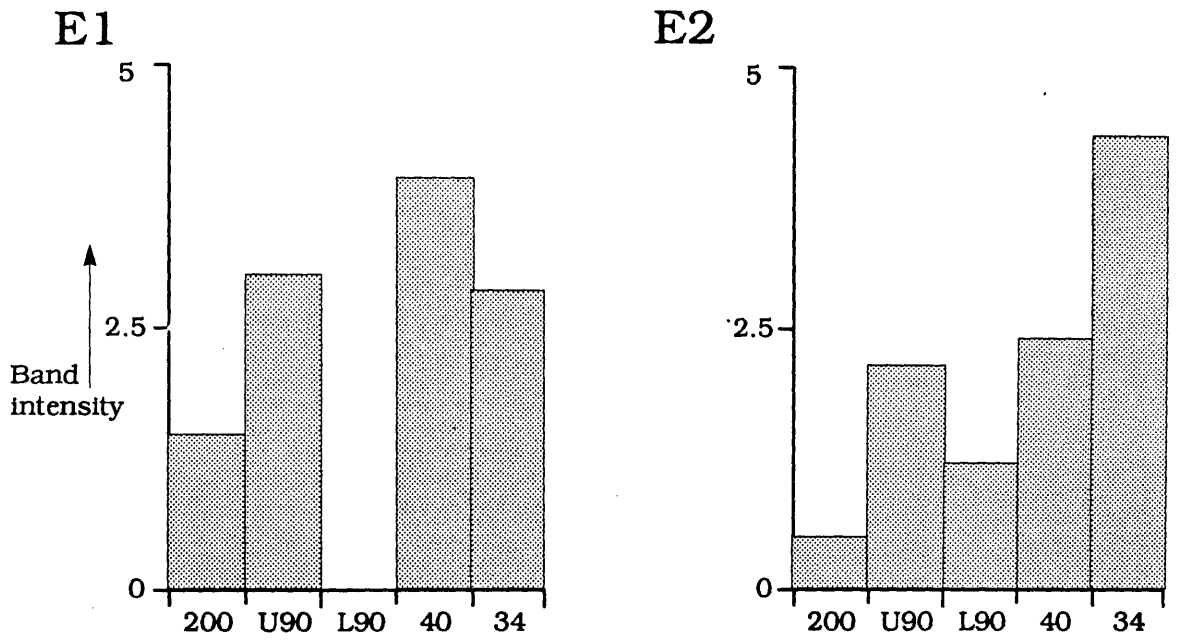
(a) Bn5T U90 polypeptide: No differences were detected between Bn5T U90 polypeptides i.p. by TBS and TG7A with either V8 protease or chymotrypsin, over a range of enzyme concentrations. In digests of the TBS and TG7A polypeptides with 1/25ug V8 protease, all of the fifteen resulting peptide bands comigrated exactly (figure 6.3), as did all of the eight bands produced by 1/25ug chymotrypsin (figure 6.4).

(b) Bn5T L90 polypeptide: Similarly, no differences were found when comparing L90 polypeptides in this way. On

Figure 6.2; Densitometric analysis of TBS and TG7A i.p.s of Bn5T cell lysate, analyzed by SDS-PAGE and autoradiography. The intensities of major bands seen on autoradiography (200kDa, U90, L90, 40kDa, 35kDa and 34kDa) are expressed graphically. Batches of TBS prepared in different animals (batches A to E) were used to i.p. the same preparation of Bn5T cell lysate, in two replicate experiments. Accordingly, graphs **A1** and **A2** represent profiles of Bn5T polypeptides i.p. by TBS batch A, in experiments 1 and 2. The same rule applies for graphs **B1** and **B2**, **C1** and **C2**, **D1** and **D2** and **E1** and **E2**. Graph **F** shows the band intensities in a typical Bn5T:TG7A i.p.


A1
5
2.5
0
Band intensity
200
U90
L90
40
34
A2
5
2.5
0
200
U90
L90
40
34
B1
5
2.5
0
Band intensity
200
U90
L90
40
34
B2
5
2.5
0
200
U90
L90
40
34
C1
5
2.5
0
Band intensity
200
U90
L90
40
34
C2
5
2.5
0
200
U90
L90
40
34


D1
5
2.5
0
Band intensity
200
U90
L90
40
34
D2
5
2.5
0
200
U90
L90
40
34


E1
5
2.5
0
Band intensity
200
U90
L90
40
34
E2
5
2.5
0
200
U90
L90
40
34

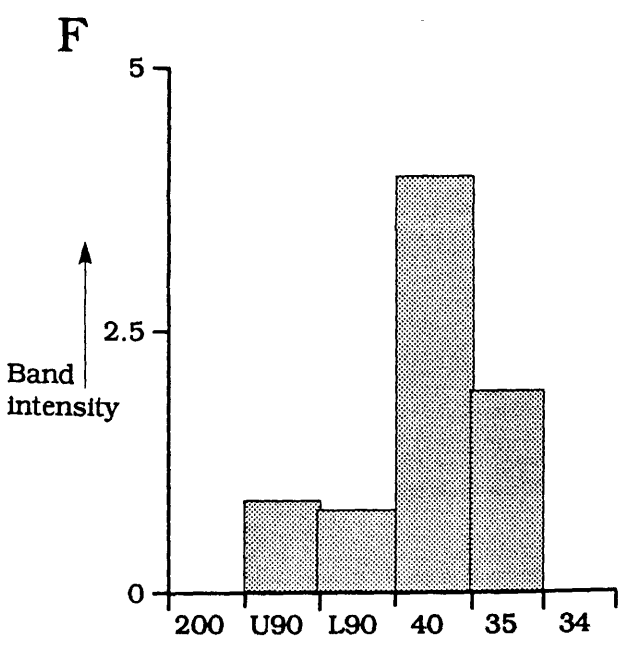

F
5
2.5
0
Band intensity
200
U90
L90
40
35
34

Figure 6.3; V8 protease peptide maps of the Bn5T:TBS:U90 and Bn5T:TG7A:U90 compared. Cleveland digest analysis of the U90 polypeptide as i.p. from Bn5T cell lysate by TG7A (tracks 1-3) and TBS (tracks 4-6). Tracks 1 and 4 contained 1/25ug, tracks 2 and 5 contained 1/5ug and tracks 3 and 6 contained 1ug of S.aureus V8 protease. MW markers are shown in track 7.

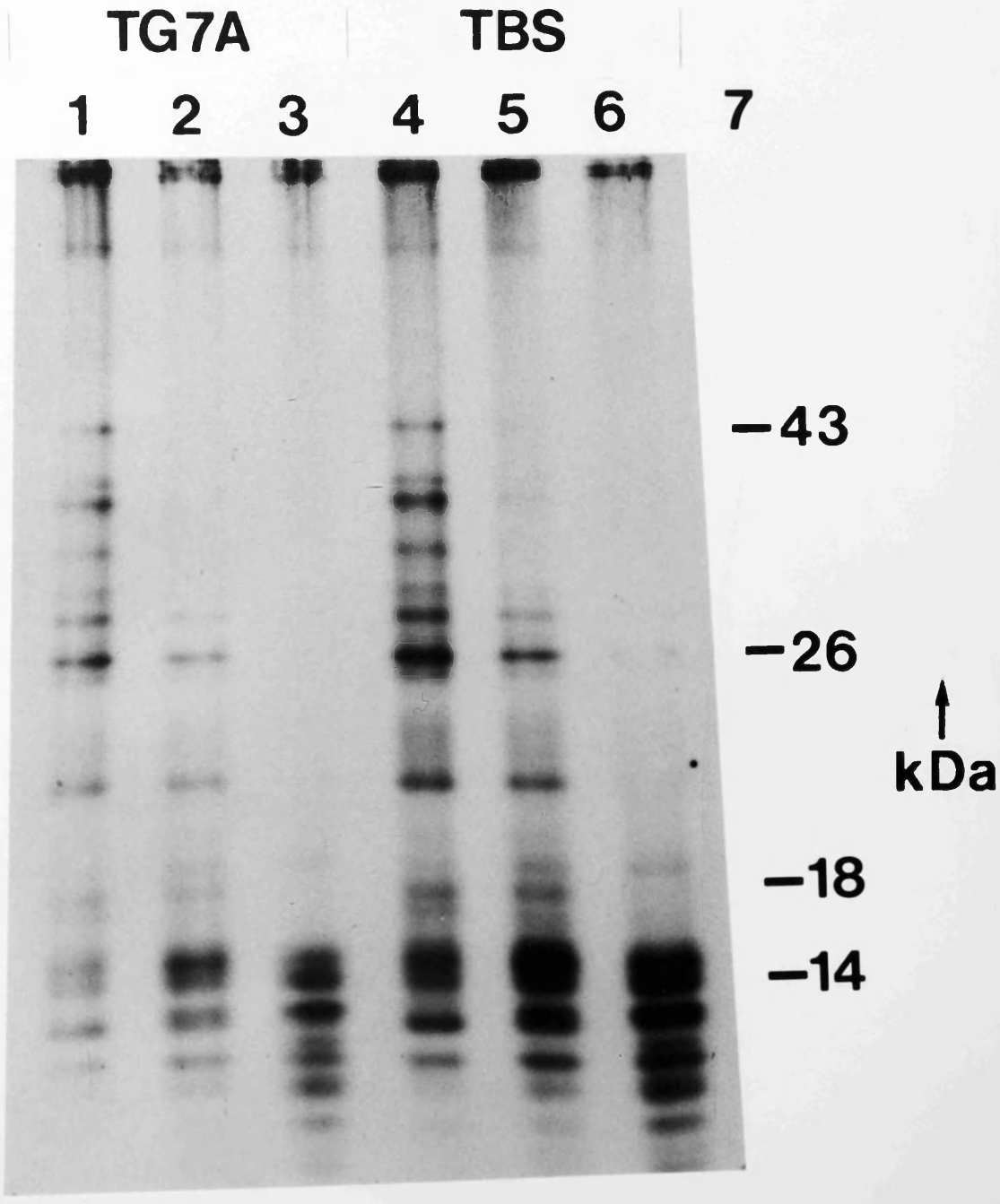
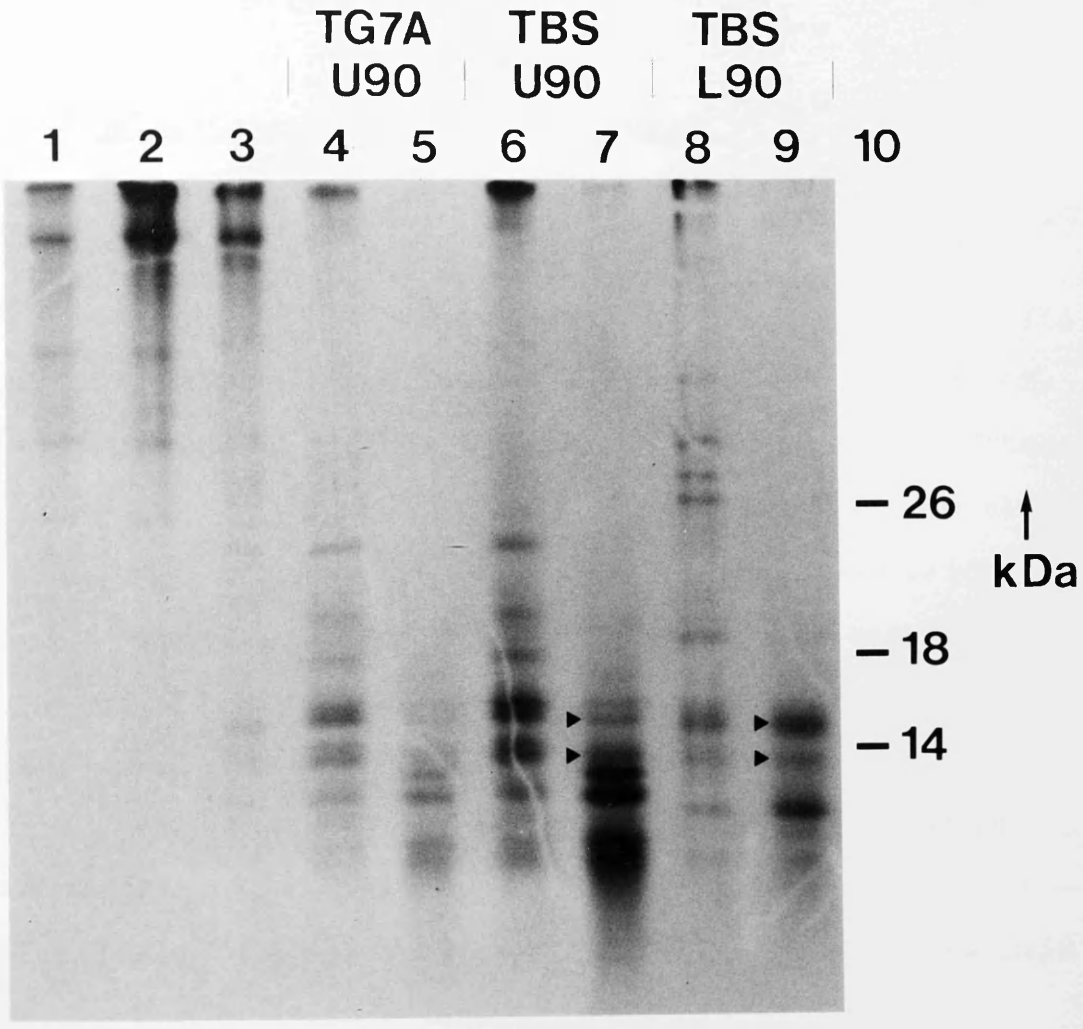

TG7A
TBS
1
2
3
4
5
6
7
43
26
18
14
kDa

Figure 6.4; Chymotrypsin peptide maps of Bn5T:TG7A:U90, Bn5T:TBS:U90 and L90. Cleveland digest analysis using chymotrypsin of U90 and L90 polypeptides i.p. from Bn5T cell lysate. The U90 polypeptide i.p. by TG7A (tracks 4 and 5) is compared with the U90 (tracks 6 and 7) and L90 (tracks 8 and 9) polypeptides i.p. by TBS. Tracks 4, 6 and 8 contained 1/25ug and tracks 5, 7 and 9 contained 1ug of chymotrypsin. Comigrating bands in digests of TBS U90 and TBS L90 are indicated by the arrows in tracks 7 and 9 respectively. Undigested TG7A U90, TBS U90 and TBS L90 are seen in tracks 1, 2 and 3 respectively. MW markers are seen in track 10.


TG7A
U90
TBS
U90
TBS
L90
1
2
3
4
5
6
7
8
9
10
26
18
14
kDa

digestion with 1/25ug V8 protease seventeen out of seventeen resulting peptides comigrated exactly (figure 6.5).

These results indicate that the 97kDa and 93kDa tumour antigens recognized by tumour bearing serum are also recognized by the TG7A monoclonal.

6.2.2 Comigrating polypeptides of 40kDa

(a) Bn5T:TBS:40 compared with Bn5T:TG7A:40: V8 protease peptide maps of Bn5T 40kDa polypeptides i.p. by TBS and TG7A are similar but not identical (figure 6.6). To examine the subtle differences between V8 protease peptide maps of these two 40kDa polypeptides, a detailed comparison has been carried out. No peptides are seen in digests of one polypeptide that do not correspond with a comigrating peptide in digests of the other. On well exposed autoradiographs, digests of both 40kDa polypeptides show seven comigrating peptides with the MWs shown in table 6.1. The exact comigration of these seven peptides strongly suggests that TBS and TG7A 40kDa polypeptides are closely related. However, the peptide maps differ in the relative intensity of the peptide bands on autoradiography. The three strongest bands in a Bn5T:TBS:40 digest with 1/25ug V8 protease, are numbers 5, 7 and 2 (table 6.2). In contrast, for the Bn5T:TG7A:40 digested with 1/25ug enzyme they are 4, 6 and 3. These findings suggest that a difference exists between the molar ratios of peptide fragments of the 40kDa polypeptides recognized by TBS and TG7A. Two possible explanations are: (a) two or more of the same 40kDa polypeptides, not resolved by SDS-PAGE, are i.p. by both TBS and TG7A. The two different antibodies i.p. these

Figure 6.5; V8 protease peptide maps of the Bn5T:TBS:L90 and Bn5T:TG7A:L90 compared. Cleveland digest analysis of the L90 polypeptide as i.p. from Bn5T cell lysate by TBS (tracks 4-6) and TG7A (tracks 7-9). Tracks 4 and 7 contained 1/25ug, tracks 5 and 8 contained 1/5ug and tracks 6 and 9 contained 1ug of S.aureus V8 protease. Tracks 1 and 2 correspond to undigested L90 polypeptides i.p. by TBS and TG7A respectively. MW markers are shown in tracks 3 and 10.

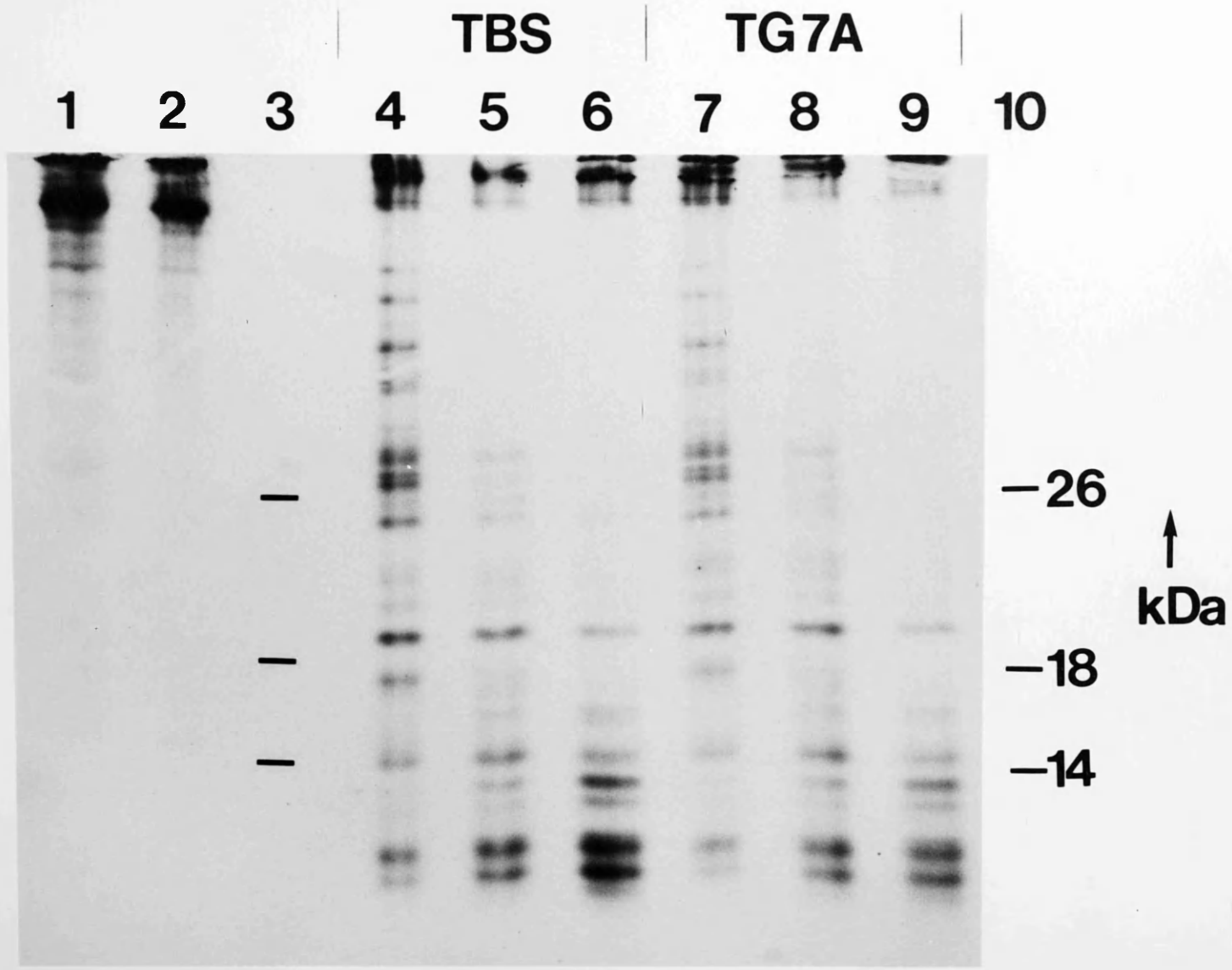
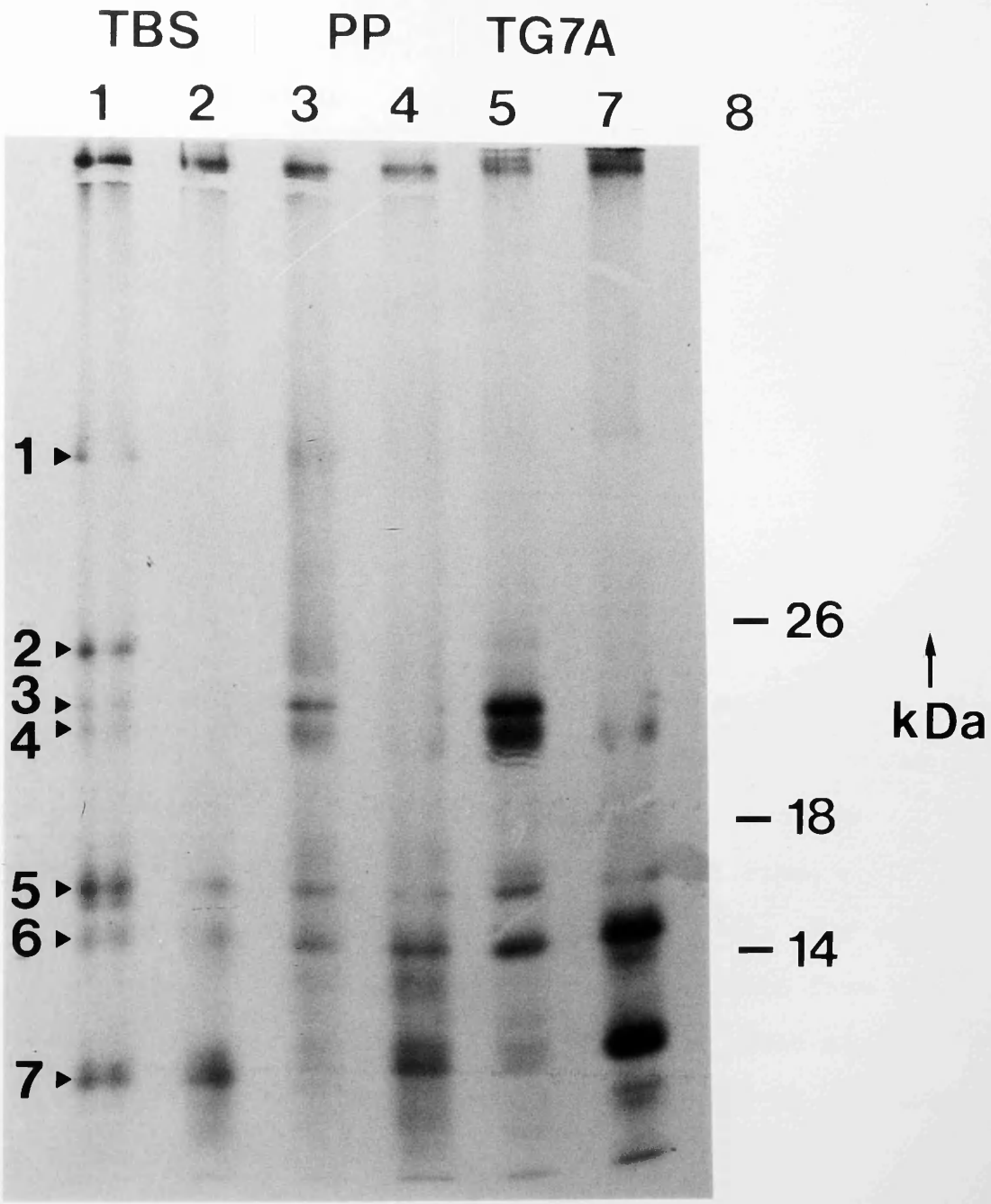

TBS
TG7A
1
2
3
4
5
6
7
8
9
10
26
18
14
kDa

Figure 6.6; V8 protease peptide maps of Bn5T:TBS:40, Bn5T:TG7A:40 and Bn5T:PP:40 compared. Cleveland digest analysis of the 40kDa polypeptides i.p. from Bn5T cell lysate by TBS (tracks 1 and 2) and TG7A (tracks 5 and 7) compared with the comigrating polypeptide seen in total Bn5T polypeptide profiles (tracks 3 and 4). Tracks 1, 3 and 5 contained 1/25ug, tracks 2, 4 and 7 contained 1ug of S.aureus V8 protease. Peptide bands are numbered in track 1 and MW markers are shown in track 8.


TBS
PP
TG7A
1
2
3
4
5
7
8
1
2
3
4
5
6
7
26
18
14
kDa

Band no.	MW(kDa)
1.	40.3 (SD=1.7)
2.	25.6 (SD=1.5)
3.	23.0 (SD=0.9)
4.	21.8 (SD=0.8)
5.	16.7 (SD=0.7)
6.	14.9 (SD=0.5)
7.	11.4 (SD=0.5)

Table 6.1; MW estimates of peptides produced on digestion of Bn5T:TBS:40 and Bn5T:TG7A:40 with V8 protease: Calibration curves were plotted for each gel using BRL MW standards (ovalbumin - 43kDa, alpha-chymotrypsinogen - 25.7kDa, beta-lactoglobulin - 18.4kDa, lysozyme - 14.3kDa). For every band, five independent MW estimates were made, each from a different gel. Mean values and SDs are given. Band no.1 probably represents undigested polypeptide as the MW estimate is approximately 40kDa.

A. Digests with 1/25ug enzyme

TBS:40	- Gel 1 :	7 > 5 > 2 > 1
	... 2 :	5 > 7 > 2 > 6 > 4 > 3
	... 3 :	5 > 7 > 2 > 6 > 4 > 1 > 3
TG7A:40	- Gel 1 :	4 > 3 > 6 > 5
	... 2 :	4 > 6 > 3 > 5 > 7
	... 3 :	4 > 6 > 3 > 7 > 5 > 2
PP:40	- Gel 3 :	2 > 6 > 5 > 4 > 1 > 3

B. Digests with 1ug enzyme

TBS:40	- Gel 1 :	7 > 5
	... 2 :	7 > 6 > 5 > 4
	... 3 :	7 > 6 > 5
TG7A:40	- Gel 1 :	7 > 6 > 4 > 5
	... 2 :	7 > 6 > 4
	... 3 :	7 > 6 > 5 > 4
PP:40	- Gel 3 :	7 > 6 > 5

Table 6.2; Analysis of V8 protease peptide maps of Bn5T:TBS:40, Bn5T:TG7A:40 and Bn5T:PP:40 polypeptides: Protease digests using two different enzyme concentrations were analyzed on three different gels. Band numbers (defined in table 6.1) are placed in order of band intensity, as measured by densitometer.

polypeptides in different molar ratios. Thus, proteolysis of 40kDa polypeptides i.p. by TBS, gives peptide fragments in different molar ratios to those resulting from proteolysis of 40kDa polypeptides i.p. by TG7A. (b) A different protein : enzyme ratio for Bn5T:TG7A:40 and Bn5T:TBS:40 digestions produces the differences in peptide map pattern. The stronger signal obtained from peptide bands in Bn5T:TG7A:40 digests compared with the Bn5T:TBS:40, suggests that the TG7A i.p. polypeptide is more abundant. Further supporting evidence is seen in table 6.2, which shows that peptide maps of different 40kDa polypeptides become more alike when digestion is closer to completion, as with the higher enzyme concentration.

Despite the differences in peptide band intensity, the above evidence suggests that TBS and TG7A recognize very similar, if not identical 40kDa polypeptides. For reasons not determined peptide mapping of these 40kDa polypeptides with chymotrypsin gave peptide maps with poor resolution which were very difficult to interpret.

(b) Bn5T:PP:40: Peptide mapping of the Bn5T:PP:40 with V8 protease gives seven peptide bands which comigrate with those produced on digestion of both Bn5T:TBS:40 and Bn5T:TG7A:40 (figure 6.6). On visual inspection the pattern of bands in Bn5T:PP:40 peptide maps appears very similar to the pattern for Bn5T:TG7A:40. However, densitometric analysis of these peptide maps indicates that the pattern for digests of Bn5T:PP:40, shares features with both Bn5T:TBS:40 and Bn5T:TG7A:40 (data not shown).

6.2.3 Polypeptides of 200, 35 and 34kDa

Peptide mapping comparisons were not possible for the

200, 35 and 34kDa polypeptides i.p. by TBS and TG7A. For successful peptide mapping, i.p. polypeptides separated on the first gel must produce strong bands on autoradiography otherwise the signal will be too weak for detection after proteolytic digestion and separation of peptides on the second gel (section 3.7.2). The 200 and 34kDa polypeptide bands in Bn5T:TG7A i.p.s and the 35kDa polypeptide band in Bn5T:TBS i.p.s were always too weak to allow successful peptide mapping.

6.3 Conclusions

TBS and TG7A recognize comigrating polypeptides of 200, 97, 93, 40, 35 and 34kDa in Bn5T cells but not in RE cells prepared from late gestation embryos. Those comigrating polypeptides for which comparison by peptide mapping was possible (97, 93 and 40kDa), show very similar digest patterns. This evidence suggests that TBS and TG7A identify the same set of transformed cell polypeptides.

7 RELATIONSHIPS BETWEEN TUMOUR ANTIGENS RECOGNIZED BY TBS

Evidence is presented in chapter 6, which indicates that TBS and TG7A recognize members of the same set of transformed cell polypeptides. Evidence will now be presented which sheds light on the inter-relationships of these polypeptides belonging to this set.

7.1 Co-expression or non-expression

Every cell type examined appears to express either all or none of the polypeptides recognized by TBS and TG7A with MWs of 200, 97, 93, 40, 35 and 34kDa.

7.1.1 Different cell types

TBS and TG7A i.p. polypeptides of the above MWs from a range of immortalized cells. These include the established cell lines Rat-1, Rat-2 and Hood, the transformed cell lines Bn5, LA24 and LA90 (data not shown) and the tumour cell line Bn5T. LA90 cells are RSV transformed mouse 3T3 cells and so expression of the polypeptides is not specific for rat cells. No polypeptides of similar MW were i.p. by TBS or TG7A from the human HeLa cell line. These polypeptides were not i.p. by TG7A from lysates of ten different preparations of rat embryo fibroblasts (Macnab et al.,1985a). Similar results were obtained with TBS. These results were obtained using cells prepared from embryos of approximately 16 to 20 days old. However, when embryos of approximately 12 days are tested, it is possible to i.p. polypeptides with TBS which co-migrate exactly with Bn5T:TBS polypeptides (data not shown). This has also been observed for two different cultures by Dr J.Macnab. To further investigate the pre-natal expression of these polypeptides, the embryonal carcinoma cell line PC1315 and

four embryonal carcinoma cell lines differentiated *in vitro* were i.p. with TBS. These cell lines were found to be the only immortalized rodent cells so far encountered which do not express the Bn5T:TBS polypeptide set. Owing to lack of time the author was unable to repeat this experiment.

7.1.2 Different culture conditions

No culture conditions were identified which consistently influenced the level of expression of any of the Bn5T:TBS polypeptides or induced the expression of any comigrating polypeptides in RE cells.

(a) Degree of confluence: No RE cell polypeptides were i.p. by TBS from either subconfluent or highly confluent cultures prepared from late gestation rat embryos. The same Bn5T cell polypeptides were i.p. by TBS from both sub- confluent and highly confluent cultures. In one experiment the intensity of the U90 was found to be greater for confluent Bn5T cell cultures, while the intensity of the 34kDa polypeptide was greater in subconfluent cultures. However, this experiment could not be repeated (Dr J.Macnab, personal communication).

(b) Serum concentration: RE cell cultures prepared from late gestation embryos were incubated in medium containing different concentrations of serum. Cells were radiolabelled for 18h with ^{35}S-methionine in Eagle's minus methionine culture medium containing 0%, 2% and 10% foetal calf serum (FCS). Neither high nor low serum concentration induced detectable levels of any polypeptides comigrating with Bn5T:TBS polypeptides (data not shown).

7.1.3 IVT polypeptides

A further characteristic shared by all Bn5T polypeptides recognized by TBS and TG7A is that none of them can be i.p. from the IVT products of Bn5T mRNA (section 5.2.2).

7.1.4 Co-expression: conclusions

The fact that Bn5T:TBS polypeptides are either expressed as a complete set or not expressed at all, indicates a common origin and/or regulation by the same factor(s). It also suggests that they may have related functions.

7.2 Co-precipitation

As stated above, every cell type examined will express either all or none of the polypeptides recognized by TBS and TG7A with MWs of 200, 97, 93, 40, 35 and 34kDa. In addition, analysis of cells expressing these polypeptides shows that polypeptides of 97, 93, 40 and 35kDa are co-precipitated in all i.p. assays with TG7A and polypeptides of 200, 97, 93, 40 and 34kDa are co-precipitated in all i.p. assays with TBS.

7.2.1 Explanations for co-precipitation of Bn5T:TBS polypeptides

Co-precipitation of Bn5T polypeptides by the TG7A monoclonal antibody suggests that they are antigenically related and/or linked in a multi-subunit complex. TG7A detects Bn5T polypeptides of approximately 90kDa, 40kDa and 35kDa on Western blotting analysis, which indicates that these polypeptides are antigenically related (Dr N.B. La Thangue, personal communication). TBS detects U90 and L90 Bn5T polypeptides on Western blots (Dr D.O. Toft, personal communication). Thus, TBS must recognize an epitope on both

the U90 and the L90 polypeptide. Western blotting studies leave unresolved the question of whether 200, 40 and 34kDa polypeptides have epitopes recognized by TBS. The alternative being, that they are i.p. due to complex formation with U90 and L90 polypeptides. The circumstantial evidence that follows suggests that 200, 40 and 34kDa polypeptides do have epitopes recognized by TBS:

(a) In Bn5T:TBS i.p.s with some batches of TBS, the L90 polypeptide gives a very weak band and yet 200, 40 and 34kDa polypeptides are easily visible (figure 6.2). This indicates that complexing to the L90 polypeptide is unlikely to account for the i.p. of the 200, 40 and 34kDa polypeptides.

(b) Analysis of Bn5T:TG7A i.p.s shows that U90 and L90 bands are easily visible, but that 200kDa and 34kDa bands are not (figure 6.2). In contrast, for most Bn5T:TBS i.p.s the 200kDa and 34kDa bands are easily visible, as well as the U90 and L90 bands. This suggests that 200 and 34kDa polypeptides are not complexed to U90 or L90 polypeptides and that the i.p. of 200kDa and 34kDa polypeptides by TBS is due to recognition of epitopes on these molecules.

(c) There was considerable variation in the specificity of sera from different tumour bearing rats as indicated by the variation in precipitation ratios shown in table 7.1. The widest range of precipitation ratios is between 34kDa and U90 polypeptides. This suggests that i.p. of the 34kDa polypeptide by TBS does not depend on complex formation with the U90 polypeptide.

(d) When developing a large scale i.p. technique (section 9.1.2) a single carefully quantitated study was carried out to test the effect of increasing the antigen concentration on the strength of the immunopurified polypeptide bands. It was

Bands compared	Ratio for TBS i.p.s **minimum - maximum**	Ratio for TG7A i.p.
200:U90	0.32, 0.34, 0.37, 0.50, 0.54	0.00, 0.00
L90:U90	0.00, 0.00, 0.27, 0.44, 0.75	0.87, 1.10
40:U90	0.46, 0.78, 0.82, 1.11, 1.30	6.06, 8.50
34:U90	0.57, 0.67, 0.69, 0.95, 2.03	0.00, 0.00

Table 7.1; Quantitative analysis of precipitation ratios of Bn5T polypeptides i.p. by TBS and TG7A: 5 batches of TBS each from a different tumour bearing rat were used to i.p. the same sample of Bn5T cell lysate and precipitates analysed on SDS-PAGE in replicate experiments. Bn5T:TG7A i.p.s were also analysed. Densitometer measurements of autoradiograph band intensity allow quantitative comparison of precipitation ratios. Ratios of band intensity measurements give a measure of precipitation ratio for polypeptides corresponding to the bands compared.

found that increasing the antigen concentration had a marked effect on the intensity of the 97kDa Bn5T polypeptide band. More dramatic than this however was the increase in intensity of the 40kDa band. Densitometric analysis showed that a 10X increase in antigen concentration gave a 1.5X increase in U90 polypeptide binding and a 3.8X increase in 40kDa binding. Thus, the U90:40 precipitation ratio does not appear to be constant with increasing antigen concentration, indicating that the 40kDa polypeptide does not need to be complexed to the U90 in order to be recognized by TBS antibodies. This implies that the 40kDa polypeptide has an epitope recognized by TBS antibodies.

7.2.2 Coprecipitation: conclusions

There is direct evidence that TG7A recognizes epitopes on U90, L90, 40 and 35kDa polypeptides and that TBS recognizes epitopes on U90 and L90 Bn5T polypeptides. In addition there is evidence which suggests that TBS also recognizes epitopes on 200, 40 and 34kDa polypeptides. Thus, shared epitopes seem a more likely explanation for co-precipitation, than the existence of Bn5T:TBS polypeptide complexes.

7.3 Peptide mapping studies

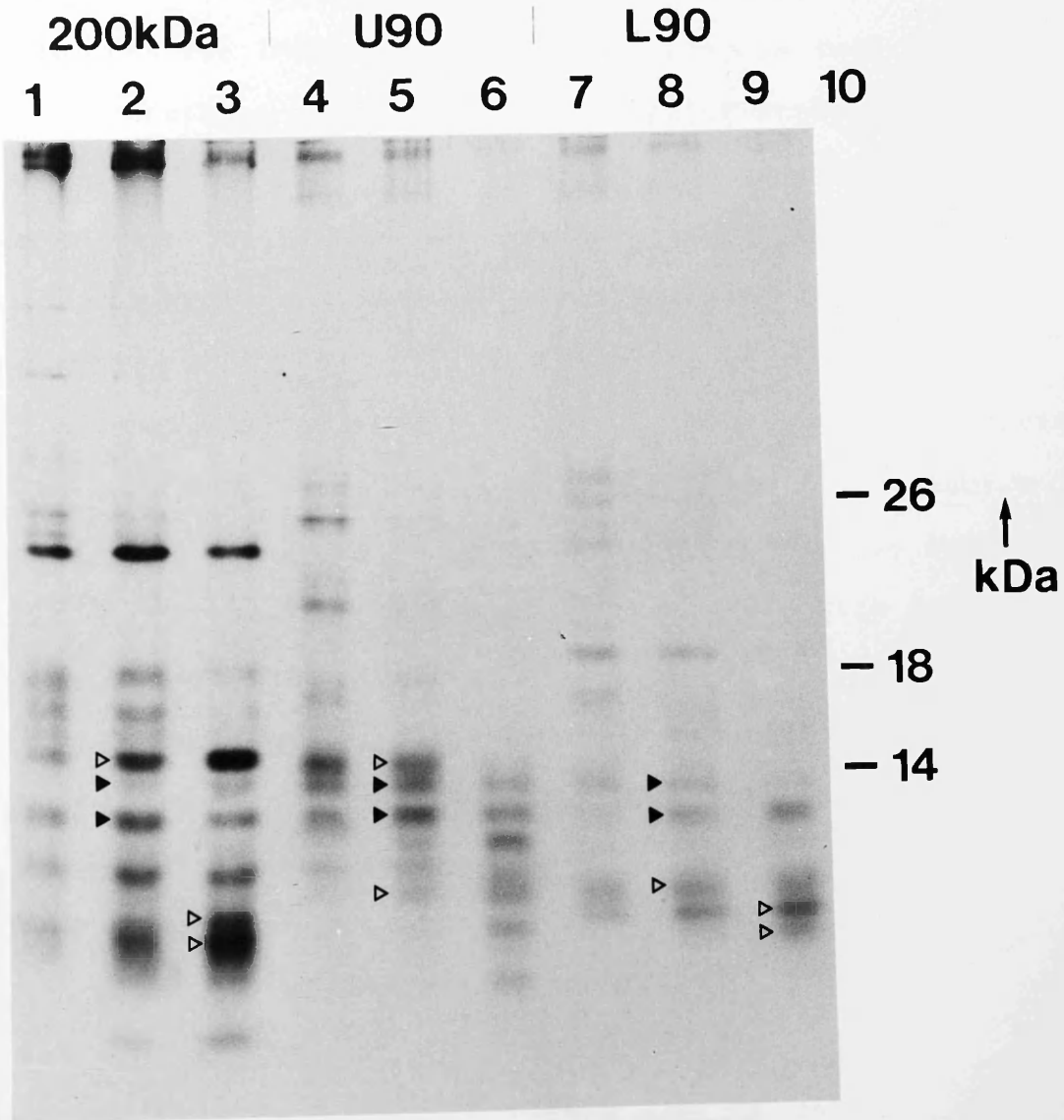
To investigate the possibility that shared domains account for the antigenic relatedness of Bn5T polypeptides i.p. by TBS and TG7A, one-dimensional peptide mapping analysis was performed. Both staphylococcal V8 protease and chymotrypsin were used in these studies and each was used at a range of concentrations giving partial proteolysis.

7.3.1 Analysis of Bn5T polypeptides i.p. by TBS

(a) U90, L90 and 200kDa polypeptides: With both enzymes and in particular with V8 protease, there were similarities between U90 and L90 polypeptides. V8 protease peptide maps were carried out on these polypeptides using three different concentrations of enzyme. Peptide maps of U90 and L90 polypeptides with 1ug V8 protease per track show major comigrating bands of 13.3kDa and 14.1kDa (mean of 3 independent MW estimates for each peptide band); (figure 7.1). V8 protease digestion of the 200kDa polypeptide gave a peptide map showing similarities with those of U90 and L90 polypeptides (figure 7.1). All show comigrating 13.3kDa and 14.1kDa peptides (observed in 3 independent experiments) which suggests that they share a similar domain. Comigrating 14.5kDa peptides are also seen in digests of the U90 and 200kDa polypeptides. Digestion of the U90 and L90 bands with 1ug chymotrypsin yielded comigrating bands of 13.5, and 15kDa (figure 6.4).

Thus, there is evidence from peptide mapping studies for shared regions of homology between the U90, L90 and 200kDa Bn5T polypeptides i.p. by TBS. Further evidence of a close relationship between U90, L90 and 200kDa polypeptides comes from a comparison of the precipitation ratios in Bn5T:TBS and Bn5T:TG7A i.p.s. These three polypeptides are all recognized more strongly by TBS than TG7A (figures 6.1 and 6.2). TBS recognizes the 200, 97 (U90 polypeptide), 93 (L90 polypeptide) and 34kDa more strongly than TG7A. Conversely, TG7A recognizes the 40 and 35kDa more strongly than TBS. This suggests that within the total set of polypeptides recognized by the two antibody preparations there are two subsets of

Figure 7.1; V8 protease peptide maps of the Bn5T:TBS:200kDa, U90 and L90 compared. Cleveland digest analysis of the 200kDa (tracks 1-3), U90 (tracks 4-6) and L90 (tracks 7-9) polypeptides as i.p. from Bn5T cell lysate by TBS. Tracks 1, 4 and 7 contained 1/25ug, tracks 2, 5 and 8 contained 1/5ug and tracks 3, 6 and 9 contained 1ug of S.aureus V8 protease. MW markers are shown in track 10.


200kDa
U90
L90
1
2
3
4
5
6
7
8
9
10
26
18
14
kDa

polypeptides which are more closely related: one subset containing 200kDa, U90, L90 and 34kDa polypeptides and the other subset containing 40kDa and 35kDa polypeptides.

(b) 40kDa and 34kDa polypeptides: No similarities are seen between peptide maps of 34 and 40kDa Bn5T polypeptides i.p. by TBS. Neither polypeptide gives a peptide map resembling that of the L90 or U90 polypeptide. A comparison of V8 protease peptide maps for 40kDa and U90 polypeptides is seen in figure 7.2, and a further detailed peptide mapping analysis of these two polypeptides with 1, 5, 10 and 25ug V8 protease per track showed no similarity with any enzyme concentration (data not shown). In addition, chymotrypsin digests of these polypeptides showed no obvious similarity (figure 7.3).

The 40kDa polypeptide gives V8 protease and chymotrypsin peptide maps which show similarities to those of the 200kDa polypeptide. Using 1ug V8 protease, two out of five bands in 40kDa polypeptide digests, appear to comigrate with two out of eight bands in 200kDa polypeptide digests (figure 7.2). These two comigrating bands are different from the comigrating bands shared by 200kDa, U90 and L90 polypeptides and have MWs of approximately 14.3kDa and 14.7kDa (estimated from a single gel). No obvious similarity was seen between peptide maps of 34kDa and 200kDa polypeptides (figure 7.3).

7.3.2 Analysis of Bn5T polypeptides i.p. by TG7A

(a) U90 and L90 polypeptides: The U90 and L90 polypeptides i.p. by TG7A from Bn5T cells give indistinguishable peptide maps to those of the comigrating polypeptides i.p. by TBS (section 6.2.1), and the relationship between peptide maps of

Figure 7.2; V8 protease peptide maps of Bn5T:TBS:200, U90 and 40kDa polypeptides compared. Cleveland digest analysis of polypeptides i.p. by TBS from Bn5T cell lysate using 1ug of S.aureus V8 protease. Peptide maps of the 200kDa polypeptide (track 1), U90 polypeptide (track 2) and 40kDa polypeptide (track 3) are shown. Bands which appear to comigrate are indicated by arrows in tracks 1 and 3.

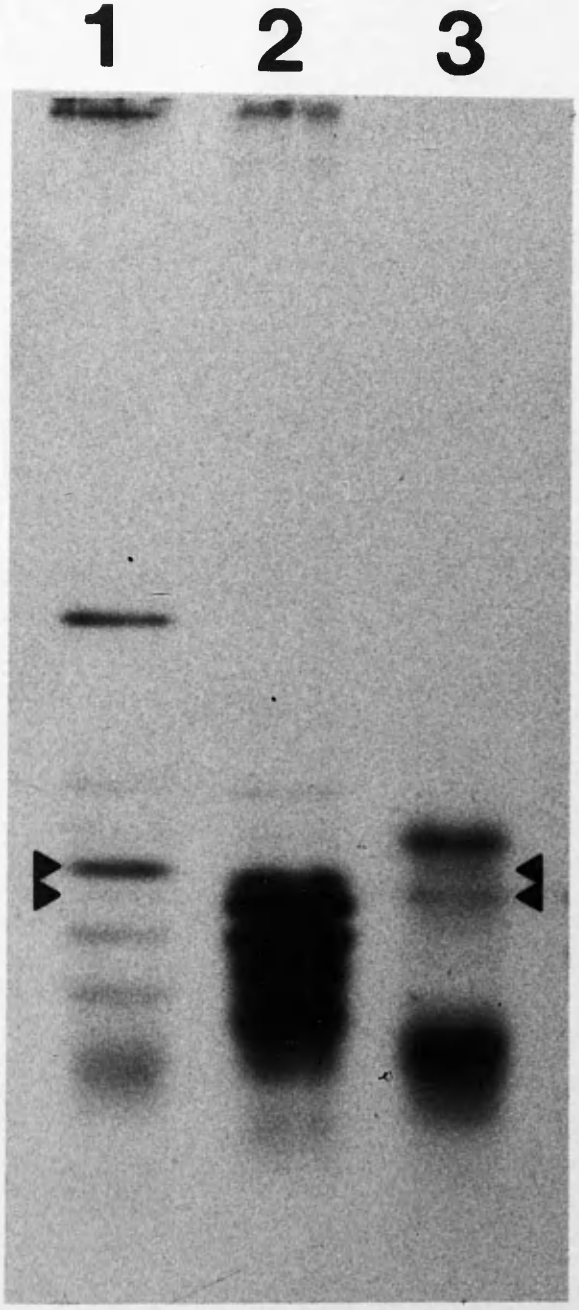
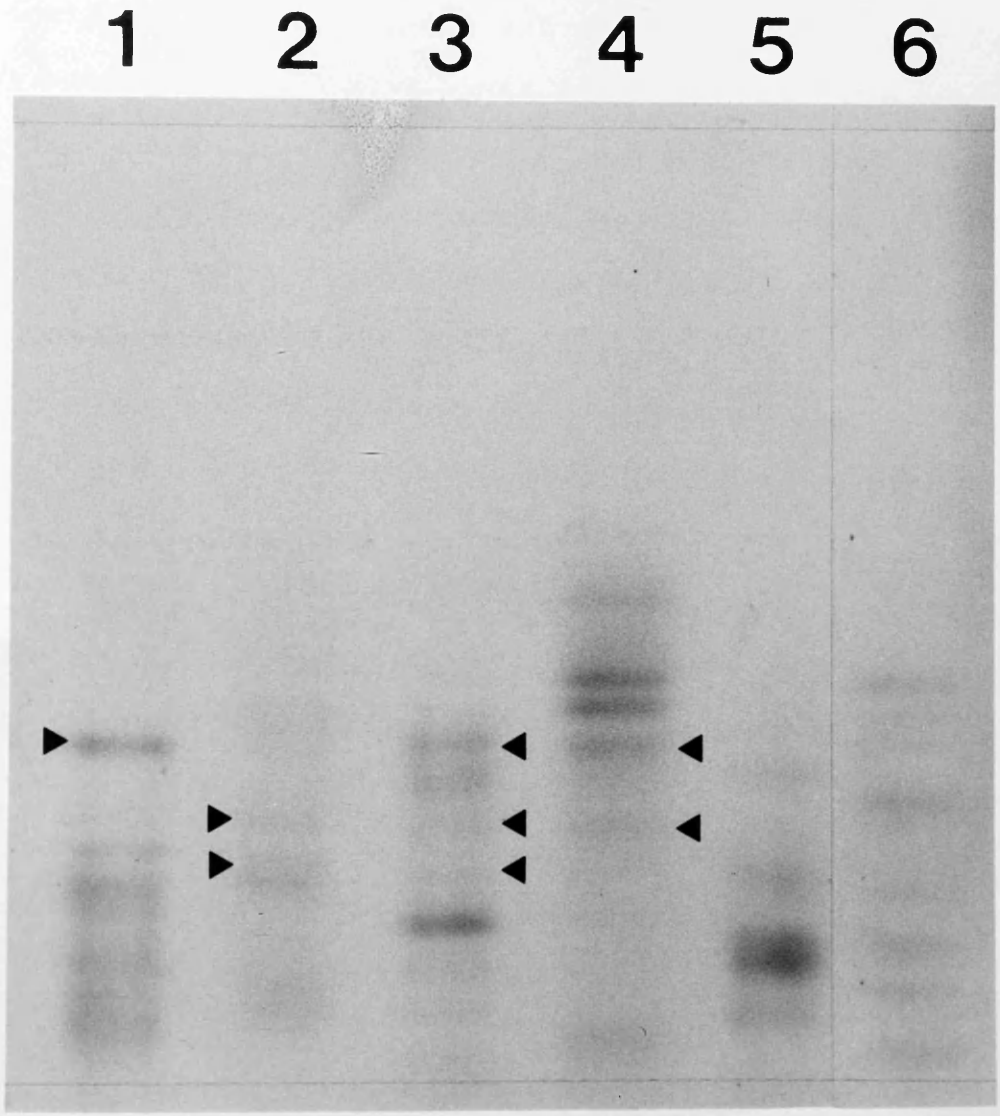

1
2
3

Figure 7.3; Chymotrypsin peptide maps of Bn5T:TBS polypeptides compared with Bn5T:AC88:90. Cleveland digest analysis using 1ug chymotrypsin of polypeptides i.p. from Bn5T cell lysate by TBS and AC88. The TBS i.p. 200kDa (track 1), U90 (track 2), L90 (track 3), 40kDa (track 5) and 34kDa (track 6) are compared with the AC88 i.p. 90kDa polypeptide (track 4). Arrows in tracks 1-4 indicate bands which appear to comigrate.


1
2
3
4
5
6

U90 and L90 polypeptides recognized by TG7A is therefore exactly the same as for corresponding polypeptides recognized by TBS (section 7.3.1).

(b) 40kDa and 35kDa polypeptides: Peptide map patterns of 35 and 40kDa polypeptides with 1/25ug V8 protease appear similar (figure 7.4), but careful inspection shows that many bands do not comigrate exactly. Thus, out of eleven bands seen for the 35kDa polypeptide and nine bands for the 40kDa polypeptide only two comigrate. No similarity was seen between 35 and 40kDa polypeptides on peptide mapping using either 10ug V8 protease or 10ug chymotrypsin (data not shown). In summary then, peptide maps of the 35kDa and 40kDa Bn5T cell polypeptides i.p. by TG7A show little true similarity to either to eachother or to other Bn5T polypeptides i.p. by TG7A.

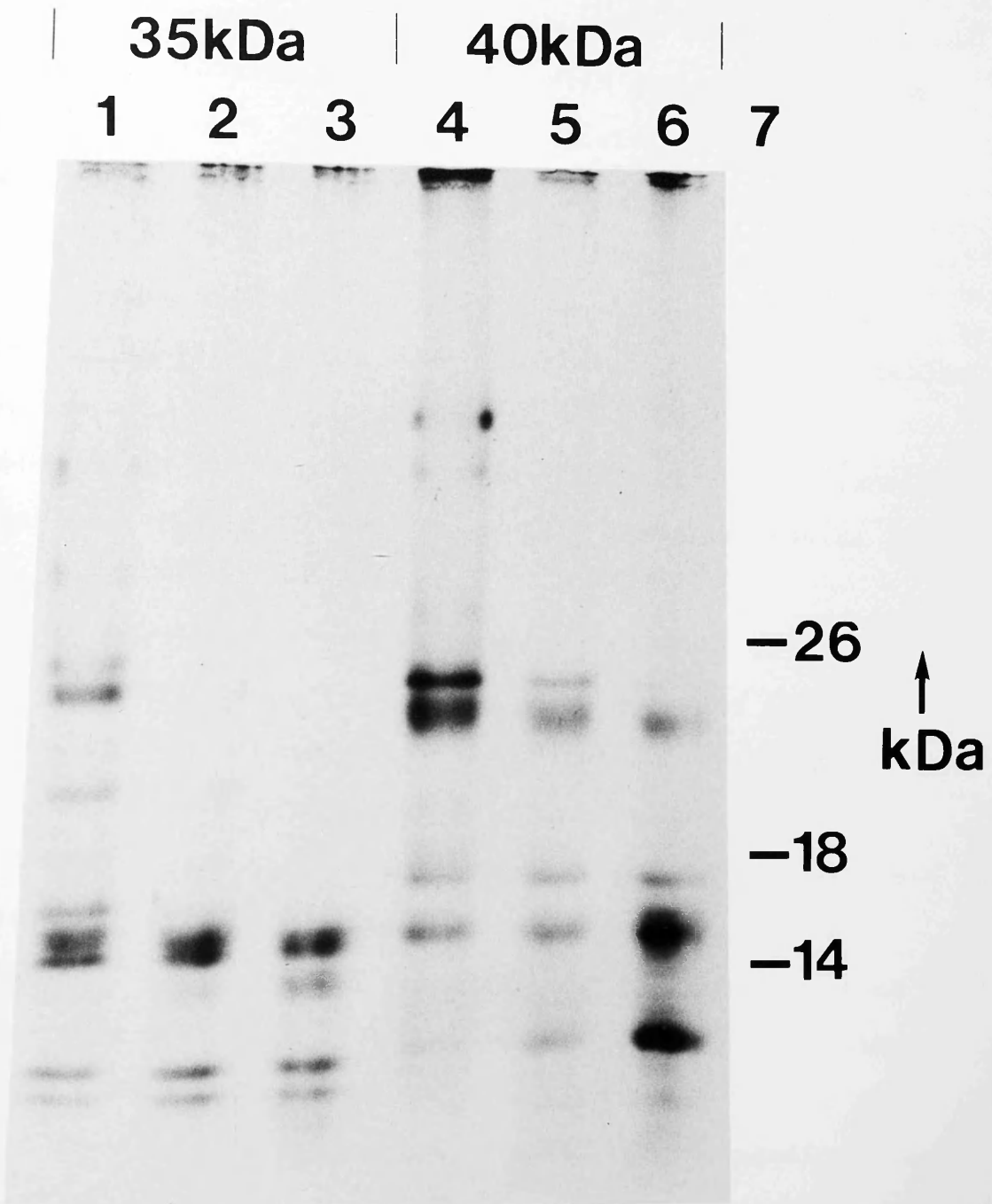
7.3.3 Peptide mapping studies: conclusions

Peptide maps of Bn5T:TBS:U90, L90 and 200kDa polypeptides share a number of comigrating peptide bands and this suggests a region of similarity in these three polypeptides. Some slight similarity was also seen between peptide maps of Bn5T:TBS:40kDa and 200kDa polypeptides.

7.4 Kinetic studies

When the author investigated the effect of radiolabelling Bn5T cells for 4h instead of the usual 18h, it was noted that in a TBS i.p. the 40kDa band was very weak relative to the U90 band. This finding was further investigated.

Figure 7.4; V8 protease peptide maps of the Bn5T:TG7A:40 and 35kDa compared. Cleveland digest analysis of the 35kDa (tracks 1-3) and 40kDa (tracks 4-6) polypeptides as i.p. from Bn5T cell lysate by TG7A. Tracks 1 and 4 contained 1/25ug, tracks 2 and 5 contained 1/5ug and tracks 3 and 6 contained 1ug of S.aureus V8 protease. MW markers are shown in track 7.


35kDa
40kDa
1
2
3
4
5
6
7
26
18
14
kDa

7.4.1 Pulse labelling experiments

Bn5T cells were labelled with ^{35}S-methionine as described in section 3.6.2. Cells were incubated with radiolabel for increasing periods of time and after harvesting, volumes of lysate corresponding to $4X10^6$ c.p.m. were i.p. with TBS and i.p.s then analysed by SDS-PAGE. With incubation periods increasing from 1 h to 45h, the intensity of the U90 band remains relatively constant, while the intensities of the 40kDa and 34kDa bands increase steadily.

Another interesting observation was the coordinate fluctuation in L90 and 200kDa band intensity. These results are shown by the autoradiograph in figure 7.5 and the densitometer scan results in figure 7.6. The pulse labelling experiment was carried out twice with the same results each time.

In figure 7.6 the intensities of a number of bands are expressed as a proportion of U90 band intensity and the reason for this is as follows: minor artefactual variations in band intensity should be expected with i.p. assays, as it is technically very difficult to remove the supernate with a pipette after each washing step, without removing small amounts of the protein-A sepharose bound immune complexes which form the pellet. Pellets at the end of the washing stages are therefore frequently of slightly different size. Apart from minor fluctuations, the U90 band intensity appears relatively constant throughout the pulse labelling experiment, and therefore by expressing the intensity of other bands as a proportion of the U90 band intensity, artefactual fluctuations can be ignored, allowing underlying trends to be seen more easily.

Figure 7.5; Pulse labelling experiment. Autoradiograph of a 6-15% gradient polyacrylamide gel containing electrophoretically separated ^{35}S-methionine labelled polypeptides extracted from Bn5T cells, radiolabelled for different time periods before harvesting for i.p. assay with TBS. Cells were pulse radiolabelled for the following times: track 3, 1h.; track 4, 2h.; track 5, 4h.; track 6, 7h.; track 7, 9.25h.; track 8, 20h.; track 9, 31h.; track 10, 45h. Track 12 shows a standard TBS i.p. of Bn5T cells radiolabelled 18h. and tracks 1, 2, 11, 13 and 14 show total Bn5T cell polypeptide profiles.

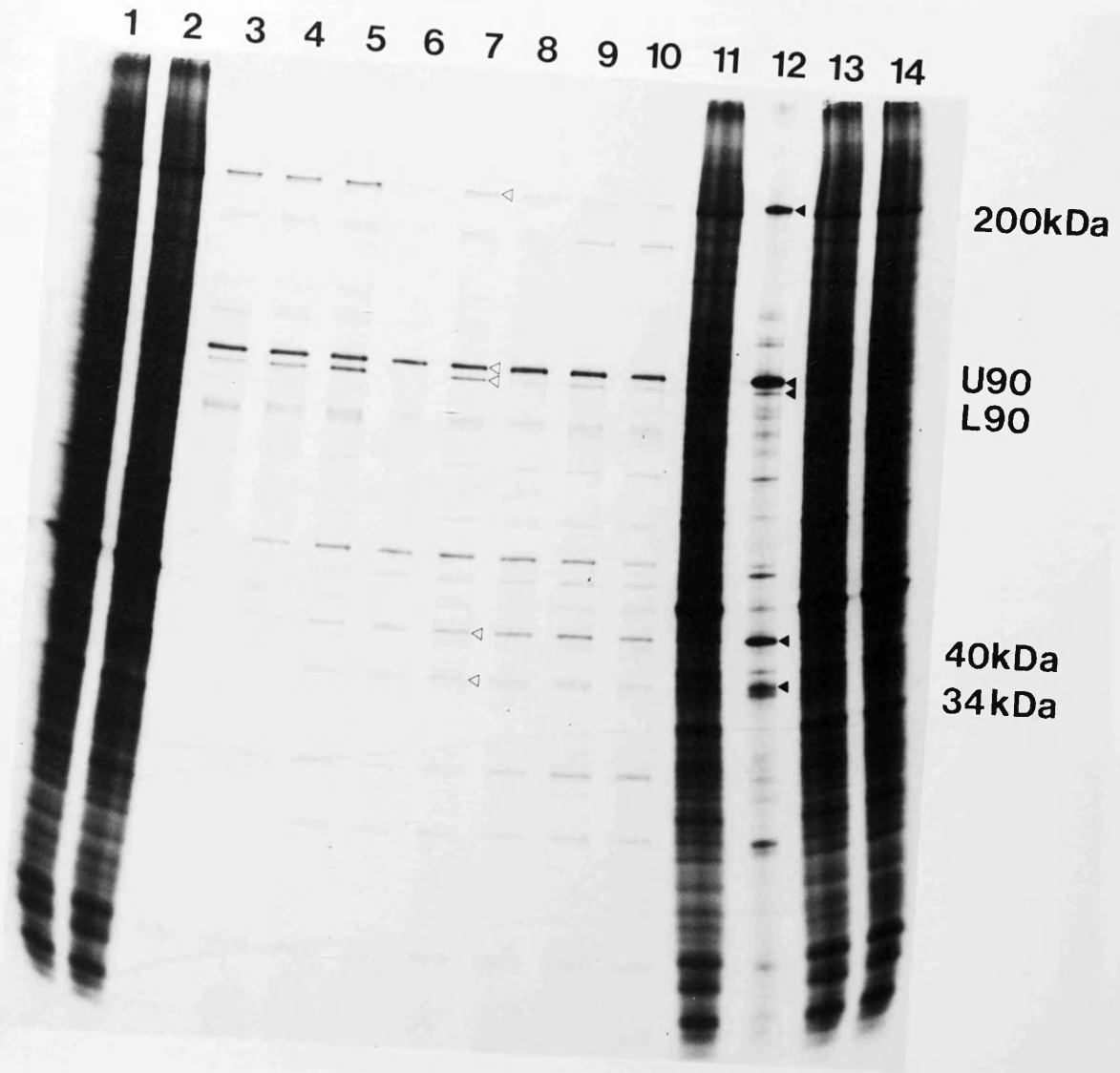
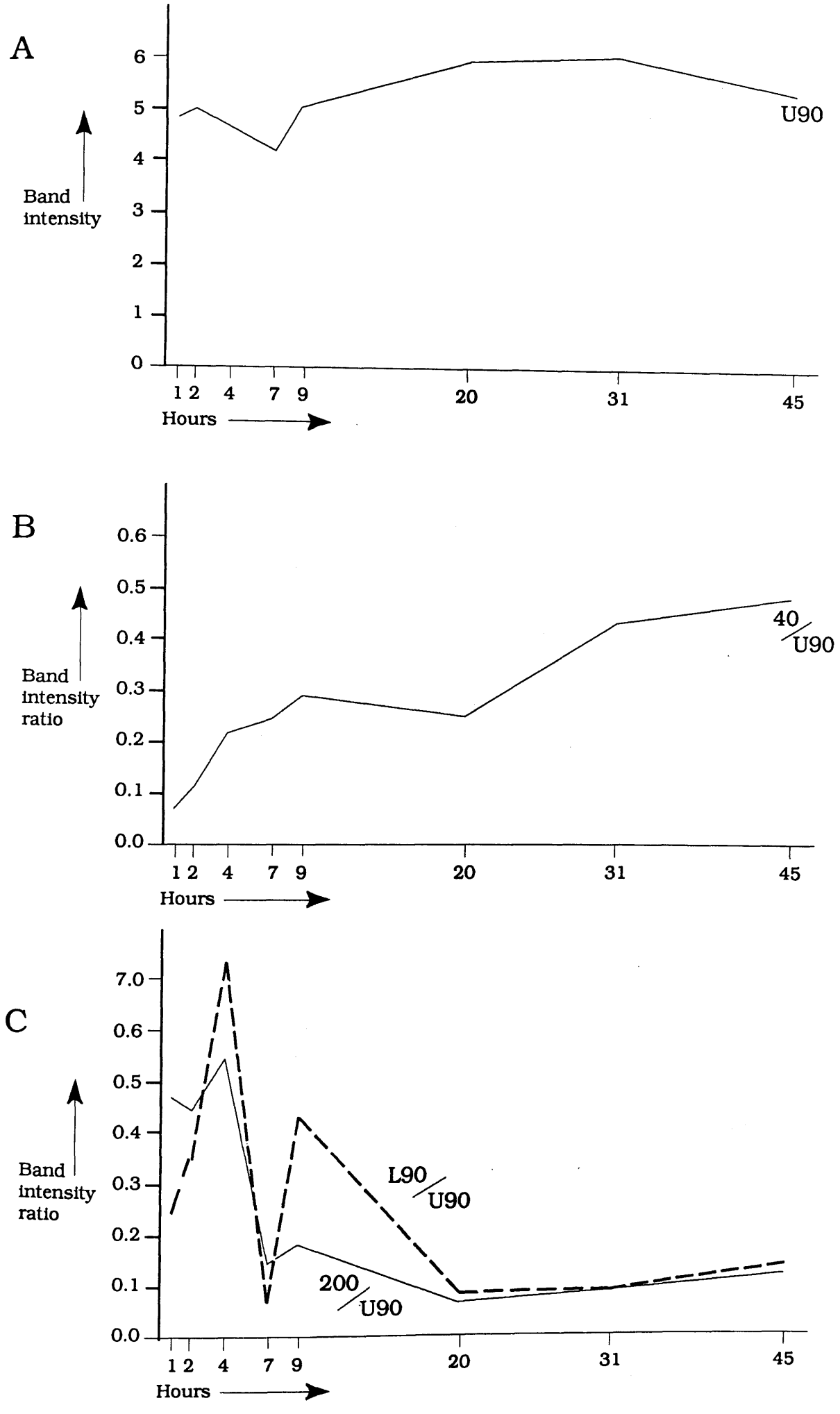

1
2
3
4
5
6
7
8
9
10
11
12
13
14
200kDa
U90
L90
40kDa
34kDa

Figure 7.6; Pulse labelling experiment. Graphs show changes in the intensity of autoradiograph bands seen in figure 7.5. U90 band intensity changes with time, are displayed in **A**. In B changes in ratio of 40:U90 band intensities are shown, and in **C** changes in 200:U90 and L90:U90 ratios are shown.


A
Band intensity
0
1
2
3
4
5
6
U90
1 2 4 7 9 20 31 45
Hours
B
Band intensity ratio
0.0
0.1
0.2
0.3
0.4
0.5
0.6
40/U90
1 2 4 7 9 20 31 45
Hours
C
Band intensity ratio
0.0
0.1
0.2
0.3
0.4
0.5
0.6
7.0
L90/U90
200/U90
1 2 4 7 9 20 31 45
Hours

7.4.2 Pulse-chase experiments

In order to explore possible precursor-product relationships within the set, pulse-chase experiments were carried out as described in section 3.6.3. Cell monolayers were radiolabelled for 1 h, then washed and incubated in EF5 for increasing periods prior to harvesting for assay by i.p. with TBS.

The U90 band is relatively constant in intensity over the first five h after the chase. However, the 40kDa polypeptide band increases steadily in intensity over this period (figures 7.7 and 7.8). The 200kDa and L90 bands show coordinate fluctuation in intensity, but show no general increase or decrease. Densitometer readings for 200kDa, L90 and 40kDa polypeptide bands are expressed in figure 7.8 as a proportion of U90 readings, for the reason explained in the above section. The 40kDa polypeptide gives a weaker band in pulse-chase experiments than in standard i.p.s because as pulse labelling experiments show, radiolabel accumulates gradually in this band over a period of 20 h or more. Radiolabel also accumulates gradually in the 34kDa polypeptide in pulse labelling experiments but this band is not visible at all in the pulse-chase experiment. In contrast to the 40kDa polypeptide therefore, insufficient radiolabelled 34kDa polypeptide seems to be produced in the 1 h pulse-chase experiment, to produce a visible band on autoradiography.

The pulse-chase experiment was repeated with very similar results including a constant U90, a steadily increasing 40kDa and coordinately fluctuating L90 and 200kDa band intensities. However, it was noted that the pattern of L90 and 200kDa coordinate fluctuation was different on the second occasion that this experiment was carried out, which suggests that the

Figure 7.7; Pulse-chase labelling experiment. Autoradiograph of a 6-15% gradient polyacrylamide gel containing electrophoretically separated ^{35}S-methionine labelled polypeptides extracted from Bn5T cells, pulse radiolabelled for 1h. and chased with non-radioactive culture medium for different time periods before harvesting for i.p. assay with TBS. The chase time periods were as follows: track 3, 0h.; track 4, 0.5h.; track 5, 1h.; track 6, 2.5h.; track 7, 5.5h.; track 8, 7.75h.; track 9, 18.5h.; track 10, 29.5h.; track 11, 43.5h. Track 13 shows a standard TBS i.p. of Bn5T cells radiolabelled 18h. and tracks 1, 2, 12 and 14 show total Bn5T cell polypeptide profiles.

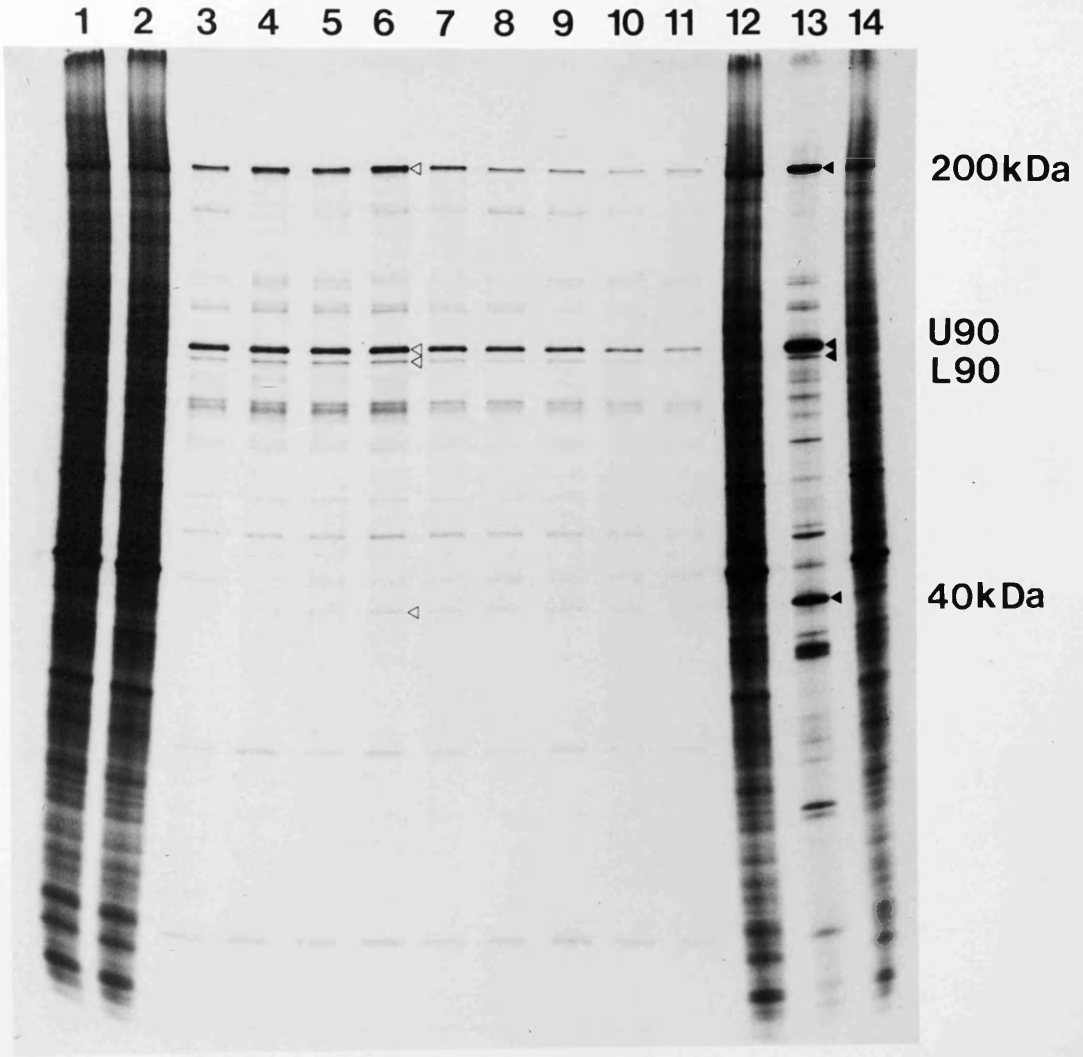
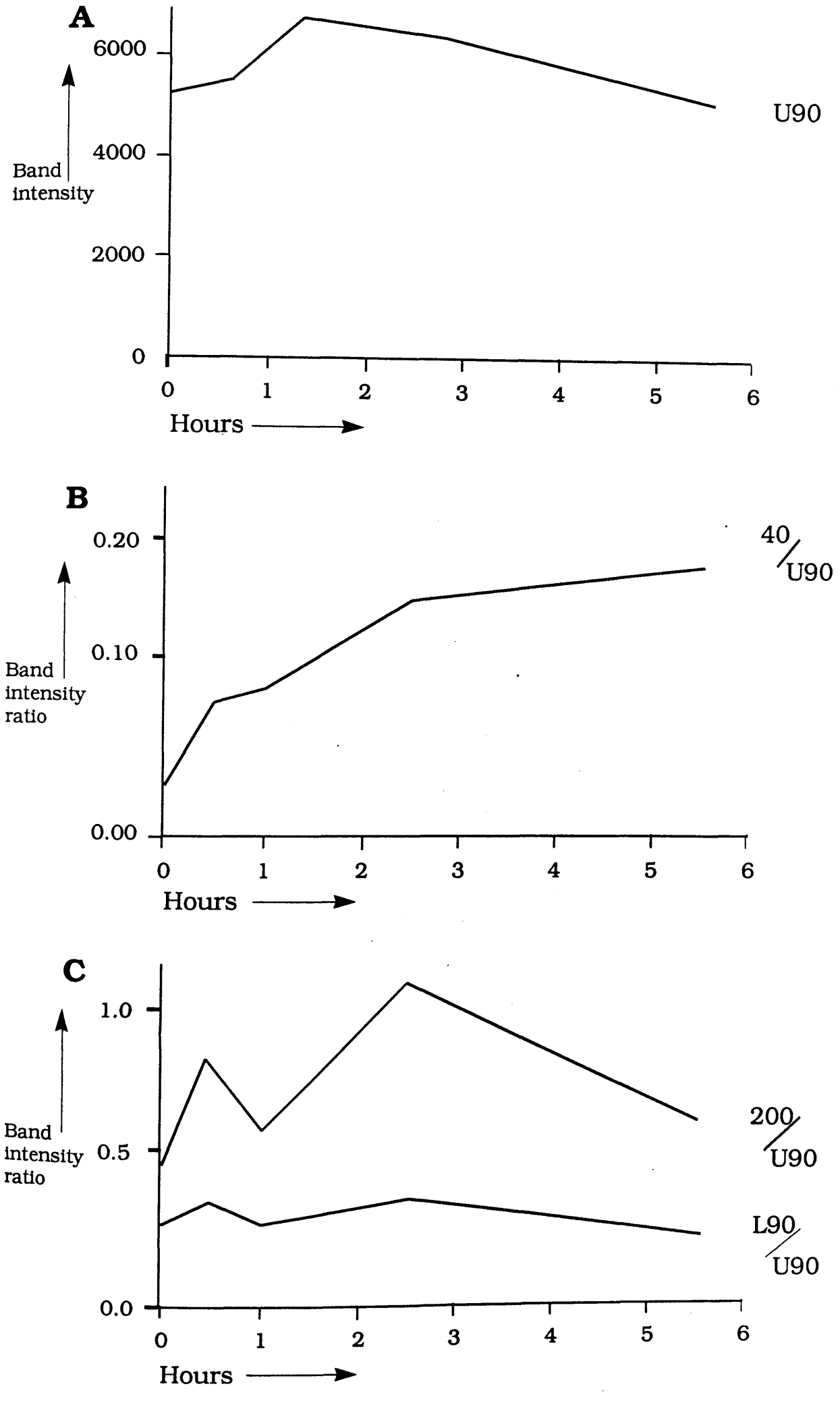

1 2 3 4 5 6 7 8 9 10 11 12 13 14
200kDa
U90
L90
40kDa

Figure 7.8; Pulse-chase labelling experiment. Graphs show changes in the intensity of autoradiograph bands seen in figure 7.7. U90 band intensity changes with time, are displayed in **A.** In **B** changes in ratio of 40:U90 band intensities are shown, and in **C** changes in 200:U90 and L90:U90 ratios are shown.


A
6000
4000
2000
0
Band intensity
U90
0
1
2
3
4
5
6
Hours
B
0.20
0.10
0.00
Band intensity ratio
40/U90
0
1
2
3
4
5
6
Hours
C
1.0
0.5
0.0
Band intensity ratio
200/U90
L90/U90
0
1
2
3
4
5
6
Hours

fluctuation is due to an artefact rather than to temporal variation in polypeptide synthesis.

With a shorter pulse of 10 min. and 2X the concentration of radiolabel (to increase the incorporation of radiolabel), the same results were obtained (data not shown). Similar results were obtained for TG7A i.p.s of the same cell lysates although the increase in intensity of the 40kDa band was less marked. A possible explanation for this is as follows: there may be a larger number of polypeptide species in the TG7A 40kDa band than in the corresponding TBS band which is consistent with the observations that the Bn5T:TG7A:40 band is stronger than the Bn5T:TBS:40 and also that it gives a slightly different peptide map (see section 6.2.2). If a 40kDa polypeptide species recognized by TG7A alone gave a signal of constant strength at all times following pulse-chase, then another 40kDa polypeptide species recognized by TG7A (and also TBS) giving a signal of increasing intensity following pulse-chase, would not produce such an obvious rise in autoradiograph band intensity as it would if it were the only polypeptide in the 40kDa band.

7.4.3 Kinetic studies: conclusions

No precursor-product relationship between Bn5T:TBS:U90 and L90 polypeptides is evident from the kinetic studies described. In the pulse-chase experiment, the delayed appearance of radiolabel in the 40kDa band following chase, suggests that a post-translational change must occur before the polypeptide can be recognized and i.p. by TBS. As no bands show any decrease in intensity concomitant with the increase in intensity of the 40kDa band after pulse-chase, no putative precursor polypeptide can be identified from this

experiment. When the pulse-chase experiment was repeated, the timing of coordinate fluctuation in L90 and 200kDa polypeptide band intensities differed from the original experiment. This, together with the irregular nature of the fluctuations indicates that these changes are not related to the time elapsed since the chase. It seems more likely therefore that the levels of these two polypeptides are influenced by minor differences in one or more of the following: (a) culture conditions, (b) cell lysate preparation and storage procedure, and (c) i.p. procedure.

7.5 Conclusions

(1) "All or nothing" expression of Bn5T:TBS polypeptides suggests that the polypeptides have a common origin and/or are regulated by the same factor(s).

(2) Evidence presented suggests that Bn5T:TBS polypeptides all have epitopes recognized by TBS.

(3) Immunological and peptide mapping studies suggest that Bn5T:TBS:200, U90 and L90 polypeptides are closely related.

(4) Kinetic studies suggest that Bn5T:TBS:40 and 34kDa polypeptides are synthesised from precursors by post-translational modification.

8 TUMOUR ANTIGENS COMPARED WITH STRESS- AND VIRUS-INDUCED POLYPEPTIDES

The TG7A monoclonal antibody recognizes a 90kDa polypeptide induced in BHK21 cells by heat-shock and HSV-2 infection (Latchman et al., 1987). This monoclonal has also been shown to recognize a polypeptide of approximately 90kDa, present in Bn5T cells but not in control RE cells (Macnab et al., 1985a). It was therefore of great interest to analyze the relationship between polypeptides recognized by TG7A in cells either infected or transformed by HSV-2, and the 90kDa heat-shock protein.

8.1 The 90kDa heat-shock protein

8.1.1 Identification of the 90kDa heat-shock protein, HSP90

The AC88 monoclonal antibody recognizes the 90kDa heat-shock protein, HSP90, in a range of avian and mammalian tissues (Riehl et al., 1985; Sánchez et al., 1985; Catelli et al., 1985). The AC88 monoclonal was used in this study to investigate alterations in the expression of HSP90 and related polypeptides, in HSV infected and transformed cells.

SDS-PAGE analysis of polypeptides i.p. by AC88 shows that the 90kDa polypeptide recognized in RE cells comigrates exactly with that recognized in Bn5T cells and gives a V8 protease peptide map which is either very similar or identical (data not shown). With 1ug of V8 protease per track, seven comigrating peptides are seen for digests of both RE and Bn5T:AC88:90 polypeptides. This indicates that the constitutive HSP90 is very similar whether it is present in normal control RE cells, or transformed Bn5T cells. On gels showing high resolution the 90kDa band i.p. by AC88 appears as

a doublet suggesting heterogeneity of the HSP90 (figure 8.1).

The identity of the 90kDa rat polypeptide i.p. by AC88 was verified by comparison of its peptide map with that of rabbit reticulocyte HSP90 kindly supplied by Dr Tim Hunt (Department of Biochemistry, Cambridge). This had been prepared by a similar method to that described by Welch and Feramisco (1982) involving gel filtration on Sepharose CL-6B and anion exchange chromatography on a Pharmacia Mono Q column, using fast protein liquid chromatography (FPLC) (Walker et al., 1985). On receipt of the protein it was subjected to SDS-PAGE, located by Coomassie blue staining of the gel and the band excised for peptide mapping. The protein had not been radiolabelled in vivo and so could not be peptide mapped and autoradiographed in the usual way. Two approaches were used to overcome this problem. One approach was to detect peptides on the peptide mapping gel by staining with Coomassie blue followed by silver stain. The other approach was to radiolabel the protein in vitro with ^{125}I prior to the initial electrophoresis step, allowing subsequent detection of peptide fragments by autoradiography (figure 8.2). The chloramine-T ^{125}I-labelling method which radiolabels tyrosine residues was used here.

Peptides separated by SDS-PAGE do not necessarily give identical patterns when detected in different ways. For example, a methionine-rich peptide that incorporates ^{35}S-methionine very efficiently in vivo may produce a strong signal on autoradiography, even if too little is present for detection of the peptide by silver staining. However, if very similar peptide map patterns are revealed for two different protein preparations using different detection techniques,

Figure 8.1; Analysis by SDS-PAGE of Bn5T polypeptides i.p. by TBS and AC88. Autoradiograph of a 6-15% gradient polyacrylamide gel containing electrophoretically separated ^{35}S-methionine labelled polypeptides extracted from Bn5T cells, i.p. by control serum (track 1), TBS (track 2) and AC88 (track 3). The bands of interest in the 90kDa region are indicated by arrows.

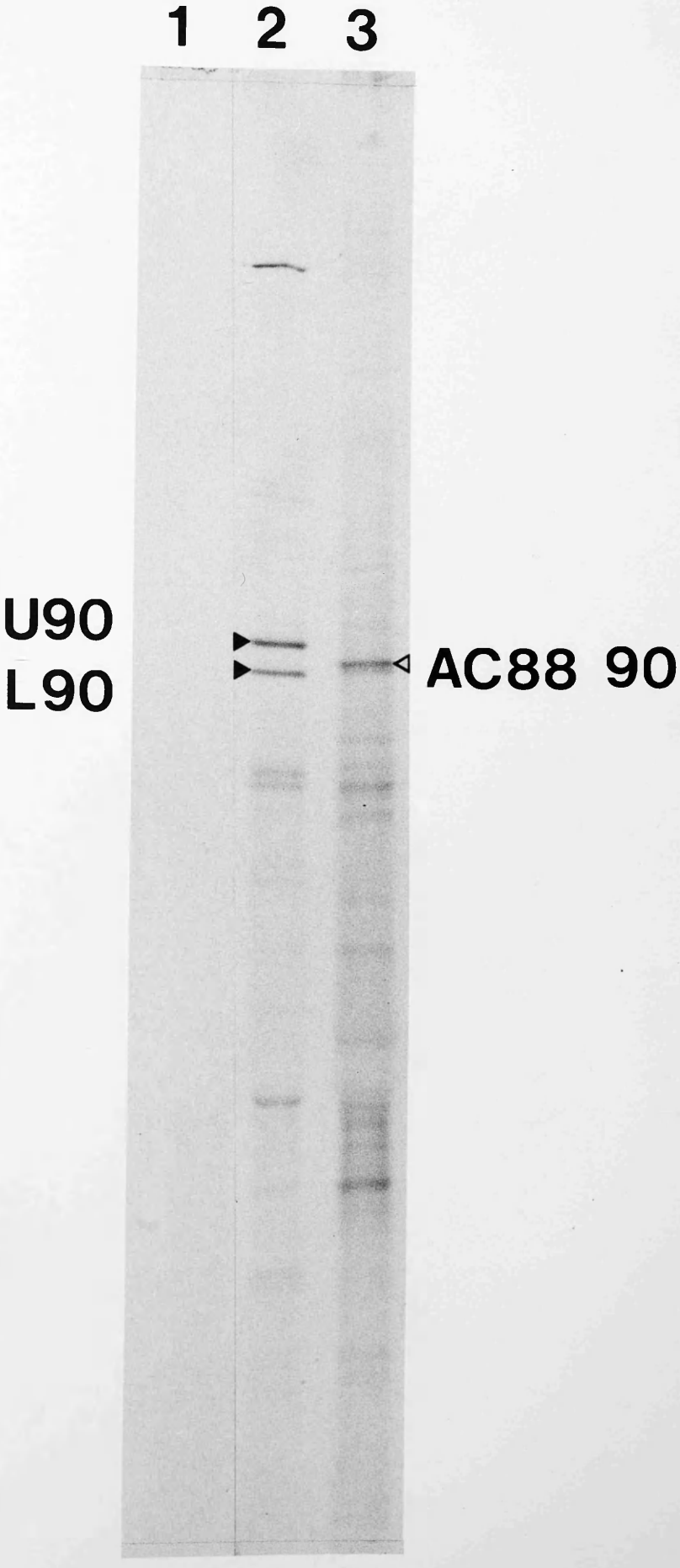
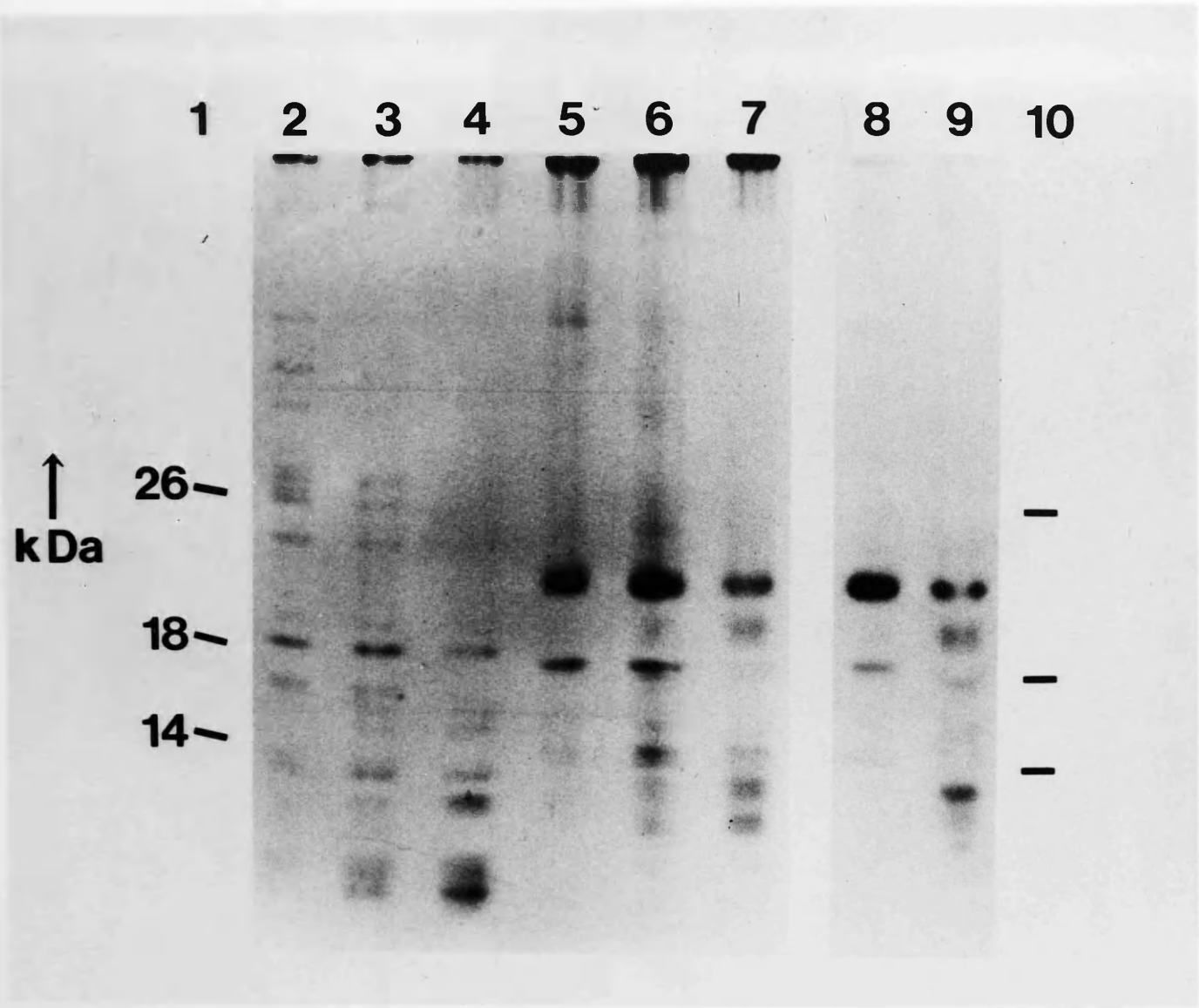

1 2 3
U90
L90
AC88 90

Figure 8.2; V8 protease peptide maps of Bn5T:TBS:L90 and HSP90 compared. Cleveland digest analysis of the L90 polypeptide i.p. by TBS from Bn5T cell lysate (tracks 2-4) compared with the 90kDa polypeptide i.p. by AC88 from Bn5T cell lysate (tracks 5-7) and in vitro radiolabelled rabbit HSP90 (tracks 8 and 9). Tracks 2, 5 and 8 contained 1/25ug, tracks 3 and 6 contained 1/5ug and tracks 4, 7 and 9 contained 1ug of S.aureus V8 protease. MW markers are shown in tracks 1 and 10.


1
2
3
4
5
6
7
8
9
10
kDa
26
18
14

this does constitute evidence of similarity. HSP90 contains 2.7% methionine and 2.9% tyrosine (Welch and Feramisco, 1982) and a peptide of 20kDa would be expected to contain approximately 180 amino acid residues. Therefore, it is likely that such a peptide would contain at least one methionine and one tyrosine residue. It is also likely that if present in sufficient quantity the peptide would produce a band on SDS-PAGE, detectable by all three approaches used in this study.

The major band in V8 protease digests of the Bn5T:AC88:90 (and RE:AC88:90) polypeptide, using both 1/25ug and 1ug enzyme, corresponds to a peptide of 21.0kDa (mean of three MW estimates from two different gels). The two other peptides giving strong bands include a 17.6kDa peptide with 1/25ug enzyme and a 19.3kDa peptide with 1ug enzyme (figure 8.2). ^{125}I-labelled rabbit reticulocyte HSP90 gives a very similar pattern for the strongest bands (figure 8.2). The major band using both 1/25ug and 1ug V8 protease, corresponds to a peptide of 22.6kDa (single estimate). The two other peptides giving strong bands include a 19.0kDa peptide with 1/25ug enzyme and a 20.0kDa peptide with 1ug enzyme. Silver staining gave a very similar result, the major band of 21.1kDa being present at both enzyme concentrations and the two next strongest bands being 18.0kDa with 1/25ug enzyme and 19.0kDa with 1ug enzyme (data not shown).

Heat-shock proteins are highly conserved in evolution (Schlesinger et al., 1982). Rabbit reticulocyte and rat fibroblast HSP90 would therefore be expected to give closely similar peptide maps. The close similarity detected here between RE and Bn5T HSP90 and rabbit reticulocyte HSP90 prepared by Dr Hunt's group, provides evidence that the

polypeptide i.p. by AC88, is the HSP90 or a closely related species.

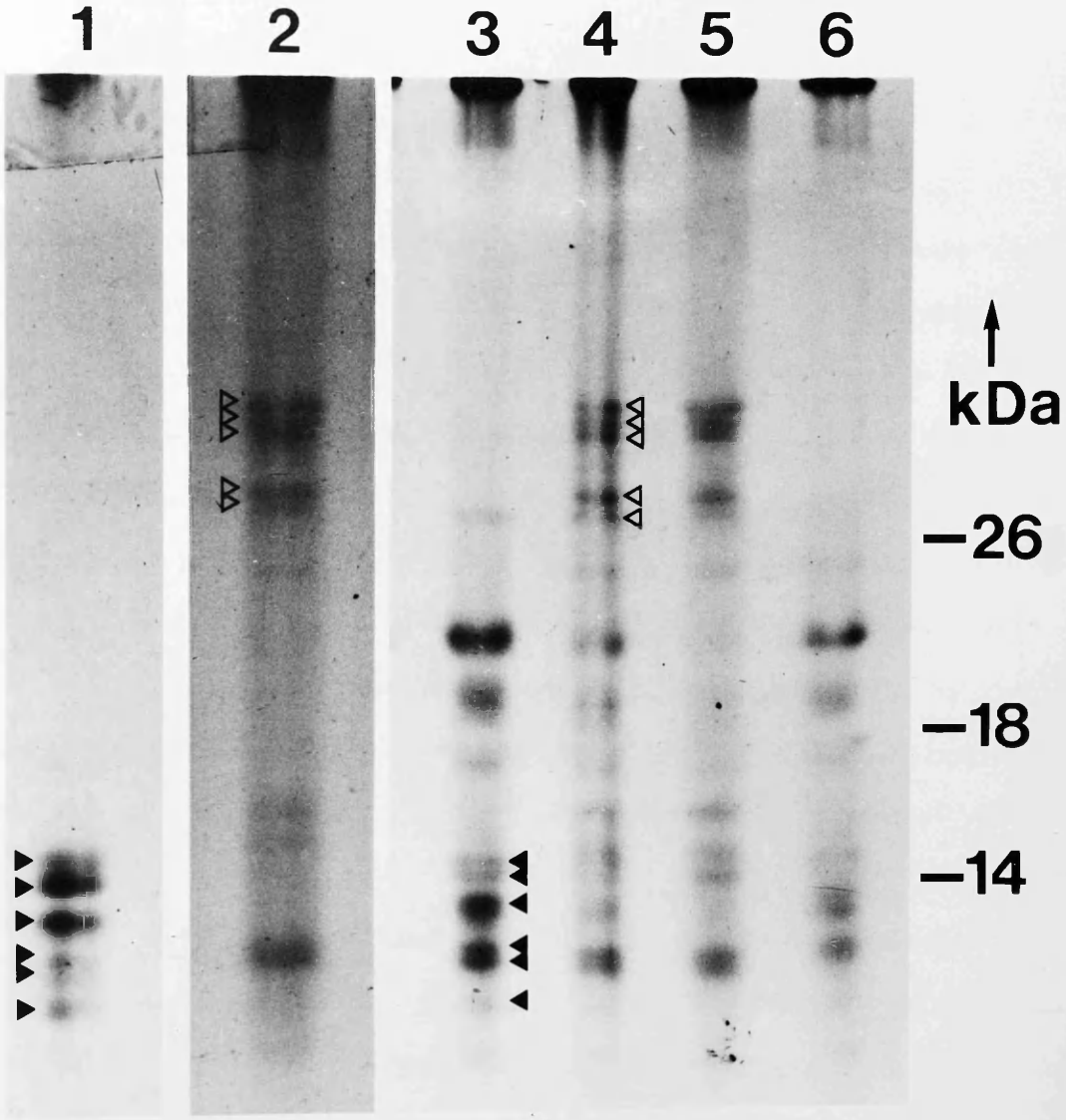
8.1.2 Comparison of HSP90 and tumour antigens

Immunofluorescence studies on a wide range of cells have shown that TG7A recognizes antigens that are induced dramatically on heat-shock treatment (Dr N.B. La Thangue, personal communication). It was therefore of interest to test whether the 90-100kDa tumour cell polypeptides recognized by TG7A are related to the HSP90. It has not been possible to detect any differences between the 90-100kDa polypeptides i.p. by TBS and TG7A (see section 6.2.1). Owing to a shortage of the TG7A monoclonal, TBS was used instead for comparison with the HSP90.

The Bn5T:TBS:L90 appears to co-migrate with the HSP90 on some 9-10% polyacrylamide gels. On 7.5% gels however, the HSP90 usually migrates slightly behind the Bn5T:TBS:L90 (figure 8.1). Two-dimensional gel electrophoresis studies have shown comigration of 90-100kDa Bn5T polypeptides i.p. by TBS and AC88 (personal communication, Dr Macnab). This indicates that they have similar isoelectric points as well as similar MWs. One-dimensional peptide mapping with V8 protease was used to compare the HSP90 with Bn5T:TBS:L90 and U90 (figure 8.3). There are obvious differences in peptide bands of over 15kDa, but comparison of peptide bands in the 11kDa to 15kDa region suggests a possible relationship between HSP90 and the Bn5T:TBS:U90 and L90 polypeptides.

In contrast to AC88, TBS fails to recognize and i.p. any Bn5T phosphoproteins in the 90kDa region, a finding confirmed by Dr D.Toft. This indicates that the HSP90 is phosphorylated in Bn5T cells, but that the 90-100kDa polypeptides recognized

Figure 8.3; V8 protease peptide maps of HSV-2 transformed and infected cell polypeptides in the 90kDa-100kDa MW range. Cleveland digest analysis using 1ug S.aureus V8 protease, of polypeptides with apparent MWs close to 90kDa (as determined by SDS-PAGE). The U90 Bn5T polypeptide i.p. by TBS (track 1) is compared with the 90kDa RE polypeptide i.p. by AC88 (track 3) which represents the HSP90. The arrows in tracks 1 and 3 indicate bands which appear to comigrate. The 90kDa polypeptide i.p. from HSV-2 infected RE cells by TG7A (track 2) is compared with the two 90kDa polypeptides i.p. from these infected cells by AC88: lower band (track 4), upper band (track 5). The arrows in tracks 2 and 4 indicate bands which appear to comigrate. The 90kDa polypeptide i.p. by AC88 from HSV-1 infected RE cells is in track 6 and MW markers are shown to the right of the gel.


1
2
3
4
5
6
kDa
26
18
14

by TBS are not.

These results indicate that while TBS and TG7A do not recognize the HSP90 in Bn5T cells, the Bn5T polypeptides they do recognize of 90-100kDa may be related to the HSP90.

8.2 HSV infected cell polypeptides recognized by TG7A and AC88

8.2.1 HSV infected cell polypeptides of 90kDa

(a) In BHK cells: Western blotting studies have shown that the TG7A monoclonal reacts with a 90kDa cellular polypeptide which accumulates during lytic infection of BHK cells with HSV-2, but not HSV-1, and also during heat-shock (La Thangue and Latchman, 1988). To demonstrate the virus-induced polypeptide, confluent BHK 21 cells were infected with HSV-2 strain 333 (m.o.i. 10). The level of the TG7A-recognized 90kDa polypeptide increased between 2h and 16h p.i., as demonstrated by western blotting (La Thangue and Latchman, 1988).

(b) In RE cells: TG7A and AC88 i.p.s of HSV-2 infected RE cell lysates, were kindly supplied by Mr A. Orr. The AC88 monoclonal i.p.s two polypeptides of approximately 90kDa from the lysate of RE cells infected with HSV-2 strain HG52 (m.o.i. 10), harvested 16h p.i. These polypeptides which will be described here as REHSV-2:**AC88**:U90 (upper band) and REHSV-2:**AC88**:L90 (lower band), migrated very close together and gave very similar peptide maps (figure 8.3), suggesting that they represent closely related polypeptides. In addition, these peptide maps show some similarity to the

RE:AC88:90 (HSP90) digest (figure 8.3). One possible explanation for this peptide map similarity is that on HSV-2 infection of RE cells, HSP90-related 90kDa polypeptides are synthesised. This might result from altered processing of HSP90 or its mRNA, following HSV-2 infection.

The 90kDa polypeptide i.p. by AC88 from HSV-1 infected RE cells, gives a peptide map which appears indistinguishable from that of the RE:AC88:90 (figure 8.3). This suggests that HSV-1 does not induce the same HSP90-related polypeptides as those induced by HSV-2.

The TG7A monoclonal also i.p.s a polypeptide of approximately 90kDa from the lysate of RE cells infected with HSV-2 strain HG52 (m.o.i. 10), harvested 16h p.i. TG7A i.p.s a comigrating polypeptide of approximately 90kDa from tsK-infected but not HSV-1-infected RE cells, which is consistent with the findings of LaThangue and Latchman (1988). The HSV-2 induced polypeptide recognized by TG7A, which will be described here as REHSV-2:**TG7A**:90, comigrates with the HSP90. REHSV-2:**TG7A**:90 was peptide mapped with V8 protease to investigate its relationship with the HSP90 and the 90-100kDa Bn5T polypeptides i.p. by TG7A and TBS (figure 8.3). The peptide map of REHSV-2:**TG7A**:90, with 1ug V8 protease per track is unlike that of either Bn5T U90 or L90. It is also unlike that of the RE:AC88:90. These results suggest that the 90kDa polypeptide i.p. by TG7A from HSV-2 infected cells, does not appear to be closely related to the 90-100kDa species i.p. by the same antibody from lysates of HSV-2 transformed cells (Bn5T cells).

The peptide map pattern for REHSV-2:**TG7A**:90 is very similar to that of the REHSV-2:**AC88**:L90 and U90. There are five peptides of 27kDa to 34kDa MW in the REHSV-2:**TG7A**:90

digest, which comigrate with peptides in the REHSV-2:**AC88**:L90 digest (figure 8.3). This strong similarity between the distinctive peptide map patterns of REHSV-2:**AC88**:L90 and REHSV-2:**TG7A**:90, suggests that on HSV-2 infection of RE cells a 90kDa polypeptide is induced, which is recognized by both AC88 and TG7A monoclonals.

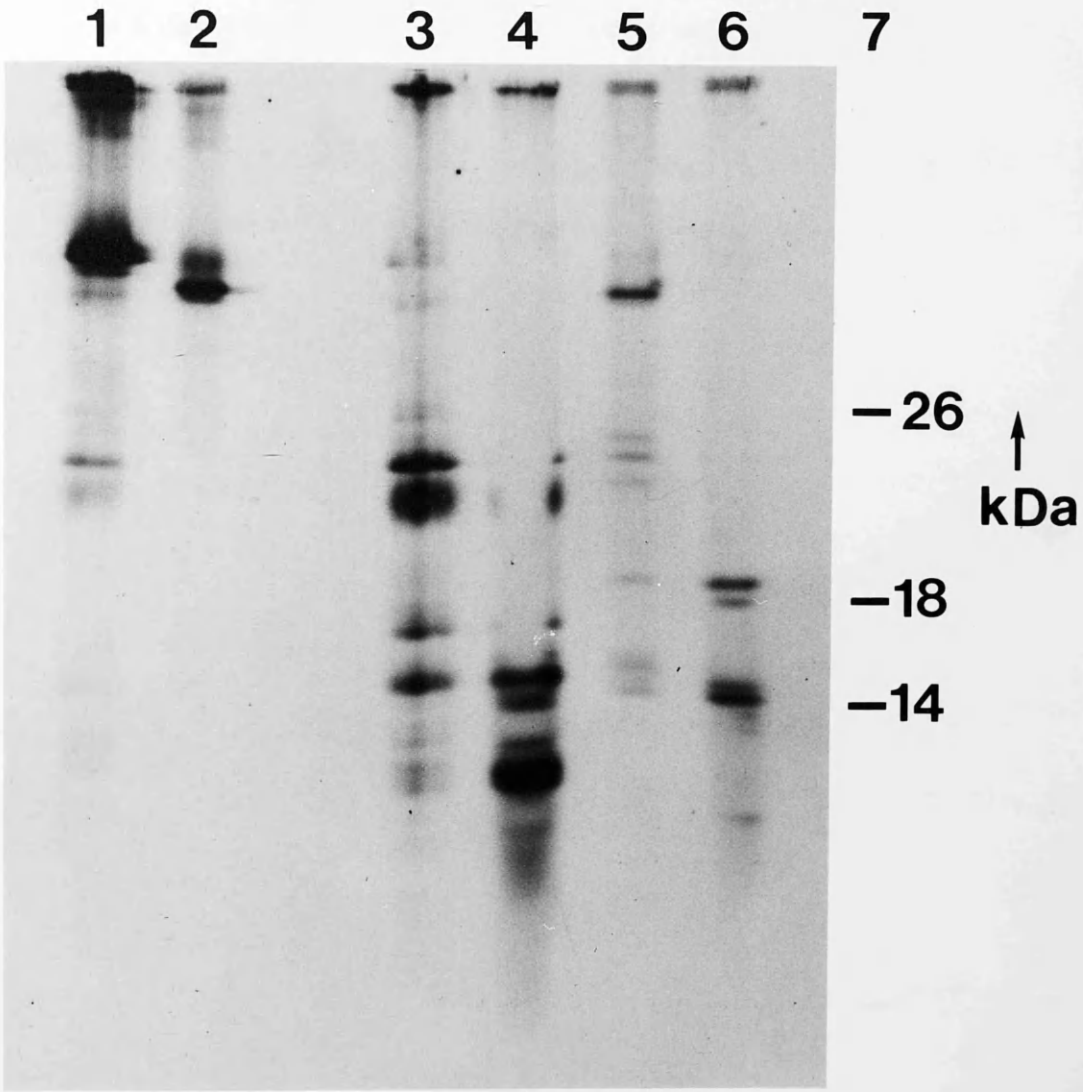
8.2.2 HSV infected cell polypeptides of 40kDa

(a) In BHK cells: Western blotting studies have shown that TG7A recognizes a 35-40kDa polypeptide induced on HSV-1 infection (LaThangue and Latchman, 1988). To demonstrate this, confluent BHK 21 cells were infected with HSV-1 strain HFEM (m.o.i. 10), and this polypeptide has been found to accumulate to high levels between 6h and 10h p.i. (Dr N. La Thangue, personal communication).

(b) In RE cells: SDS-PAGE analysis of TG7A i.p.s of lysates prepared from RE cells infected with HSV-1 strain 17 syn^+ (m.o.i. 10) harvested 8h p.i., gave a strong 38kDa polypeptide band. Peptide mapping of this 38kDa band with V8 protease gave a pattern unlike that of either Bn5T:TG7A:35 or 40 (data not shown). TG7A i.p.s of lysates prepared from RE cells infected with HSV-2 strain HG52 (m.o.i. 10) harvested 8h p.i., gave a 40kDa polypeptide band which comigrated exactly with the Bn5T:TG7A:40. However, on V8 protease peptide mapping the REHSV-2:TG7A:40 gave a pattern unlike the Bn5T:TG7A:40 (figure 8.4).

These results indicate that the TG7A recognized 38kDa and 40kDa polypeptides induced on HSV infection of RE cells differ from the TG7A recognized 40kDa polypeptide present in

Figure 8.4; A comparison of the S.aureus V8 protease peptide map of the Bn5T cell 40kDa polypeptide i.p. by TG7A, with that of the comigrating HSV-2 infected RE cell polypeptide i.p. by TG7A. Cleveland digests of the 40kDa polypeptides i.p. from Bn5T cell lysate by TG7A (tracks 3 and 4) are compared with digests of the 40kDa HSV-2 infected cell polypeptide i.p. by TG7A (tracks 5 and 6). Tracks 3 and 5 contained 1/25ug and tracks 4 and 6 contained 1ug of V8 protease. Tracks 1 and 2 correspond to undigested 40kDa polypeptides i.p. by TG7A, from Bn5T cells and HSV-2 infected cells respectively. MW markers are shown in track 7.


1
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7
26
18
14
kDa

transformed RE cells.

8.3 Conclusions

(1) The HSP90 and Bn5T:TBS polypeptides of 93kDa and 97kDa are different polypeptides. However, they have similar MW and pI values and also give similar V8 protease peptide maps.

(2) HSV-induced RE cell polypeptides seen in TG7A i.p.s give different V8 protease peptide maps from comigrating Bn5T:TG7A polypeptides.

(3) A 90kDa polypeptide induced on HSV-2 infection of RE cells is seen in both TG7A and AC88 i.p.s.

9 AMINO ACID SEQUENCE ANALYSIS

Two approaches were taken by the author to obtain sequence data for Bn5T:TBS polypeptides for the purposes of: (i) identification and (ii) the preparation of DNA and antibody probes. The first of these approaches was to use the technique of polysome immunoselection, but as described in chapter 5, this was not successful. The second approach taken was to purify the Bn5T:TBS:U90 polypeptide for amino acid sequence analysis, as from Coomassie blue staining experiments, this appears to be the most abundant Bn5T:TBS polypeptide. A purification protocol was developed for preparing large amounts of Bn5T:TBS:U90 and as a result, amino acid sequence data was obtained.

9.1 Polypeptide purification for sequence analysis

9.1.1 The standard Bn5T:TBS i.p.

A standard Bn5T:TBS i.p. carried out on 100ul undiluted lysate does not produce an U90 band on SDS-PAGE that is visible after Coomassie blue staining of the gel. To produce a visible U90 band it is necessary to run at least four such i.p.s in a single track (figure 4.3).

In general 0.2ug of a protein running in a tight band is just sufficient to be visible on Coomassie blue staining of polyacrylamide gels, but this does vary to some extent as Coomassie blue is an anionic dye which binds most strongly to more basic proteins (Hames, 1981). From two-dimensional gel electrophoresis studies of Dr J. Macnab and Mr A. Orr Bn5T:TBS:U90 is known to have an isoelectric point of 5.0-5.3 while the majority of proteins have isoelectric points in the 5-7 range (Walker and Gaastra, 1983), and so it is not a

highly acidic polypeptide. Therefore the above quantitation guide is probably fairly accurate. Thus, if four standard i.p.s contain about 0.2ug of the 97kDa polypeptide (roughly 2 picomoles), then 1 standard i.p. contains about 0.5 picomoles of polypeptide. For sequencing on the Applied Biosystems 470A gas phase sequencer, 20-30 picomoles are usually required so that over forty standard i.p.s were needed to provide sufficient material for sequencing. Carrying out so many identical standard i.p.s was extremely tedious and it was therefore decided to scale up the procedure.

9.1.2 The large-scale immunoprecipitation

A large-scale i.p. protocol was devised by the author, and sequence data was obtained on the Bn5T:TBS:U90 polypeptide so prepared.

In a standard i.p. (see section 3.6.7) a volume of Bn5T cell lysate corresponding to $4X10^6$ c.p.m. is made up to 100ul with RIPA buffer, then incubated with 5ul TBS for 1 h at 4^oC. Immune complexes are precipitated by addition of a suspension of protein-A sepharose and incubated for 1 h on a rotating mixer. Immune complexes are washed by cycles of centrifugation, removal of supernate and resuspension of pellet in fresh buffer. Addition of electrophoresis sample buffer (e.s.b) to the final pellet is followed by heating to 100^oC for 3 min. releasing bound polypeptides into the supernate. This supernate was then collected and analysed by SDS-PAGE.

The following alterations were made for the scaled up procedure (see section 3.8.1):

(a) The volume of lysate used was increased to 10ml and the volume of TBS increased to 1ml. Thus, the scale of the

reaction was approximately 200X that of a standard i.p.

(b) Protein-A sepharose was maintained in suspension during incubation periods by gentle mixing in a 50ml conical flask with a small magnetic stirring bar. For the increased volumes used this was found to be more effective than incubating on a rotating mixer.

(c) Protein-A sepharose and bound complexes were washed by transfer to a column, over which 3ml of first RIPA buffer, then tris saline were run. This was simpler for the scaled-up volumes used than the washing cycles of a standard i.p.. It also required much less washing buffer and gave cleaner results than standard i.p.s, as judged by autoradiography after SDS-PAGE analysis.

(d) Initially, polypeptides were eluted from the protein-A sepharose column using electrophoresis sample buffer (e.s.b.). The e.s.b was run into the column until the bromophenol blue dye front reached the bottom. The column was then sealed in a polythene bag and heated to 100^{o}C in a boiling water bath for 5 min. The column was then unsealed and polypeptides eluted by application of further e.s.b to the column. This proved an efficient method of eluting polypeptides. However, e.s.b. contains 2% SDS and the content of SDS was further increased on concentration of the eluted fractions (see section 3.8.1). The resulting high concentration of SDS was thought to cause distortion of polypeptide bands on subsequent SDS-PAGE (Dr J. Walker, personal communication). Alternative elution buffers tested include: 0.1M glycine-HCl pH2.5; 1M acetic acid; 20mM diethylamine pH10.8, 10%(v/v) glycerol and 3M KSCN. 3M KSCN gave the best results and was as efficient as e.s.b. in eluting polypeptides from the column.

The author was kindly helped by Dr J. Macnab and Mr D.

McNab in optimizing the following aspects of this procedure:

(e) Lysate was made 10X more concentrated to maximize antigen concentration in the reaction. In a preliminary study, this modification was shown to increase the intensity of U90 and 40kDa bands by 1.5X and 3.8X respectively, as measured by densitometer.

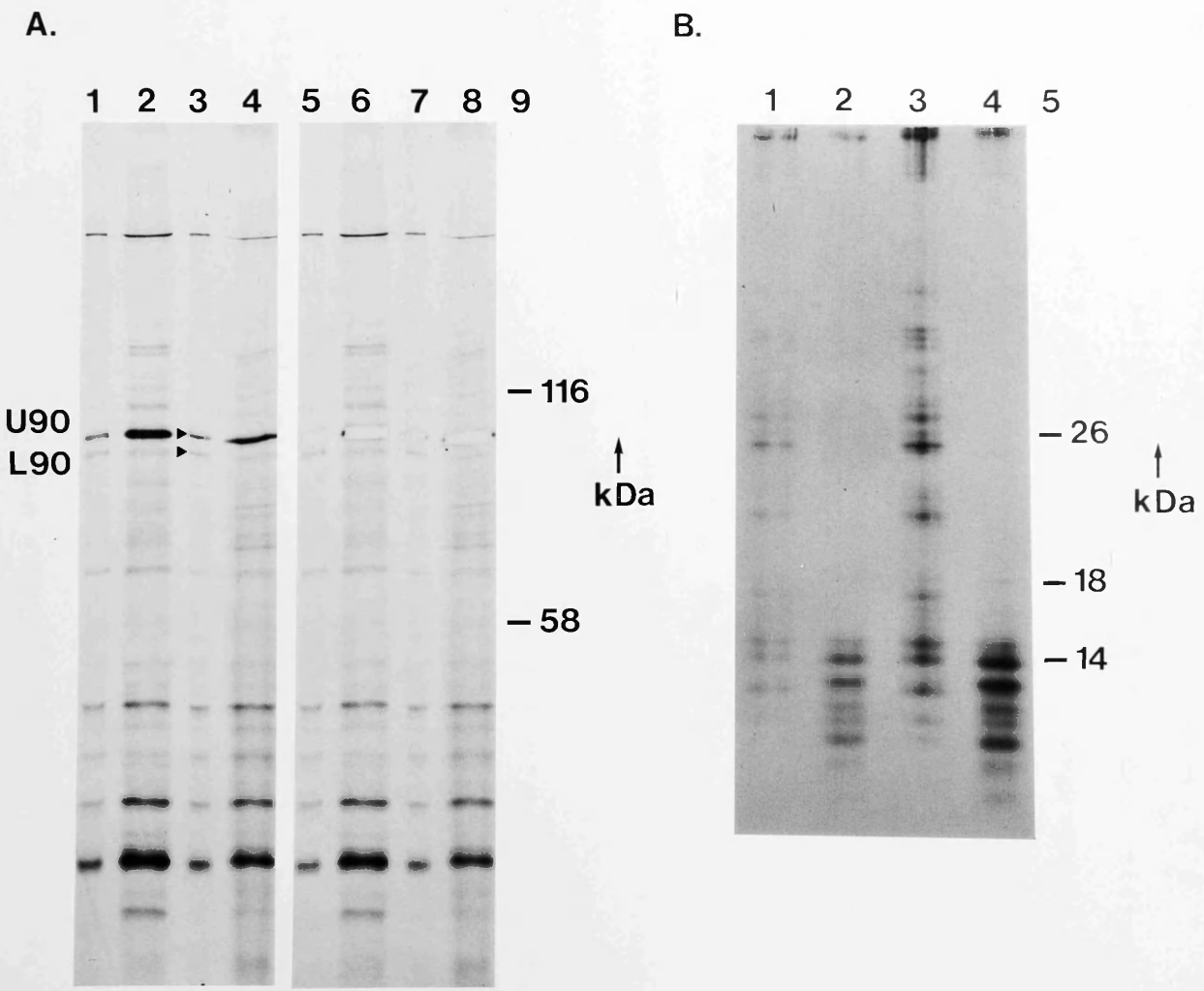
(f) Antibody was incubated with protein-A sepharose before the addition of cell lysate to minimize the binding of large numbers of antibody molecules to each antigen molecule, thus improving the efficiency of the reaction (Dr H. Marsden, personal communication).

(g) Incubations were at carried out at room temperature instead of 4^{o}C, which gave slightly improved yields of i.p. polypeptides.

(h) Eluted polypeptides in 3M KSCN were desalted on a Pharmacia PD-10 column (sephadex G-25 M), eluted in 10mM ammonium bicarbonate, freeze-dried and then dissolved in electrophoresis sample buffer. Removal of KSCN appears to improve the stability of the polypeptide on storage.

SDS-PAGE analysis of large-scale i.p.s shows that the 97kDa polypeptide comigrates exactly with the Bn5T:TBS:U90 polypeptide of a standard i.p. (figure 9.1). Peptide mapping of the 97kDa band from two independent scaled-up i.p.s gave an identical pattern to the Bn5T:TBS:U90 from standard i.p.s (figure 9.1), and is therefore assumed to represent the same polypeptide. On analysis of large-scale i.p.s there is sometimes a faint band visible on autoradiographs which comigrates with the Bn5T: TBS:L90. The reason that the intensity of this band differs between standard Bn5T:TBS i.p.s and large-scale i.p.s is not known, but it may relate to the

Figure 9.1; Comparison of the 97kDa polypeptides in standard and large-scale Bn5T:TBS i.p.s, by SDS-PAGE and Cleveland digest analysis. A. Autoradiographs of a gel before (1-4) and after (5-8) excision of 97kDa polypeptide bands for the Cleveland digest analysis seen in B. The 97kDa or U90 polypeptide in standard i.p.s (tracks 1 and 3) is seen to comigrate with the strong 97kDa polypeptide band in large scale i.p.s (tracks 2 and 4). The absence of 97kDa bands in tracks 5-8 indicates that these bands were accurately excised for peptide mapping. **B.** Comparison by Cleveland digest analysis of 97kDa Bn5T polypeptides i.p. by TBS in standard (tracks 1 and 2) and large-scale i.p.s (tracks 3 and 4). Tracks 1 and 3 contained 1/25ug and tracks 2 and 4 contained 1ug of S.aureus V8 protease. MW markers are shown in track 5.


A.
1 2 3 4 5 6 7 8 9
U90
L90
116
58
kDa
B.
1 2 3 4 5
26
18
14
kDa

different washing procedures used. In autoradiographs of large-scale i.p.s analysed by SDS-PAGE, the signal:background ratio of 97kDa and 40kDa polypeptides is greater than for standard i.p.s. This suggests that washing of protein-A sepharose-bound immune complexes is more thorough in the large-scale i.p. procedure than in the standard i.p. procedure. Hence, polypeptides bound with lower affinity would be more likely to be removed in the large-scale i.p than in the standard i.p. This may explain why on SDS-PAGE analysis of large-scale i.p.s, the L90 (93kDa) polypeptide is either weak or absent.

Figure 9.2 shows a Coomassie blue stained track containing a large-scale Bn5T:TBS i.p., together with the autoradiograph of the same track. The strong 97kDa band seen on autoradiography, can be seen to correspond to a strong Coomassie blue stained band.

9.1.3 Transfer of polypeptides onto Immobilon membrane

Polypeptides were separated out by SDS-PAGE on a 7% acrylamide gel and transferred electrophoretically onto an Immobilon membrane as described in section 3.8.2. Polypeptide bands of interest were excised from this membrane, and the accuracy of this excision is demonstrated by the autoradiograph in figure 9.3).

Optimum transfer conditions were determined using MW markers separated by SDS-PAGE on a 7% acrylamide gel. A current of 0.3mA/cm^2 for 2h gave best results as it transferred all detectable phosphorylase b (97.4kDa) and ovalbumin (43kDa) from gel to membrane. Only a small proportion of the ovalbumin passed through the membrane to be deposited on a second Immobilon membrane placed on the anode

Figure 9.2; Analysis of a large-scale Bn5T:TBS i.p. by SDS-PAGE. ^{35}S-methionine labelled polypeptides from a Bn5T:TBS large scale were subjected to SDS-PAGE on a 7.5% polyacrylamide gel and the gel was then Coomassie blue stained (track 1) and autoradiographed (track 2). MW markers are shown to the right of track 2.

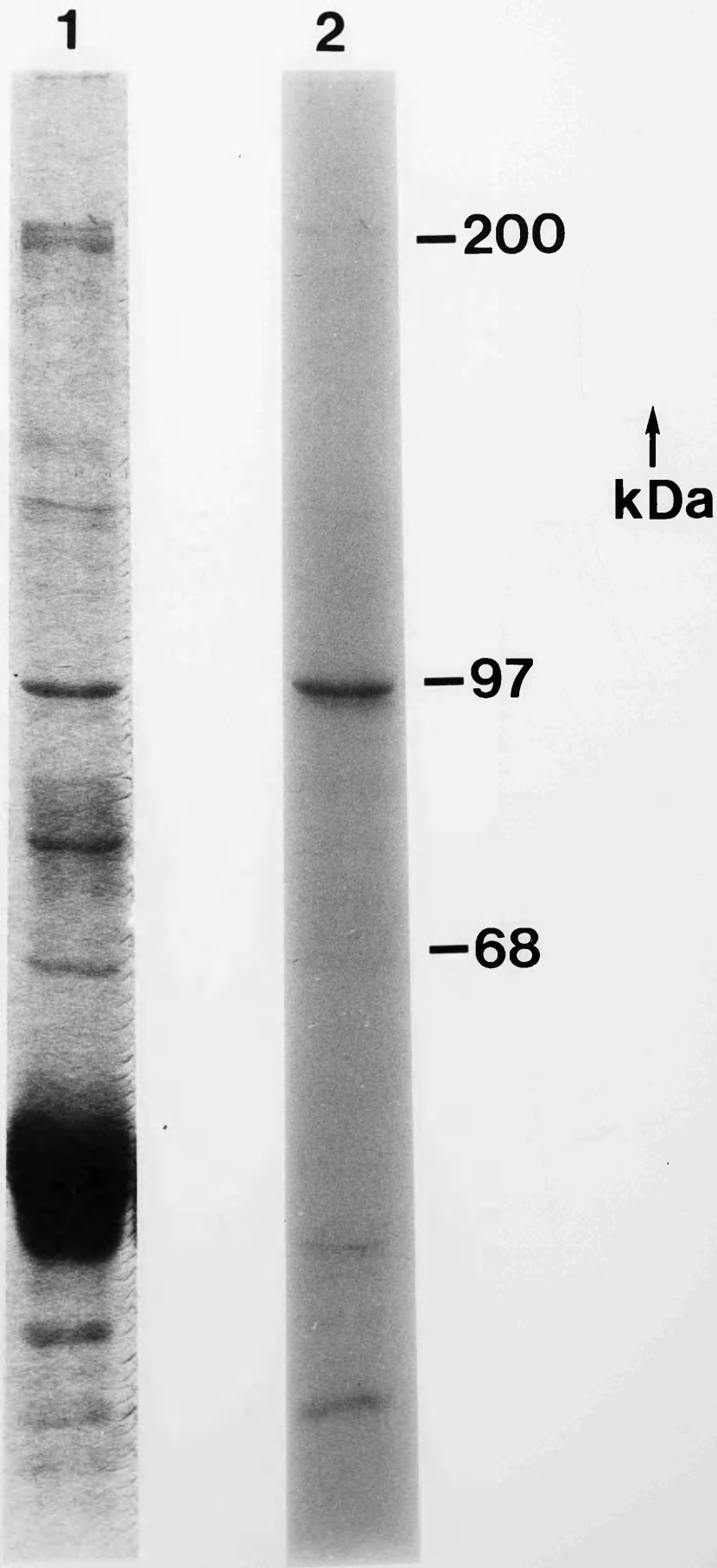
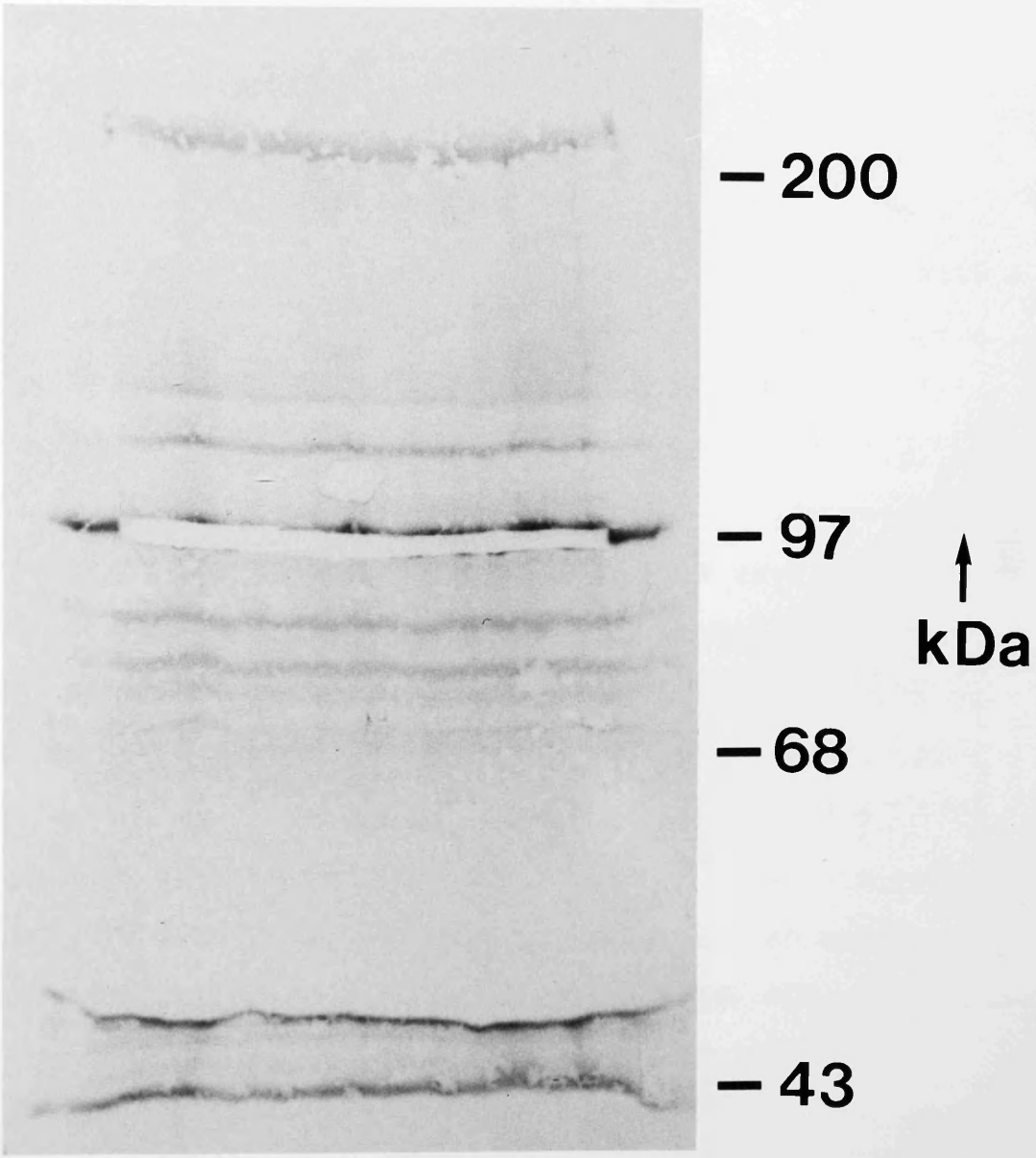

1
2
200
97
68
kDa

Figure 9.3; Testing the accuracy of band excision for sequence analysis: A single large-scale i.p. of Bn5T cell lysate using TBS was subjected to SDS-PAGE, then electrophoretically transferred onto Immobilon PVDF transfer membrane. An autoradiograph of the Immobilon transfer membrane was used to locate the 97kDa band for excision. The Immobilon membrane was re-autoradiographed to demonstrate that the 97kDa band had been accurately excised for sequence analysis.


200
97
68
43
kDa

side of the first (data not shown).

9.2 Amino acid sequence analysis

Sequence analysis was performed by Mr F. Northrop and Dr I. Fearnley. Peptides were prepared by Dr J. Macnab and the author, under the direction of Dr J. Walker (Laboratory of Molecular Biology, Cambridge). The techniques used are described in section 3.8.

9.2.1 Sequence analysis of intact Bn5T:TBS:U90

Comparison of the Coomassie blue stained U90 band with a known amount of phosphorylase b MW marker, indicated that a minimum of 25ug (approximately 250 picomoles) of U90 polypeptide is precipitated in a single large-scale i.p., an amount which should be sufficient for sequence analysis of most proteins. It was surprising then, that when Bn5T:TBS:U90 resulting from a single large-scale i.p. prepared by the author, was sequenced by Mr F. Northrop, very low levels of PTH-amino acid derivatives were obtained in the first two sequencer cycles. The levels suggested that only 1-2 picomoles of polypeptide had been sequenced, which is less than $1/100^{th}$ the quantity of polypeptide that was estimated from Coomassie blue staining. In addition the PTH-amino acids obtained on sequencing were derived from more than one polypeptide. This result was interpreted to mean that the major polypeptide present in the U90 band has a blocking group at the N-terminus which prevents sequencing and that minor unblocked polypeptide components of the band account for the small amount of sequenceable polypeptide detected. Examples of blocking groups include cyclized glutamine, acetylated glycine and N-formyl methionine (Fearnley, I., 1987, Ph.D.

thesis, University of Cambridge).

9.2.2 Sequence analysis of peptide fragments of the Bn5T:TBS:U90

Preparation of peptide fragments of a polypeptide allows sequence data to be obtained even if the polypeptide N-terminus is blocked. This approach also has the advantage that a lot more sequence data can be obtained for the polypeptide as a whole, as amino acid sequencing does not usually allow sequencing of more than 20 to 30 residues in from the N-terminus. This is because the recovery of all PTH-amino acids decreases as the Edman degradation proceeds, due to chromatography and sequencer artifacts which increase the system noise level and the accumulation of protein/peptide breakdown products which are also sequenced (Fearnley, I., Ph.D. thesis). Large amounts of Bn5T:TBS:U90 and 40 were prepared by Dr J. Macnab and Mr D. McNab, using the large-scale i.p. procedure and peptides were prepared from these polypeptides at the LMB, Cambridge, by Dr J. Macnab and the author.

Separate preparations of the U90 polypeptide were cleaved by treatment with trypsin or by treatment with cyanogen bromide. Peptides were separated by HPLC on a 0% - 80% acetonitrile gradient and then sequenced separately on an Applied Biosystems sequencer by Mr F. Northrop and Dr I. Fearnley. Sequence data obtained on these peptides, is shown in table 9.1. The evaluation of sequencing data by computer analysis is discussed in the next chapter.

9.3 Summary

(a) A protocol was developed for the large-scale preparation

Table 9.1; Sequence analysis of peptide fragments of the 97kDa polypeptide: Peptides A11-22 were derived by tryptic digestion and peptides C10-40 by cyanogen bromide cleavage. The sequences obtained were analysed using EMBL databases for protein and DNA sequences (section 10.4.1).

* indicates no sequence data obtained on the peptide.

---- indicates no homology detected to sequence data on the EMBL database.

Peptides C14, C36 and C37 have been compared by Dr Macnab with sequence data for HSP90 and GRP94 in the literature, but no homology has been detected.

One letter amino acid code: A = alanine, C = cyseine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = glycine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, N = asparagine, P = proline, Q = glutamine, R = arginine, S = serine, T = threonine, V = valine, W = tryptophan, X = not determined, Y = tyrosine

Peptide	Sequence	Source
A11	IVGGY	Trypsin
12	IVG	Trypsin
14	APILSXSSXK + SAYPGQITSN	Trypsin
15	TIVPPSY	-------
16	*	
17	*	
18	*	
19	SSGTSYPXVLK	Trypsin
20	*	
21	XVGGYT	Trypsin
22	IVGGYTXGANTVPK	Trypsin
C10	XVXK	
11	KEGI	bovine $alpha_{s1}$-casein
12	*	
13	*	
14	APFPE	bovine $alpha_{s1}$-casein
16	*	
17	XQPHNV	-------
18	*	
19	*	
20	XPXILEKL	-------
21	*	
22	*	
25	XPXIGXKTXVQXYK	
29	*	
36	PIQAFLLY	bovine beta-casein
37	XXVKQELAYFYPELFXQF	bovine $alpha_{s1}$-casein
40	*	

of the 97kDa polypeptide recognized by TBS.

(b) Sequence data was obtained on the 97kDa polypeptide prepared by this protocol.

10 DISCUSSION

The results will be discussed under four main headings. Firstly, data on general features of Bn5T:TBS polypeptides will be examined and a model presented to explain the inter-relationships of these polypeptides. After this, the terms "tumour antigen" and "oncofoetal antigen" will be discussed in relation to Bn5T:TBS polypeptides. Next studies indicating the existence of an HSP90-related protein family will be reviewed and finally, the preliminary sequence data on the Bn5T:TBS:U90 will be discussed.

10.1 PROPERTIES OF Bn5T:TBS POLYPEPTIDES

10.1.1 The cell type specificity of polypeptides immunoprecipitated by TBS and TG7A

Bn5T cells express a group of polypeptides which are detected in i.p. assays using serum from rats bearing tumours induced by injected Bn5T cells and also using the TG7A monoclonal antibody. They are not detected by i.p. assay in control RE cells prepared from embryos of 16-20 days gestation. However, comigrating polypeptides are detected in RE cultures prepared from younger embryos of approximately 12 days gestation. Hence, Bn5T:TBS polypeptides appear to show oncofoetal expression.

In all established and transformed rat and mouse cell lines tested, with the exception of mouse embryonal carcinoma (EC) derived cell lines, polypeptides can be i.p. with TBS which comigrate with Bn5T:TBS polypeptides. It is interesting that TBS did not recognize the characteristic set of polypeptides in any of four differentiated EC-derived cell lines. One of these, EB28/10n has myoblast-like culture

morphology and produces tumours containing muscle-like cells, cartilage and bone (Morgan et al., 1983). EB28/10n therefore has a mesodermal origin, as does the RE fibroblast-derived Bn5T cell line. Perhaps then, the polypeptide set recognized by TBS is expressed specifically by immortalized fibroblastic cells, rather than being characteristic of immortalized mesoderm derived cells in general. It may be relevant that, unlike all other rodent cells tested, EC-derived cell lines are always trypsinized in the presence of 1% (v/v) chick serum to protect against trypsin toxicity (Dr M. Hooper, personal communication). Hence, it is possible that the expression of Bn5T:TBS polypeptides is a consequence of trypsin toxicity. However, the fact that Bn5T:TBS polypeptides are not detected in control RE cells suggests otherwise, because RE cells like Bn5T cells, are trypsinized without the addition of chick serum.

10.1.2 The implications of Bn5T:TBS polypeptide co-expression

When members of the Bn5T:TBS polypeptide set have been detected for a particular cell type, they have always been found to be co-expressed in an "all or nothing" manner, regardless of culture conditions. Possible explanations for this include: (a) that Bn5T:TBS polypeptides have a common origin (all members of the set being encoded by a single primary transcript, with differential post-transcriptional or post-translational processing accounting for the different polypeptides produced), and (b) that expression of all Bn5T:TBS polypeptides may be regulated by common control mechanisms at transcriptional or post-transcriptional levels.

Whether co-expression is due either to a common origin or common control mechanism, it may still indicate a shared

purpose for Bn5T:TBS polypeptides. Interestingly, these polypeptides share epitopes recognized by TG7A and TBS, which may represent related domains important for a common function. Latchman et al., (1987) have found that TG7A recognizes an epitope which is highly conserved, indicating that it represents part of a functionally important domain. The only clue to the function of this domain so far, is that TG7A recognizes a protein in E. coli which has protease activity (Latchman et al., 1987).

10.1.3 Developing a model to explain Bn5T:TBS polypeptide inter-relationships

Kinetic studies were carried out to examine the inter-relationships between Bn5T:TBS polypeptides. Very similar experiments to those reported in this thesis were performed in a different system by Khosravi et al., (1985). These authors studied a human melanoma-associated oncofoetal antigen of 87kDa by pulse labelling and pulse-chase experiments and identified an 83kDa precursor polypeptide in this way. The 83kDa precursor was shown to be converted over a 4h period to the 87kDa polypeptide and tunicamycin treatment showed that the processing involved glycosylation (Khosravi et al., 1985).

The results of pulse-chase labelling experiments described in section 7.4.2, suggest that the Bn5T:TBS:40 polypeptide is also synthesised from a precursor polypeptide, as the corresponding autoradiographic band shows a gradual increase in intensity at increasing time periods following chase. However, results from metabolic labelling studies with tritiated mannose and treatment of cells with tunicamycin indicate that the Bn5T:TBS:40 is unlikely to be a

glycoprotein. Bn5T:TBS:40 may be a processing/breakdown product of a precursor polypeptide, which may or may not be recognized by TBS. In the pulse-chase labelling experiment, no Bn5T:TBS polypeptide bands show a fall in intensity on autoradiography, concomitant with the rise in intensity of the Bn5T:TBS:40 band. However, if the quantity of precursor greatly exceeds the quantity of breakdown product formed during the pulse-chase experiment, a decrease in precursor band intensity would not necessarily be detectable. Peptide mapping studies showed no similarity between the Bn5T:TBS:40 and other members of the Bn5T:TBS set, except for a slight similarity with the 200kDa polypeptide. Another link between 200kDa and 40kDa polypeptides is that they are the only phosphorylated members of the Bn5T:TBS set. If the 40kDa were a breakdown product of the 200kDa, then given the fact that it is only 20% of the size of the 200kDa, a close similarity between peptide maps would in fact be surprising. The slight peptide map similarity between Bn5T:TBS:200 and 40kDa may therefore reflect a precursor-product relationship.

In pulse-chase experiments the Bn5T:TBS:34 band is too faint to be visible, but in pulse labelling experiments it is visible and like the Bn5T:TBS:40 shows a gradual rise in intensity with increasing labelling periods. This suggests that the 34kDa polypeptide may also be a breakdown/processing product of some precursor polypeptide, but peptide mapping studies did not demonstrate any obvious similarity to any other Bn5T:TBS polypeptides. However, owing to the small size of the 34kDa polypeptide relative to other members of the Bn5T:TBS set, the possibility still remains that the 34kDa polypeptide is derived from another Bn5T:TBS polypeptide.

Very similar patterns of variation in L90 and 200kDa band

intensity were seen for both pulse labelling and pulse-chase labelling experiments, suggesting some relationship between these two polypeptides. Perhaps both are bound by antibody with low affinity, so that they are more sensitive than other Bn5T:TBS polypeptides to the minor inevitable differences that occur in the handling of replicate tubes in an i.p. assay.

The fact that U90, L90 and 200kDa polypeptides are i.p. strongly as a group by TBS and weakly as a group by TG7A, together with the fact that they give similar peptide maps, suggests that they form a closely related subgroup of the Bn5T:TBS set. Precursor-product relationships between these three polypeptides were not evident from pulse-chase experiments and no evidence for post-translational processing of these polypeptides has been found apart from phosphorylation of the 200kDa polypeptide. U90, L90 and 200kDa polypeptides may therefore be the products of related genes, or may be the products of a single gene like the calcitonin gene, for which there are a number of alternative modes of post-transcriptional modification. The calcitonin gene generates distinct mRNAs by differential splicing, which encode either the hormone calcitonin, or a novel neuropeptide (Amara et al., 1984).

One model which explains the assembled data on Bn5T:TBS polypeptide inter-relationships is as follows: (a) the 200kDa, U90 and L90 polypeptides are encoded by the same gene or similar genes, (b) the 40kDa polypeptide is a breakdown/ processing product of the 200kDa polypeptide, and (c) the 34kDa polypeptide is a breakdown/ processing product (perhaps also a product of the 200kDa polypeptide).

10.1.4 Explaining why IVT Bn5T polypeptides are not recognized by TBS and TG7A

An unusual feature of the Bn5T:TBS polypeptides is that it has not been possible to demonstrate their synthesis *in vitro* by i.p. assay using TBS and TG7A. This finding was thoroughly investigated because it suggested that Bn5T:TBS polypeptides might require post-translational processing to be recognized by TBS and TG7A, a possibility which would make the immunoselection of Bn5T polysomes unlikely to work with these antibodies.

On IVT of total cytoplasmic Bn5T RNA for the standard period of 1 h, no 40kDa polypeptide could be detected by i.p. with TBS. This is not surprising in the light of results from pulse-labelling studies (section 7.4.1) which showed that the 40kDa band in TBS i.p.s of Bn5T cell lysate is much weaker if cells are radiolabelled 1 h than for the usual 18 h incubation period. However, the kinetic studies do not explain the fact that 200kDa, U90 and L90 are not detected in the IVT products of Bn5T RNA.

A 40kDa band has been seen in profiles of the total polypeptide products of IVT Bn5T polysomes (only if IVT for a shorter time period than usual) and of IVT poly A^+ RNA (on one out of three occasions); (section 5.2). The IVT 40kDa bands do not necessarily represent the same 40kDa polypeptide seen in total protein profiles of Bn5T cell lysate, even though both comigrate exactly with the Bn5T:TBS:40. Unfortunately, peptide mapping comparisons were not possible because the IVT 40kDa bands did not give a strong enough signal on autoradiography.

The following are possible explanations why no IVT Bn5T polypeptides were i.p. by TBS:

(a) The mRNAs encoding Bn5T:TBS polypeptides are unstable: Unstable mRNA may be degraded during RNA preparation, or may have a short half-life in the cytoplasm-like environment of reticulocyte lysate. On examination of polypeptides resulting from IVT of total cytoplasmic Bn5T RNA, clearly defined bands are visible in the 30kDa to 100kDa range, indicating that at least some mRNAs which encode polypeptides within this MW range had been prepared intact. Thus, it would not have been unreasonable to expect to be able to i.p. Bn5T:TBS:U90, L90, 40 and 34 polypeptides. When this was found not to be possible, total cell polysomes were substituted for total cytoplasmic RNA. The quality of the mRNA in the polysome preparations was better than in cytoplasmic RNA preparations, judging by the efficient translation of high MW polypeptides from the former. However, no members of the Bn5T:TBS set were i.p. by either TBS or TG7A from the products of IVT polysomes. These results suggest that if mRNA degradation does account for our inability to detect IVT Bn5T:TBS polypeptides, then it is due to the unstable nature of the corresponding transcripts rather than to being a reflection of the overall quality of RNA or polysome preparations.

Short mRNA half-life in reticulocyte lysate, does not seem to provide a very satisfactory explanation for the inability to i.p. IVT Bn5T polypeptides with TBS. Firstly, the half-life would need to be extremely short. An mRNA half-life of 1 h is relatively short (Theodorakis and Morimoto, 1987), yet to account for our inability to detect any polypeptide after IVT for 1 h, the mRNA would need to have a half-life of a few minutes or seconds. Secondly, one would also have to argue that the polypeptides translated within the short life of the mRNA are so unstable that within 1 h of

synthesis they are undetectable.

(b) The mRNAs encoding Bn5T:TBS polypeptides are either not IVT efficiently, or not IVT at all: Observations on profiles of total IVT polypeptides suggest that this explanation accounts, at least partly, for the fact that no members of the Bn5T:TBS set could be i.p. with TBS. In the special circumstances when a 40kDa polypeptide could be IVT, it still only gave a faint band compared with that seen for Bn5T cell lysates. It must be remembered that although peptide mapping studies have shown that the 40kDa polypeptide seen in Bn5T cell lysate profiles is closely related to the Bn5T:TBS:40, the only evidence that it is also closely related to the IVT 40kDa polypeptide is that the two bands comigrate on SDS-PAGE. Thus, despite the fact that a 40kDa Bn5T polypeptide has been IVT, there is still no strong evidence that any Bn5T:TBS polypeptides can be IVT from Bn5T cell RNA or polysomes.

(c) The IVT products of Bn5T RNA and polysomes require post-translational processing in order to be recognized by TBS: A problem with this explanation is that the inclusion of microsomal membranes in IVT reactions (which allows for post-translational processing including removal of signal peptides and glycosylation), did not result in the IVT of Bn5T polypeptides recognized by TBS. Further, the only evidence found of post-translational processing, for members of the Bn5T:TBS set, was the phosphorylation of 200kDa and 40kDa polypeptides and possible glycosylation of the 34kDa polypeptide. No evidence was detected for a type of processing common to all members of the Bn5T:TBS set. Perhaps then, the recognition of Bn5T:TBS polypeptides by TBS depends

on some unanticipated type of processing, or depends on the formation of complexes which are not formed *in vitro*.

In conclusion, we can only speculate as to why it was not possible to i.p. Bn5T:TBS polypeptides from the products of IVT RNA and polysomes. From the above discussion the two following explanations seem to be the most likely: (a) Bn5T:TBS polypeptides may be encoded by mRNAs which are extremely fragile and/or translated extremely inefficiently in the reticulocyte lysate system, and (b) polypeptides i.p. by TBS and TG7A may require some unusual kind of post-translational processing before they can be recognized by TBS and TG7A.

10.2 Tumour antigens and oncofoetal antigens

Before defining Bn5T:TBS polypeptides as either "tumour antigens" or "oncofoetal antigens" and examining their relationship with other members of these groups, the definitions of these terms will first be considered in detail.

10.2.1 Evaluation of definitions for tumour antigens and oncofoetal antigens

Tumour antigens have been defined as immunological determinants present in tumour tissue, which elicit an antibody response in a tumour bearing animal (Maltzman *et al.*, 1981), while oncofoetal antigens have been defined as antigenic substances expressed exclusively as phase-specific autoantigens in the developing embryo or foetal tissues, of metazoans and their tumours (Coggin, 1986a).

Tumour-specificity is an important feature in both of the above definitions and the fact that there are no problem-free criteria for deciding whether an antigen is tumour-specific,

gives rise to problems with both definitions. The two criteria used in the cited definitions are; the ability to induce an antibody response in a tumour bearing animal, and the exclusive expression in embryo/foetal tissues and tumours. These criteria will now be discussed:

(a) Induction of an antibody response in a tumour bearing animal: Induction of an immune response in a syngeneic tumour bearing animal suggests that the antigen is not expressed by normal adult tissues, but failure to induce antibodies does not necessarily suggest the opposite. The antigen may: (i) have low intrinsic immunogenicity, resemble a self component to which the host is tolerant, or not be expressed in a cellular location accessible to the immune system, (ii) induce antibodies of a subtype not detected in the assay system used (e.g. IgM would not be detected in i.p. assays), or (iii) induce a cell mediated immune response instead of an antibody response (Roitt, 1984). Therefore, the detection of an antibody response is a useful, though not infallible indicator that an antigen is tumour-specific.

(b) Exclusive expression in embryo/foetal tissues and tumours: It is not possible to state with certainty that an embryo/foetal antigen is not expressed in any non-neoplastic adult tissue, given the diversity of adult tissues and the very wide range of non-neoplastic disease. Hence, the classification of an antigen as an oncofoetal antigen can only be provisional.

A further problem with the cited definition of oncofoetal antigens: "antigenic substances expressed exclusively as

phase-specific autoantigens in the developing embryo or foetal tissues, of metazoans and their tumours" (Coggin, 1986a), is the use of the term "autoantigen". This is presumably intended to mean an antigen that induces autoantibodies, but in the context of an embryo or foetus, the meaning of this term is less clear. An embryo/foetus would not be expected to produce antibodies against antigens which are normally expressed in pre-natal development and it would be incorrect to describe maternal antibodies produced against embryonic/foetal antigens as autoantibodies. The most important problem in the application of this definition is the difficult or impossible task of finding out whether an antigen is expressed exclusively in embryonic and neoplastic tissues.

In contrast, the cited definition for tumour antigens: "immunological determinants present in tumour tissue, which elicit an antibody response in a tumour bearing animal" (Maltzman et al., 1981), is a useful working definition, although it is not problem-free.

10.2.2 Classification of Bn5T:TBS polypeptides

(a) Can Bn5T:TBS polypeptides be defined as tumour antigens?

Polypeptides of the Bn5T tumour cell line i.p. by TBS (the Bn5T:TBS set), are not detectable in control RE cells. The presence of antibodies to these polypeptides in TBS indicates that they are recognized by the tumour bearing animal as being immunologically foreign and distinct from normal self antigens. On this basis, it seems correct to call Bn5T:TBS polypeptides, tumour antigens.

(b) Can Bn5T:TBS polypeptides be defined as oncofoetal

antigens?

Bn5T:TBS polypeptides are not expressed by primary and secondary cultures of late gestation RE cells, but we do not know whether this also applies to all normal adult tissues and adult tissues with non-neoplastic disease. It is therefore not possible to decide whether Bn5T:TBS polypeptides can be defined as oncofoetal antigens. However, Bn5T:TBS polypeptides do appear to show oncofoetal expression, as polypeptides comigrating with these tumour cell polypeptides were detected in RE cells prepared from embryos of approximately 12 days, but not in cells prepared from embryos of 16-20 days gestation.

10.2.3 Comparison of Bn5T:TBS polypeptides with other polypeptides which show oncofoetal expression

A number of tumour-associated antigens show oncofoetal expression as discussed in section 1.3. Examples are p53, the OFAs of 44kDa and 200kDa described by Payne and Coggin (1985) and the SDEAs of 35kDa and 40kDa described by Gerhards and Mehnert (1986). These antigens share a number of features with Bn5T:TBS polypeptides which include:

(a) Expression in transformed/tumour cells independent of the oncogenic agent: The p53 tumour antigen is expressed in cells transformed by a range of viruses including SV40 and in cells transformed by chemical carcinogens (DeLeo et al., 1979; Linzer and Levine, 1979; Rotter et al., 1981). It is also detected in a variety of human tumour cell lines (Crawford et al., 1981). Similarly the 44kDa and 200kDa OFAs (Payne and Coggin, 1985) are detected in cell lines derived from SV40- and methylcholanthrene-induced mouse fibrosarcomas, as well as

from a range of human tumours, including ovarian, colonic and rectal carcinomas. In the same way, the 35kDa and 40kDa SDEAs (Gerhards and Mehnert, 1986) are detected in SV40 and polyomavirus transformed cells and cell lines derived from a variety of human tumours including HeLa and rhabdomyosarcoma cell lines.

(b) Phase specific expression in embryonic/foetal life: The expression of p53 occurs in primary cell cultures prepared from 12-14 day mouse embryos, but not 16 day embryos (Mora et al., 1980). Similarly the 44kDa and 200kDa OFAs are present in cell cultures of 12-13 day mouse embryos, but not 19-21 days (Payne and Coggin, 1985). The 35kDa and 40kDa SDEAs are expressed in primary cell cultures of 16 day BALB/c mouse embryos, but not 12, 14 or 18 day embryos (Gerhards and Mehnert, 1986).

The p53 tumour antigen has been shown to be the product of an immortalizing oncogene (Jenkins et al., 1984; Eliyahu et al., 1984; Parada et al., 1984) and it will be interesting to find out whether these other oncofoetally expressed tumour antigens are also encoded by oncogenes. The expression of these polypeptide antigens in transformed and tumour cells independent of the oncogenic agent, suggests that their induction is a common secondary event and not the primary oncogenic event.

It has been suggested that gene products important in prenatal development might confer a survival advantage in the tumour environment (Gerhards and Mehnert, 1986). If this is correct the reactivation of embryonic genes may be a common event in tumour progression or evolution rather than being a

direct consequence of the primary oncogenic event. However, embryonic antigens are expressed by tissue culture cells transformed in vitro, which indicates that the possibility that they aid tumour cell survival can not be the only reason for their reactivation in neoplasia.

10.3 HSP90-related proteins provide a possible link between lytic infection by HSV-2 and oncogenic transformation

TG7A monoclonal was raised in mice to DNA-binding proteins in HSV-2 infected cells and it recognizes an HSV-2 induced 90kDa polypeptide and an HSV-1 induced 40kDa polypeptide in lytically infected cells. It has been shown to recognize heat-shock induced cell surface antigens by immunofluorescence studies (Dr LaThangue, personal communication) and also recognizes polypeptides of 97kDa, 93kDa and 40kDa in HSV-2 transformed cells.

10.3.1 Stress induction of TBS- and TG7A-recognized polypeptides

It was very difficult to induce a heat-shock response in RE cells. However, incubation of RE cells for 1.5-5 h at 44°C, followed by 0.5 h at 37°C prior to labelling for 18 h with ^{35}S-methionine, induced the appearance of HSP bands in total cell polypeptide profiles (Dr Macnab, personal communication). On two occasions a 90kDa polypeptide induced in these conditions was i.p. by TBS and TG7A and gave a faint band on autoradiography. TBS and TG7A do not recognize a 90kDa polypeptide in control RE cells. A 90kDa polypeptide i.p. by TBS and TG7A was also induced in RE cells both by incubation in medium lacking glucose for 18-24 h and by incubation in medium containing the calcium ionophore A 23187

(Dr Macnab, personal communication). This is thought to be the GRP94. In the same conditions a TBS- and TG7A-recognized polypeptide of 70kDa was induced, which may represent the GRP78. Taken together these results suggest that the heat-shock induced HSP90, the GRP94 and the GRP78, all express epitopes recognized by TBS and TG7A.

10.3.2 Peptide map comparisons of TG7A- and AC88-recognized polypeptides

As described in section 8.1.1, the AC88 monoclonal i.p.s the constitutively expressed HSP90 from control RE cells. On close examination of RE:AC88 i.p.s on SDS-PAGE, two bands of approximately 90kDa are seen, of which the upper band is the strongest. This suggests that there is more than one form of constitutively expressed HSP90. Two polypeptide bands of approximately 90kDa are also seen in AC88 i.p.s of HSV-2 infected RE cells. These bands do not migrate as close together as for RE:AC88 i.p.s, so that it was possible to excise the two bands separately for peptide mapping analysis.

Peptide mapping studies with V8 protease, show that the constitutive HSP90 gives a peptide map which bears some similarity to peptide maps of the polypeptides i.p. by AC88 from HSV-2 infected cells. The two REHSV-2:**AC88**:90 polypeptide bands give peptide maps which are very similar to eachother and which in addition share some similarity with the constitutive HSP90. The REHSV-2:**AC88**:90 polypeptides are therefore likely to represent HSV-induced HSP90s.

TG7A and TBS do not i.p. the constitutive HSP90 from RE cells, but do i.p. a comigrating polypeptide from HSV-2 infected RE cells. On peptide mapping analysis the REHSV-2:**TG7A**:90 polypeptide gave a very similar pattern to the

REHSV-2:**AC88**:90 polypeptides. TG7A and TBS also i.p. 93kDa and 97kDa polypeptides from transformed Bn5T cells, both of which give peptide maps showing some similarity to the constitutive HSP90.

10.3.3 HSV type-specific induction of the HSP90-related polypeptide

Immunoprecipitation experiments carried out by Mr Orr, Dr Macnab and the author, have shown that a 90kDa polypeptide i.p. by TBS and TG7A is induced by infection with HSV-2, but not by HSV-1. Immunoblotting studies by Latchman et al., (1987) agree with these results, and show that TG7A recognizes a 90kDa polypeptide induced by HSV-2, but not HSV-1. In addition, we have demonstrated the induction of a TG7A-recognized 90kDa polypeptide on tsK infection. This agrees with results of Notarianni and Preston (1982) who demonstrated HSP90 induction by tsK. It is interesting that tsK is an HSV-1 mutant, yet the TG7A-recognized 90kDa polypeptide is induced by wt HSV-2, but not wt HSV-1.

Russell et al., (1987) have analyzed the induction of HSP90 by tsK and have concluded that the induction is due to the abnormal Vmw175 polypeptide encoded by the mutant. Our studies indicate that HSV-2 also induces a form of HSP90, which suggests that like the Vmw175 encoded by tsK, HSV-2 Vmw175 differs from HSV-1 Vmw175 in such a way as to induce the HSP90-related protein. The involvement of Vmw175 in the expression of the virus-induced HSP90 may result from: (1) trans-activation by Vmw175 of a gene encoding HSP90-related polypeptide; and (2) complex formation between Vmw175 (particularly abnormal forms of the protein) and the HSP90-related polypeptide. This might increase the level of

the HSP90-related polypeptide by a stabilizing effect.

Thus, HSV type-specific induction of HSP90-related polypeptide may be due to differences in the DNA binding specificity or protein binding activity of the Vmw175 polypeptides encoded by HSV-1 and HSV-2.

10.3.4 Classification of HSP90-related proteins

Our findings suggest the existence of a range of HSP90-related proteins and supporting evidence for this, comes from several other sources. Hickey et al. (1986) have identified two non-cross hybridizing mRNAs which encode 89kDa heat-shock induced proteins. In addition, Ullrich et al. (1986) appear to have shown that the TSTA of a mouse sarcoma induced by methylcholanthrene is an HSP90-related protein. This group has also shown that the TSTA belongs to a highly polymorphic family of TSTAs, which vary between individual tumours induced by the same or different carcinogens (DuBois et al., 1982, 1984; DeLeo et al., 1977). From these studies the potential number of HSP90-related proteins would seem to be almost limitless.

We have shown that AC88 recognizes constitutive and HSV-induced forms of the HSP90, while TG7A and TBS recognize HSV-induced and transformation-induced forms of the HSP90. In addition, TG7A and TBS appear to recognize the heat-shock induced HSP90. From this data there seem to be at least three groups of HSP90-related polypeptide which are: (i) constitutively expressed HSP90, e.g. RE:AC88:90, (ii) stress-induced HSP90, e.g. REHSV-2:**AC88**:90 and REHSV-2: **TG7A**:90 and RE-heat-shocked:**TG7A**:90, and (iii) transformation-related HSP90, e.g. Bn5T:TG7A:U90 and Bn5T:TG7A:L90. It is not yet known whether different HSP90 forms are induced by the

different stresses of HSV-2 infection, tsK infection and heat-shock, but it will be interesting to compare the peptide maps of these polypeptides.

Recognition of the HSV-2 induced HSP90 by TBS suggests that this protein, or an antigenically related form, is expressed by tumour cells and induces antibodies in the tumour bearing animal. In addition, Macnab et al., (1985a) showed that rat antisera raised against HSV-2 infected cells, contain antibodies which i.p. the transformation-related HSP90 from Bn5T cells. This suggests that HSP90-related proteins, which show altered expression in HSV-2 infected and transformed cells, are antigenically closely related and perhaps share domains with important functions in oncogenesis.

10.4 Sequence data

Sequence data discussed here was obtained for peptide fragments of 97kDa Bn5T polypeptide, prepared by the author and Dr J. Macnab (section 9).

10.4.1 Evaluation of amino acid sequence data

Sequence data on peptide fragments the 97kDa Bn5T cell polypeptide was analyzed by computer-assisted comparisons with sequence data on other polypeptides. This analysis was carried out by Dr J. Walker, Mr M. Brunswick and Dr J. Macnab with the help of Dr P. Taylor. Of the peptides obtained by trypsin digestion, sequence data was obtained on seven (see table 9.1) and of these, six showed homology to trypsin and one showed no homology to stored sequence data on EMBL databases.

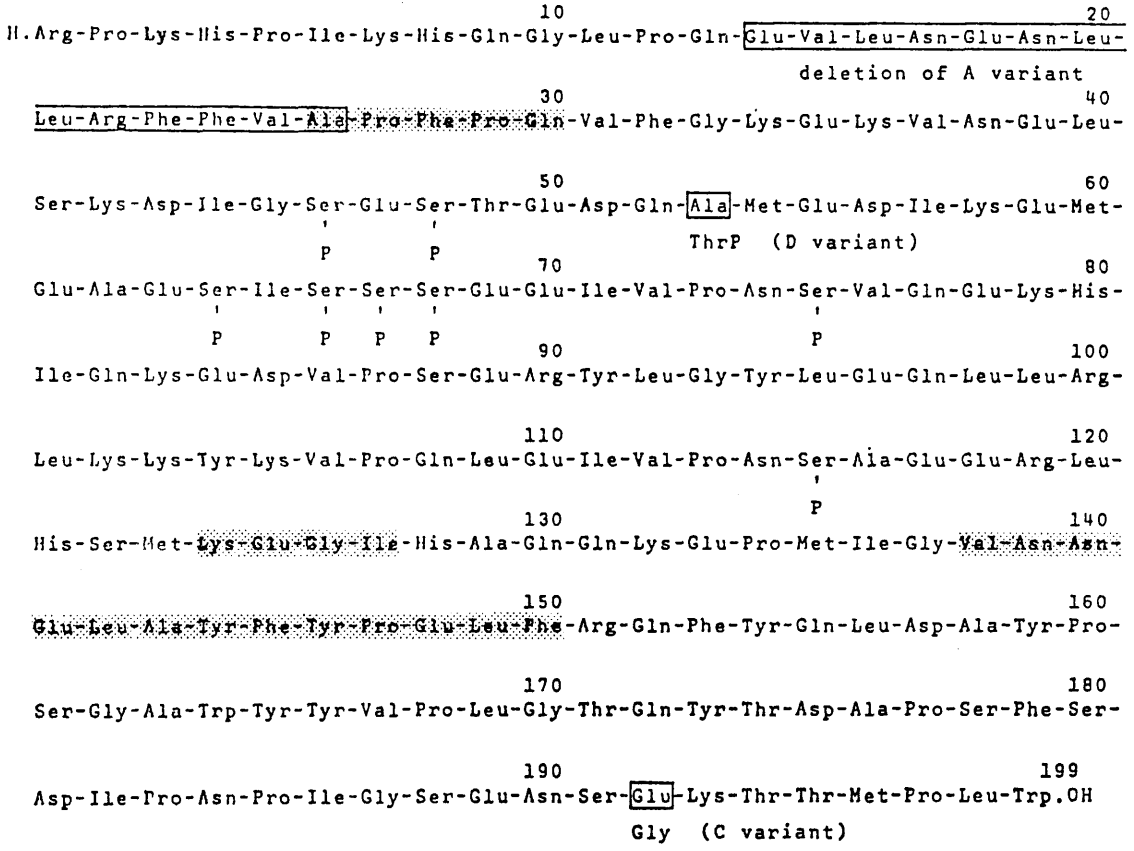
Of peptides obtained by cyanogen bromide cleavage, sequence data was obtained on eight (table 9.1) and of these

three showed homology to bovine alpha$_{s1}$-casein (C11, C14 and C37), one showed homology to bovine beta-casein (C36), one was not evaluated (C25) and three showed no homology to stored sequence data on EMBL databases (C10, C17 and C20). Three peptides (C14, C36 and C37) were compared with all available data on the HSP90 and GRP94, but no homology was detected (Dr Macnab, personal communication). The conclusion regarding the peptides yielded by trypsin digestion, was that the enzyme had yielded peptides by autodigestion and that these peptides had been sequenced.

Bovine alpha$_{s1}$-casein has a relative MW of 23.6kDa (reviewed by Ribadeau Dumas et al., 1975) and contains 199 amino acid residues (figure 10.1). Our three peptides that show sequence homology to this protein, correspond to the following residues: residues 26-30 (peptide C14); residues 124-127 (peptide C11); and residues 138-150 (peptide 37). As bovine alpha$_{s1}$-casein is approximately 1/4 the MW of the polypeptide prepared for sequencing, which has a MW of 97kDa as determined by SDS-PAGE, it is possible that bovine casein represents one domain on this 97kDa molecule. As the three sequenced peptides correspond to widely spaced regions of the primary sequence of bovine alpha$_{s1}$-casein protein, it seems likely that a major portion of bovine alpha$_{s1}$-casein would be contained within the 97kDa molecule.

Explaining why a bovine protein has been detected in the transformed rat cell line, Bn5T, would not appear to present a problem. It is quite possible that a bovine protein could have entered the system, as the rat cells were cultured in a medium containing foetal calf serum. The bovine protein must adhere to or be incorporated by the rat cells, as these were washed 3X with PBS before preparation of the cell lysate for

Figure 10.1; Amino acid sequence of bovine alpha$_{s1}$-casein variant B (Mercier et al., 1971). Enclosed amino acid residues are those corresponding to substitutions which differentiate the known genetic variants. Sequences showing homology to peptides C11, C14 and C37 (table 9.1) are highlighted.


10
20
H.Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-Glu-Val-Leu-Asn-Glu-Asn-Leu-
deletion of A variant
30
40
Leu-Arg-Phe-Phe-Val-Ala-Pro-Phe-Pro-Gln-Val-Phe-Gly-Lys-Glu-Lys-Val-Asn-Glu-Leu-
50
60
Ser-Lys-Asp-Ile-Gly-Ser-Glu-Ser-Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-Ile-Lys-Glu-Met-
P
P
ThrP (D variant)
70
80
Glu-Ala-Glu-Ser-Ile-Ser-Ser-Ser-Glu-Glu-Ile-Val-Pro-Asn-Ser-Val-Gln-Glu-Lys-His-
P
P
P
P
P
90
100
Ile-Gln-Lys-Glu-Asp-Val-Pro-Ser-Glu-Arg-Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg-
110
120
Leu-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-Ser-Ala-Glu-Glu-Arg-Leu-
P
130
140
His-Ser-Met-Lys-Glu-Gly-Ile-His-Ala-Gln-Gln-Lys-Glu-Pro-Met-Ile-Gly-Val-Asn-Asn-
150
160
Glu-Leu-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu-Phe-Arg-Gln-Phe-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-
170
180
Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-
190
199
Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-Leu-Trp.OH
Gly (C variant)

large-scale i.p. If the 97kDa polypeptide that we have sequenced does represent bovine casein bound to another molecule (giving it the apparent MW of 97kDa) then this binding would have to be strong, probably covalent binding, as otherwise the molecules would have been separated by electrophoresis under the reducing conditions used. As described in section 1.4 of the Introduction there is an HSP called ubiquitin which binds covalently to other proteins, targeting them for proteolysis. It is possible that radiolabelled rat ubiquitin or a similar rat HSP might bind to bovine casein taken up by the transformed rat cell, targeting this foreign protein for proteolytic degradation. Such an explanation may also account for recognition of the 97kDa polypeptide by TG7A, a monoclonal known to react with HSPs in eukaryotic and bacterial cells. Bovine beta-casein has a relative MW of 24.0kDa and comprises 209 amino acids (reviewed by Ribadeau Dumas et al., 1975). Why this protein might also be present in the 97kDa band is not clear, but it is possible that beta-casein is processed in the rat cell in the same way as $alpha_{s1}$-casein, giving a molecule of similar MW.

10.4.2 The reliability of the sequence data

The sequence data would be unreliable if either: (a) the wrong polypeptide was prepared for sequencing, or (b) the sequence data corresponded to a major contaminant. These two possibilities will now be considered.

(a) Could the wrong polypeptide have been prepared for sequencing? The 97kDa polypeptide prepared for sequence analysis was carefully compared with the 97kDa polypeptide in standard TBS i.p.s of Bn5T cell lysate to establish that it

represented the same polypeptide. Apart from the comigration on SDS-PAGE of the 97kDa polypeptide bands in large scale and standard i.p.s, as detected both by Coomassie blue staining and autoradiography, the 97kDa polypeptides were indistinguishable on peptide mapping. Peptide mapping with V8 protease shows that for four independent large-scale preparations of the 97kDa polypeptide each prepared on a different day and each mapped with two different enzyme concentrations, the peptide map pattern obtained was indistinguishable from that of the 97kDa polypeptide seen in standard i.p. Further, as the large scale preparation technique is essentially a scaled-up version of the standard i.p. reaction, it would be unlikely to bring down a different 97kDa polypeptide if the same antibody and antigen are used.

(b) Could the sequence data correspond to a contaminant polypeptide? One possible explanation for the sequencing result is that a contaminant 97kDa polypeptide was sequenced rather than the Bn5T:TBS:U90 which is the polypeptide of interest. As bovine proteins are not synthesised in rat cells, they would not incorporate ^{35}S-methionine in the same way as rat polypeptides during translation. It is possible then that a 97kDa bovine protein could be present in Bn5T:TBS i.p.s prepared by either or both of the techniques used (standard and large-scale i.p.s), without giving any evidence of its presence on autoradiography.

From peptide mapping studies, the 97kDa polypeptide of interest is clearly present in large-scale TBS i.p.s of Bn5T cell lysate. It is possible however that this radiolabelled 97kDa polypeptide has an undetected companion polypeptide which accounts for the sequence data. The unseen companion

polypeptide must co-migrate exactly with the polypeptide of interest and co-purify on i.p. It must also be present in at least comparable quantity, as four of the eight cyanogen bromide peptide fragments of the polypeptide prepared showed homology to bovine casein. The sequence data may correspond to a contaminant 97kDa polypeptide band which by unfortunate coincidence, overlaps the band of interest. The putative contaminant polypeptide would presumably be abundant enough to give a significant Coomassie blue stained band (as this is a criterion used to judge whether one has sufficient material for sequencing) and the only significant Coomassie blue stained band in the 80kDa to 150kDa range can be seen from figure 9.2, to be a 97kDa band which exactly overlaps the 97kDa band on autoradiography.

From 2-D gel electrophoresis studies by Dr J. Macnab and Mr A. Orr, in which separated radiolabelled polypeptides were detected by autoradiography, we know that the Bn5T:TBS:U90 band is composed of a number of polypeptides which incorporate ^{35}S-methionine. This result suggests caution in the interpretation of our sequence data. However, it can also be argued that as RE:TBS i.p.s subjected to SDS-PAGE, do not show any 97kDa band on autoradiography, all radiolabelled polypeptide components of the Bn5T:TBS:U90 band must be transformation-specific and therefore of interest.

10.4.4 Sequence data: conclusions

While it seems very unlikely that the polypeptide of interest was not present in the polypeptide sample subjected for sequence analysis, it is possible that the bovine casein sequence data corresponds to a contaminant polypeptide. However, if the correct explanation for the sequencing result

is that the Bn5T:TBS:U90 contains a bovine alpha$_{s1}$-casein domain, this may help to explain why it was not possible to demonstrate Bn5T:TBS polypeptides after IVT of Bn5T cell RNA and polysomes and also why it was not possible to immunoselect Bn5T polysomes with TBS and TG7A.

Whether or not the sequence data corresponds to the Bn5T:TBS:U90 polypeptide, the sequence data still poses two interesting questions: (a) why is a culture medium protein of bovine origin, present in abundance, in immunoselected fractions of a rat cell lysate, and (b) why does bovine casein present in our preparations, migrate under reducing conditions on SDS-PAGE, as a protein with an apparent MW of approximately 4X its known MW?

One possible explanation is that the Bn5T:TBS:U90 polypeptide is synthesised in Bn5T cells by the covalent attachment of rat cell polypeptides (which would incorporate radiolabel) to bovine alpha$_{s1}$-casein which has been taken up from the culture medium. Ubiquitin is an example of a polypeptide which attaches in this way to certain polypeptides to mark them for proteolysis (section 1.4.3). Furthermore, ubiquitin is a heat shock protein and therefore a candidate target antigen for the monoclonal antibody, TG7A.

10.5 Conclusions and future prospects

The oncofoetal expression of members of the Bn5T:TBS polypeptide set suggests that they have a role in cell growth and differentiation and the invariable co-expression of these polypeptides suggests that they may have related functions.

Evidence is presented which indicates that HSV-2 infection induces a 90kDa stress protein which is related to, but distinct from the HSP90. Antibody and peptide mapping

studies presented here also indicate that the 97kDa Bn5T polypeptide recognized by TG7A (and TBS) is related to the constitutive HSP90. However, initial amino acid sequencing studies indicate that the 97kDa Bn5T:TBS polypeptide has a casein domain. The explanation for this paradoxical finding remains to be determined.

The sequencing results suggest that the 97kDa polypeptide consists of a bovine casein moiety derived from a culture medium protein, and a rat moiety which is covalently attached. The rat component may be a polypeptide such as ubiquitin, which is a heat-shock protein known to attach covalently to abnormal proteins. If this is the correct interpretation of the data then it poses many new questions such as: (i) why do immortalized and transformed rat cells differ from control primary rat embryo cell cultures with respect to the production of the 97kDa Bn5T:TBS polypeptide, (ii) are the other members of the Bn5T:TBS set derived from foreign proteins, and (iii) are some other "tumour antigens" in fact derived in the same way from foreign proteins taken up from the culture medium by transformed cells in culture?

Further studies will be necessary to establish the nature of the 97kDa Bn5T:TBS polypeptide with more certainty. These include the analysis of Bn5T cell polypeptides from cells grown in culture media that do not contain bovine components. In addition, the purity of the Bn5T:TBS:U90 polypeptide used for sequence analysis will need to be thoroughly assessed before repeated sequence analysis is carried out. In particular, the presence of non-radiolabelled contaminant polypeptides in the 97kDa band will need to be excluded.

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