AN INVESTIGATION OF THE INTERACTIONS OF THE HERPES SIMPLEX VIRUS TYPE 1 IMMEDIATE-EARLY PROTEIN VMW110 WITH CELLULAR PROTEINS

by

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SUMMARY

HSV-1 is a human pathogen which initially infects epithelial cells (usually causing 'cold sores' around the mouth) and then establishes life-long latent infection in sensory neurons. A knowledge of the mechanisms by which latent infection is established and by which reactivation occurs is likely to be a key factor in understanding the biology of the virus.

The HSV-1 virion contains a double stranded linear genome of 152kD which encodes at least 77 genes. These genes are expressed in a temporally regulated cascade during lytic infection in tissue culture. The five immediate-early genes are transactivated by a component of the virion tegument (Vmw65) in a complex with cellular proteins. The expression of at least three,Vmw175, Vmw63 and Vmw110, is necessary for the efficient transactivation of early and late genes. Vmw175 is the major viral transactivator and Vmw63 is thought to act at the post-transcriptional level. The mechanism of action of Vmw110 is unclear.

Vmw110 is a 775aa phosphoprotein which is localised to discrete domains in the nucleus, called ND10, at early times of infection. The protein is encoded by gene IE1 which yields one of the five HSV-1 transcripts that are spliced. Vmw110 is non-essential in tissue culture but deletion mutant viruses exhibit a cell type and multiplicity dependent defect in plaque formation as a result of inefficient induction of early and late gene expression. Whilst Vmw110 is also non-essential for infection in mouse model systems, deletion mutant viruses reactivate inefficiently from latency. In an *in vitro* latency system, Vmw110 is both necessary and sufficient to reactivate quiescent viral genomes. In transfection assays, Vmw110 has been shown to be a potent and promiscuous transactivator of gene expression, particularly in synergy with Vmw175, and five functional regions have been identified on the basis of mutational analysis; one of these encompasses the RING finger and another the C-terminal end of Vmw110. The C-terminal end is important for the initial interaction of Vmw110 with ND10; this provided circumstantial evidence for Vmw110 interacting with cellular proteins via C-terminal residues. Given the potential involvement of Vmw110 in the switch between lytic and latent infection, it was considered important to pursue any potential interactions between Vmw110 and cellular proteins.

To investigate the interactions of the C-terminal end of Vmw110 with cellular proteins, a series of Vmw110 C-terminal GST fusion proteins were made and used in 'pull-down' experiments with radiolabelled whole cell extracts. Vmw110 residues 594-775 (GEXE52) were found to bind strongly and specifically to a 135kD protein from all cell types tested and to a 150kD protein from some cell types. A shorter fusion protein containing Vmw110 residues 633-775 (GEXE58) did not interact with the 135kD protein indicating that residues 594-633 of Vmw110 are necessary for the interaction in

this system. GEXE58 bound the 150kD cellular protein whilst a fusion protein containing Vmw110 residues 680-775 (GEXR5) did not, showing that Vmw110 residues between 633 and 680 are necessary for this interaction.

Immune precipitation experiments carried out by other members of the group showed that purified, intact Vmw110 bound a 135kD protein and a collaborative reciprocal depletion experiment indicated that the 135kD proteins in the two systems were likely to be the same. In order to further understand the mechanism by which Vmw110 acts during infection, it was considered important to purify the 135kD protein and attempt the cloning of a cDNA.

The 135kD protein was purified in a large scale GST 'pull-down' experiment and the fusion protein bound samples containing the 135kD protein were blotted onto a PVDF membrane. The 135kD protein band was isolated and submitted for microsequence analysis by Dr. P. Matsudaira (MIT). Six peptide sequences were obtained and the information employed in the design of redundant PCR primers for use in cloning a cDNA. This approach was unsuccessful and a long probe (53 bases) was designed based on one of the longer peptides, taking into account the optimum codon usage in the human genome (Lathe rules), for use in screening a cDNA library. This approach was also unsuccessful during the course of these studies but another member of the group was successful with this method later on.

Indirect evidence from another laboratory indicated that C-terminal residues of Vmw110 contain a multimerisation domain; it was possible that such a domain could include or overlap with the residues required for 135kD protein binding, so the multimeric nature of Vmw110 was examined more closely. The Vmw110 E52 and E58 sequences were cloned into a T7 expression system and following expression, the Vmw110 peptides (T7E52 and T7E58) were purified by ion exchange chromatography. Analysis of the purified peptides by gel filtration chromatography, glycerol gradient centrifugation and glutaraldehyde cross-linking showed that both formed multimers of higher order than dimers although their precise nature could not be resolved. These experiments clearly separated the Vmw110 residues required for 135kD binding (594-633) from those containing an independent multimerisation domain.

The significance of the Vmw110:135kD protein:protein interaction and the function of Vmw110 multimerisation during the life cycle of the virus are unknown, as yet, but undoubtably, this work has opened a new door for studying the mechanism of action of Vmw110 during HSV-1 infection.

ABBREVIATIONS

Standard chemical symbols are used for elements and salts and compounds thereof.

A:	adenine, absorbance, Amps
Ac:	acetate
APL:	acute promyelocytic leukaemia
APS:	ammonium persulphate
ATP:	adenosine-5'-triphosphate
BHK:	baby hamster kidney
bp:	base pairs
BSA:	bovine serum albumin
C:	cytosine or carboxy (-terminal end of protein)
CA:	carbonic anhydrase
CHAPS:	2-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate
Ci:	Curie
cm:	centimetre
cpm:	counts per minute
CsCl:	caesium chloride
C-terminal:	carboxy-terminal
D:	dalton
dATP:	2'-deoxyadenosine-5'-triphosphate
dCTP:	2'-deoxycytidine-5'-triphosphate
ddATP:	2'-, 3'-dideoxyadenosine-5'-triphosphate
ddCTP:	2'-, 3'-dideoxycytidine-5'-triphosphate
ddGTP:	2'-, 3'-dideoxyguanosine-5'-triphosphate
ddNTP:	2'-, 3'-deoxyribonucleoside
ddTTP:	2'-, 3'-dideoxythymidine-5'-triphosphate
dGTP:	2-deoxyguanosine-5'-triphosphate
DEPC:	diethylpyrocarbonate
DMSO:	dimethylsulphoxide
DNA:	2'-deoxyribonucleic acid
DNase:	deoxyribonuclease
DRG:	dorsal root ganglion
DTT:	dithiothrietol
dTTP:	2'-deoxythymidine-5'-triphosphate
E:	early (beta)
EBV:	Epstein-Barr virus
ECL:	enhanced chemiluminescence

E. coli:	*Escherichia coli*
EDTA:	ethylenediaminotetra-acetic acid
EHV:	equine herpes virus
EtBr:	ethidium bromide
FITC:	fluorescein isothiocyanate
FPLC:	fast protein liquid chromatography
g:	gram or glycoprotein
G:	guanine
GST:	glutathione-S-transferase
HCMV:	human cytomegalovirus
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HFL:	human foetal lung
HHV:	human herpesvirus
HSV:	herpes simplex virus
I:	inosine
IE:	immediate-early (alpha)
IPTG:	isopropyl-D-thiogalactoside
IR_L:	long internal repeat
IR_S:	short internal repeat
kb:	kilo base(s)
kbp:	kilo base pairs
KSHV:	Kaposi's sarcoma herpesvirus
L:	late (gamma)
LAT:	latency associated transcript
MAb:	monoclonal antibody
MCR:	multicloning region
2-ME:	β-mercaptoethanol
MHC:	major histocompatibility complex
min:	minutes
M-MuLV:	Moloney-Murine Leukaemia Virus
N:	nucleoside or asparagine or amino terminal of protein
(n/p)mol:	(nano/pico)moles
NP40:	Nonidet P40
OD:	optical density
ORF:	open reading frame
RT:	room temperature
^{32}P:	phosphorus-32 radioisotope
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline

PCR: polymerase chain reaction

pfu: plaque forming unit

Pfu polymerase: cloned *Pyrococcus furiosus* DNA polymerase

PMSF: phenylmethylsulphonyl fluoride

RAR-α: retinoic acid receptor alpha

RGB: resolving gel buffer

RNA: ribonucleic acid

RNase A: ribonuclease A

rpm: revolutions per minute

RT: room temperature

^{35}S: sulphur-35 radioisotope

SDS: sodium dodecyl sulphate

SGB: stacking gel buffer

SV40: simian virus 40

sec: seconds

T: thymine

Taq polymerase: *Thermus aquaticus* DNA polymerase

TBS: Tris buffered saline

TE: Tris EDTA

TEMED: N, N, N', N'-tetramethylethylene diamine

tk: thymidine kinase

Tris: tris (hydroxymethyl) aminomethane

TRITC: tetramethylrhodamine isothiocyanate

TWEEN-20: polyoxyethylene-sorbitanmonolaurate

ts: temperature sensitive

U: unit

U_L: unique long (region of HSV genome)

U_S: unique short (region of HSV genome)

USP: ubiquitin specific protease

UV: ultra-violet

V: volts

Vmw: apparent molecular weight of virion polypeptide (determined by SDS PAGE)

VP: virion polypeptide

VZV: varicella-zoster virus

X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Amino acid symbols and codons:

One letter symbol	Three letter symbol	Amino acid	Codons
A	Ala	Alanine	GCA GCC GCG GCT
C	Cys	Cysteine	TCG TCT
D	Asp	Aspartic acid	GAC GAT
E	Glu	Glutamic acid	GAA GAG
F	Phe	Phenylalanine	TTC TTT
G	Gly	Glycine	GGA GGC GGG GGT
H	His	Histidine	CAC CAT
I	Ile	Isoleucine	ATA ATC ATT
K	Lys	Lysine	AAA AAG
L	Leu	Leucine	TTA TTG CTA CTC CTG CTT
M	Met	Methionine	ATG
N	Asn	Asparagine	AAC AAT
P	Pro	Proline	CCA CCC CCG CCT
Q	Gln	Glutamine	CAA CAG
R	Arg	Arginine	AGA AGG CGA CGC CGG CGT
S	Ser	Serine	AGC AGT TCA TCC TCG TCT
T	Thr	Threonine	ACA ACC ACG ACT
V	Val	Valine	GTA GTC GTG GTT
W	Trp	Tryptophan	TGG
Y	Tyr	Tyrosine	TAC TAT

CHAPTER 1 INTRODUCTION

The research presented in this Thesis concerns the interactions of the HSV-1 immediate-early protein Vmw110 with cellular proteins. In Sections 1A and 1B, general features of the biology of herpesviruses and in particular an outline of the life-cycle of HSV-1 are discussed. In Section 1C, the regulation of HSV-1 gene expression is discussed further and Section 1D covers current knowledge of Vmw110 to date and the related cellular processes with which it may be involved.

1A Herpesviruses

1A1 Distribution and clinical aspects

The Herpesviridae form a large family of viruses; almost 100 members with diverse biological properties have been characterised. Most animal species examined have yielded at least one herpesvirus and the host range covers humans, horses, cattle, pigs and fowl. Thus, not only are these viruses relevant to the clinician but also to the veterinarian with particular reference to western economy. Molecular studies of these viruses have provided, and continue to provide, valuable insights into the molecular mechanisms of gene expression and its control. In addition, increasing interest has been taken recently in the use of human viruses as vectors for gene therapy.

Over recent years, human herpesvirus infections have become more relevant to the clinician with advances in organ transplantation techniques and with increased incidences of HIV infection. Infections are more prevalent in immune compromised individuals, whether this is drug induced, such as in transplant patients, or if it occurs as a result of the onset of AIDS. Seven herpesviruses have been isolated from humans; herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus (VZV), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), human herpes virus 6 (HHV6) and human herpes virus 7 (HHV7). Herpes virus-like sequences have been found in over 90% of AIDS-related Kaposi's sarcoma tissue, indicating that an eighth human herpes virus may exist (Chang *et al.*, 1994). More recently these sequences have also been found in Kaposi's sarcomas of HIV negative individuals which has resulted in the speculation that a herpes virus, designated Kaposi's sarcoma herpes virus (KSHV), may be involved in the pathogenesis of the tumour (Schalling *et al.*, 1995).

The ***herpes simplex viruses*** primarily infect epithelial tissue through the mucosa and abraded skin. Virions are transported to neuronal ganglia where latent infection is established. Of the two serotypes, HSV-1 is historically responsible for the common 'cold-sore' and HSV-2 for genital lesions. However, this distinction is no longer valid as HSV-1 is now the causative agent of 30-50% of genital infections (Kinghorn, 1993) and

HSV-2 the causative agent of 5-20% of oral infections (Wiedbrauk and Johnston, 1993). HSV-1 is widespread and is usually acquired in childhood; over 90% of western populations are seropositive by the age of sixty (Nahamias *et al.*, 1970). HSV-2 infection occurs later in life and is generally acquired by sexual transmission (Johnson *et al.*, 1989; Kinghorn, 1993). The herpes simplex viruses also cause conjunctivitis, herpetic whitlow, keratitis and encephalitis. Neonatal infections are often life threatening and are acquired during birth from mothers with genital lesions.

Primary infection with ***varicella zoster virus*** (VZV) generally occurs in childhood with the appearance of a rash after 14-15 days which is often accompanied by fever. Herpes zoster, resulting from reactivation of latent virus from neuronal ganglia, is characterised by the appearance of lesions at the involved dermatome and is frequently accompanied by severe pain which can persist for months following healing of the lesions (reviwed by Gelb, 1990).

Human cytomegalovirus infection is widespread with up to 60% of western children and up to 100% of children in developing countries acquiring the virus before the age of six years. Primary infection is generally asymptomatic and cytomegaly (enlargement and fusion of macrophages) is a feature. As with other herpes viruses, persistent and recurrent infection can occur as a result of reactivation of latent virus. Reinfection with other genetically distinguishable isolates of differing serology is known to occur. Infection is a problem in immune compromised hosts and symptoms include gastroenteritis and retinitis. In severe infections, widespread cytomegaly occurs (cytomegalic inclusion disease) and whilst this can be asymptomatic to a degree, it can be fatal, particularly in the newborn (reviewed by Alford and Britt, 1990).

Epstein-Barr virus is usually acquired during childhood when infection is often asymptomatic. Primary infection in older children and adults results in infectious mononucleosis. The virus has been associated with the pathogenesis of Burkitt's lymphoma and nasopharyngeal carcinoma (reviewed by Kieff and Liebowitz, 1990).

In comparison with the human herpesviruses discussed above, little is known about ***human herpesvirus 6***. It is clear that infection is widespread in the adult population and that it is acquired in childhood when it is associated with roseola (exanthem subitim). The virus may play a role in the pathogenesis of AIDS but this has yet to be verified (reviewed by Lopez and Honess, 1990).

Human herpesvirus 7 was isolated from T lymphocytes of a healthy individual (Frenkel *et al.*, 1990) and, like human herpes virus 6, has been associated with roseola in infants (Burns *et al.*, 1994; Hidaka *et al.*, 1994; Tanaka *et al.*, 1994; Ueda *et al.*, 1994; Ablashi *et al.*, 1995).

1A2 Herpesvirus classification and distinctive features

Under the Baltimore classification system, the Herpesviridae fall into group I as double stranded DNA viruses. Inclusion in the family is based on the structure of the virion (Figure 1A2(i)) (reviewed by Dargan, 1986 and Rixon, 1993). Distinctive features are: the core containing a linear, double stranded genome of 100-230kb (these are among the largest DNA viruses) (Epstein, 1962a) within an approximately 125nm diameter icosahedral capsid made up of 162 capsomeres (Wildy *et al.*, 1960); the tegument which is an amorphous structure surrounding the capsid (Roizman and Furlong, 1974); and the envelope containing viral glycoproteins (Spear and Roizman, 1972).

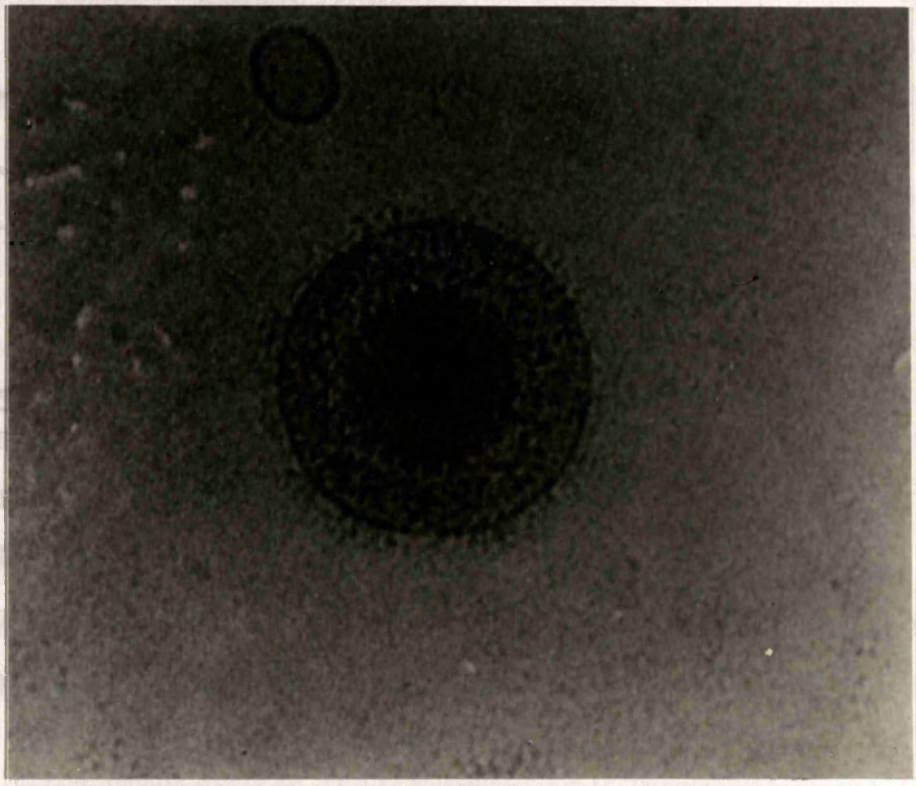

Figure 1A2(i) Electron micrograph of a herpes simplex virus type 1 virion. The micrograph shows the characteristic features of a herpes virus virion; the envelope containing viral glycoproteins and a central, inner capsid, which is 125nm diameter, are visible. The tegument is between the capsid and the envelope. The photograph is shown with the permission of Dr. F. Rixon.

On the basis of a number of early electron micrographs, the ***virion core*** was thought to contain the DNA arranged as a toroid around a central protein 'plug' (Furlong *et al.*, 1972; Nazerian, 1974). It was later suggested that this was an artifact of specimen preparation (Puvion-Dutilleul *et al.*, 1987) and there is now general agreement that the core contains only DNA which is present in a liquid crystalline state (Booy *et al.*, 1991).

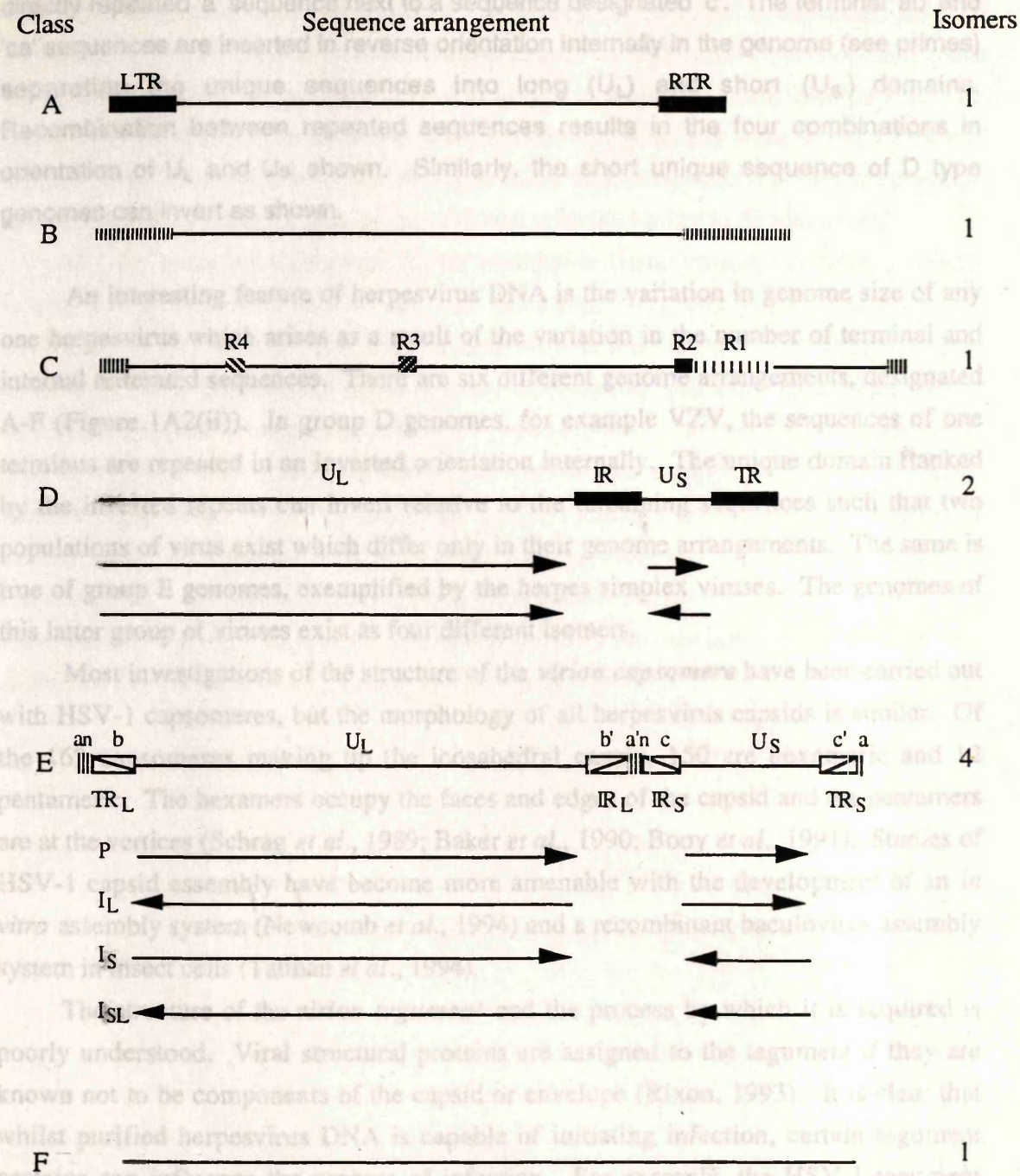

Figure 1A2(ii) A schematic diagram of the sequence arrangements in the six classes of genomes of the viruses comprising the family Herpesviridae. The six classes, A-F, are exemplified by channel catfish herpesvirus, herpesvirus saimiri, EBV, VZV, HSV and tupaia herpesvirus respectively. In the diagram, horizontal lines represent unique or quasi-unique regions. Reiterated domains are represented by rectangles, designated as follows; left and right terminal repeats (LTR and RTR) for class A; repeats R1 to R4 for the internal repeats of class C; and internal terminal repeats of class D. At the termini of classes B and C the repeats are reiterated numerous times. The termini of class E consist of two elements; one terminus contains 'n' copies of sequence 'a' next to a larger sequence designated 'b'. The other terminus has one

directly repeated 'a' sequence next to a sequence designated 'c'. The terminal 'ab' and 'ca' sequences are inserted in reverse orientation internally in the genome (see primes) separating the unique sequences into long (U_L) and short (U_S) domains. Recombination between repeated sequences results in the four combinations in orientation of U_L and Us shown. Similarly, the short unique sequence of D type genomes can invert as shown.

An interesting feature of herpesvirus DNA is the variation in genome size of any one herpesvirus which arises as a result of the variation in the number of terminal and internal reiterated sequences. There are six different genome arrangements, designated A-F (Figure 1A2(ii)). In group D genomes, for example VZV, the sequences of one terminus are repeated in an inverted orientation internally. The unique domain flanked by the inverted repeats can invert relative to the remaining sequences such that two populations of virus exist which differ only in their genome arrangements. The same is true of group E genomes, exemplified by the herpes simplex viruses. The genomes of this latter group of viruses exist as four different isomers.

Most investigations of the structure of the ***virion capsomere*** have been carried out with HSV-1 capsomeres, but the morphology of all herpesvirus capsids is similar. Of the 162 capsomeres making up the icosahedral capsid, 150 are hexameric and 12 pentameric. The hexamers occupy the faces and edges of the capsid and the pentamers are at the vertices (Schrag *et al.*, 1989; Baker *et al.*, 1990; Booy *et al.*, 1991). Studies of HSV-1 capsid assembly have become more amenable with the development of an *in vitro* assembly system (Newcomb *et al.*, 1994) and a recombinant baculovirus assembly system in insect cells (Tatman *et al.*, 1994).

The structure of the ***virion tegument*** and the process by which it is acquired is poorly understood. Viral structural proteins are assigned to the tegument if they are known not to be components of the capsid or envelope (Rixon, 1993). It is clear that whilst purified herpesvirus DNA is capable of initiating infection, certain tegument proteins can influence the process of infection. For example, the HSV-1 tegument protein Vmw65 (the gene product of UL48) is required for efficient expression of immediate-early (IE) genes. Vmw65 enhances productive infection at low multiplicities (Ace *et al.*, 1989).

The ***virion envelope*** has a trilaminar appearance in electron micrographs (Epstein, 1962b) and has been observed to be derived from cellular membranes (Armstrong *et al.*, 1961; Morgan *et al.*, 1968). A number of viral glycoproteins present in the HSV-1 envelope are known to be necessary for fusion of the virion envelope with the plasma membranes of host cells, a process which results in delivery of the capsid to the cytoplasm. At least ten distinct glycoproteins are present on the surface of HSV-1 virions (reviewed by Spear, 1993).

The herpesviruses have a number of biological features in common; they all encode enzymes for nucleic acid metabolism and DNA replication occurs in the nucleus of the infected cell. Capsid assembly also occurs in the nucleus and capsids bud from the nuclear membrane. In addition, virion production generally leads to cell death and all members investigated have the ability to cause latent infection, i.e. they may remain in a quiescent, undetected state within the host with the ability to be reactivated.

On the basis of differences in the biology of these viruses, the Herpesviridae family has been further subdivided into the alpha, beta and gamma herpesvirinae (Roizman *et al.*, 1981). The alpha herpesvirinae are characterised by their short productive cycle (less than 24 hours in tissue culture) and their establishment of latency in sensory neurons. Examples include HSV-1 and HSV-2. The beta group have a longer productive cycle and include the human and murine cytomegaloviruses. Viruses in the gamma group infect B or T lymphocytes and all members replicate in lymphoblastoid cells *in vitro*. An example is EBV. As with any classification system, the divisions are not completely adequate and some viruses do not fit clearly into any one group. For example, HHV-6 is biologically a gamma herpesvirus but has been assigned to the beta subfamily on the basis of its genome structure and sequence.

1B HSV-1 biology

HSV-1 was the first human herpesvirus to be discovered and has also been the most intensely investigated. The virus is usually acquired by close physical contact with an infected person during childhood. Infectious virus replicates in susceptible epithelial cells and initiates the formation of an inflamed lesion, usually around the mouth, called a 'cold-sore'. Virions enter sensory neuron endings and are transported to the cell bodies in sensory ganglia where latent infection is established.

In this Section, aspects of both lytic (productive) and latent infection are briefly described. Where information is relevant to HSV-1 and HSV-2, the term HSV is used.

1B1 The HSV genome

The HSV DNA genome is linear and double stranded (Becker *et al.*, 1968). It is approximately 150kbp with a G+C content of 68% (HSV-1) or 69% (HSV-2). HSV-1 strain 17^+ has a genome size of 152 kilo base pairs (McGeoch *et al.*, 1986; McGeoch *et al.*, 1988a; Perry and McGeoch, 1988). The structure is that of the class E genome and four isomers exist (see above and legend to Figure 1A2(ii)). The HSV-1 genome encodes at least 77 genes, fifty nine mapping to U_L, thirteen to U_S and two copies of four genes to the repeated sequences.

1B2 The lytic life cycle

In tissue culture, the HSV-1 lytic life cycle is completed during a period of less than 24 hours. The stages involved are: attachment (to the cell plasma membrane), viral envelope fusion with the plasma membrane and entry into the cell, transport to the nucleus and release of DNA, gene expression and DNA replication, and virion production. Cell death inevitably ensues.

1B2.1 Adsorption, penetration and uncoating

The initial stage of infection involves attachment of the virion to the cell surface. The cell surface receptors are heparan sulphate proteoglycans (reviewed in Spear, 1993; Shieh and Spear, 1994). Enzymatic removal of heparan sulphate from cell plasma membranes renders cells resistant to HSV infection. Chinese hamster ovary cells, which are defective in heparan sulphate synthesis, are also resistant to infection. The initial interaction of an HSV-1 virion with the cell is the binding of gC, one of at least ten virion glycoproteins present on the external surface of the virion, to heparan sulphate. This interaction can be inhibited and reversed by the addition of exogenous heparin. The non-essential nature of this interaction is demonstrated in gC null mutant viruses which still bind to the cell surface with only a slight reduction in binding efficiency and infectivity compared to wild-type virus. It is evident that another viral

glycoprotein, gB, also mediates heparan sulphate binding; mutant viruses which express neither gC or gB bind to cells in tissue culture extremely inefficiently. Unlike gC mutant viruses, gB null mutant viruses fail to penetrate into the cell, despite the ability of the virions to interact with heparan sulphate (reviewed by Spear, 1993).

The initial and relatively non-specific interaction at the cell surface mediated by gC and gB is followed by a heparin resistant interaction which involves gD. Glycoprotein gD is essential for virus penetration; it has been shown that binding to cells of inactivated virions containing gD can inhibit the penetration but not the initial binding of a homologous challenge virus and that a truncated soluble form of gD which binds to cells can also inhibit HSV-1 entry but not binding to the cell surface. Cells expressing gD are resistant to HSV-1 penetration but virions bind to the cell surface. These results indicated that there is a specific cell receptor for gD; it has been shown that gD is modified by mannose-6-phosphate and can bind to two different mannose-6-phosphate receptors (Brunetti *et al.*, 1994; 1995). Blockage of the mannose-6-phosphate receptors in tissue culture does not completely inhibit viral penetration, indicating that another cell surface receptor facilitates this activity. This is reflected in the necessity, not only for gB and gD in viral penetration, but gH also. Glycoprotein gH forms heterodimers with another glycoprotein, gL, an association that appears to be essential for the processing and intracellular transport of both proteins. It is therefore not surprising that gL null mutants are also non-viable. Viable gK null mutant viruses are unobtainable as well, indicating that this protein is also essential for penetration (reviewed by Spear, 1993).

Following envelope fusion with the plasma membrane and subsequent penetration, viral capsids are transported to the nuclear membrane and DNA is released into the nucleus through pores in the nuclear membrane in a process requiring a viral function (Tognon *et al.*, 1981; Batterson *et al.*, 1983). The transport process is thought to be mediated by the cellular cytoskeleton (Kristensson *et al.*, 1986). Viral gene transcription and DNA replication occur in the nucleus.

1B2.2 Viral gene expression

HSV gene expression occurs in a temporally regulated cascade (Section 1C) and genes are classified as α, β and γ (Honess and Roizman 1974, 1975) or immediate-early (IE), early (E) and late (L) (Clements *et al.*, 1977). Genes are transcribed by cellular RNA polymerase II with the participation of viral factors (Ben-Zeev and Becker, 1977; Costanzo *et al.*, 1977). The mRNAs produced are capped, methylated and polyadenylated and are translated on bound and free polyribosomes. Viral proteins can be extensively processed by cleavage, phosphorylation, sulphation or poly(ADP) ribosylation (reviewed in Roizman and Sears, 1990).

The HSV-1 virion tegument protein, Vmw65, is required for transactivation of the five IE genes. As detailed later (Section 1C) the products of at least three IE genes are necessary for fully efficient expression of E and L genes. IE gene expression is necessary for efficient E gene expression which peaks 5-7 hours post infection, and L gene expression follows the onset of DNA replication at approximately 3 hours post infection. There are two classes of L gene; leaky-late (or $\gamma 1$) genes are expressed at low levels prior to viral DNA replication and require the onset of replication for maximal expression and true late (or $\gamma 2$) genes are expressed only following the onset of replication. Specific aspects of the regulation of gene expression are discussed in Section 1C.

1B2.3 DNA Replication

HSV-1 DNA replication can be detected as early as 2 hours post infection and continues for at least 9-14 hours (Roizman *et al.*, 1963; Roizman and Roane, 1964). Replication occurs by a rolling circle mechanism and head to tail concatemers accumulate in the nucleus (reviewed by Roizman and Sears, 1990) in so-called 'replication compartments' (Quinlan *et al.*, 1984; de Bruyn Kops and Knipe, 1994).

There are three origins of replication in the HSV-1 genome and their positions were initially deduced from the structures of defective genomes. Two, designated ori_{S1} and ori_{S2}, are identical and map in the 'c' inverted repeat of the S component of the genome. The third, ori_L, is found in the L component.

Seven genes have been found to be necessary and sufficient for origin dependent DNA synthesis. The products of UL5, UL8 and UL52 form a primase and helicase complex. The product of UL9 is an origin specific DNA binding protein and the UL29 gene product binds single stranded DNA. The UL30 and UL42 genes encode the catalytic and accessory subunits of the viral DNA polymerase respectively (reviewed by Challberg, 1991). Other virally encoded enzymes are also involved in nucleic acid metabolism; for example, thymidine kinase, ribonucleotide reductase, uracil-DNA glycosylase and dUTPase (reviewed in Roizman and Sears, 1990).

1B2.4 Virion assembly

The mechanism of capsid assembly and DNA packaging in HSV-1 virion assembly is not fully understood. Three types of capsid can be isolated from infected cells designated A (empty), B (containing a proteinaceous core) and C (mature virions). It is thought that B capsids are the precursors of A and C capsids.

Capsids are made from seven viral proteins encoded by six genes (Table 1B2.4). During assembly, which occurs in the nucleus of infected cells, it is thought that the products of six genes assemble to form a B type capsid; the outer icosahedral shell is formed by VP5, VP19C, VP23 and VP26 and an internal scaffold is formed by VP22a

and the product of gene UL26; the UL26 gene product undergoes autoproteolysis to give VP24 and VP21 and the proteolytic activity also modifies VP22a. DNA packaging then occurs with removal of VP21 and VP22a to generate C capsids. Type A capsids may be the products of abortive packaging events (reviewed in Rixon, 1993).

PROTEIN	M_r (X10^{-3})	GENE	LOCATION
VP5	155	UL19	Icosahedral shell
VP19C	53	UL38	Icosahedral shell
VP21	42	UL26	Scaffold
VP22a	38	UL26.5	Scaffold
VP23	33	UL18	Icosahedral shell
VP24	25	UL26	Scaffold
VP26	12	UL35	Icosahedral shell

Table 1B2.4 Table showing the proteins involved in capsid assembly and their locations within the capsid.

Packaging of concatemeric DNA arising from replication (see above) occurs by a procedure during which concatemers are processed into genome length molecules by cleavage at 'a' sequences. There is evidence that cleavage and packaging are linked processes as viruses with mutations in the genes encoding VP5, VP19C, VP23 and the protease fail to cleave concatemeric DNA (reviewed by Roizman and Sears, 1990).

Little is known of the precise details by which the tegument and envelope are acquired. It is apparent from electron microscopy that there are two possible pathways. In the first model, capsids bud through the inner nuclear membrane and the newly acquired membrane fuses with the outer nuclear membrane and capsids are released into the cytoplasm. The tegument is then assembled and the capsids are enveloped as they bud through the plasma membrane, so releasing mature virions. In the second model, the tegument is acquired in the nucleus and capsids bud through the inner nuclear membrane and leave the perinuclear cisterna in a vacuole formed by the outer nuclear membrane. The vacuole and plasma membranes eventually fuse and mature virions are released from the cell. Both procedures involve a traverse through the Golgi apparatus (reviewed by Rixon, 1993).

1B2.5 The effect of HSV infection on the cell

Cells productively infected with HSV do not survive. Major structural alterations occur; the nucleolus disintegrates during the course of infection and host chromatin is dispersed to the edge of the nucleus and degraded. The nuclear envelope becomes distorted and multilobed as infection proceeds, and other cellular membranes form

thickened patches along their surfaces. This could be due to the deposition of tegument proteins (reviewed by Roizman and Sears, 1990).

Immediately upon infection, a rapid shut-off of host macromolecular synthesis occurs. Host DNA synthesis is shut off (Roizman and Roane, 1964), host protein synthesis declines very rapidly (Sydiskis and Roizman, 1966; Read and Frenkel, 1983), host ribosomal RNA synthesis is reduced (Wagner and Roizman, 1969) and glycosylation of host proteins ceases (Spear *et al.*, 1970). However, it is apparent that some cellular promoters are transactivated and that this requires the presence of Vmw175 (Latchman *et al.*, 1987; Kemp and Latchman, 1988). Vmw110 may also be involved (Everett, 1985).

HSV-1 host shut-off occurs in two stages. An early shut-off mechanism involves a protein present in the virion and a delayed mechanism requires viral protein synthesis (Nishioka and Silverstein, 1977, 1978a, 1978b; Fenwick and Walker, 1978; Fenwick and Clark, 1982).

The rapid shut-off function was mapped to the UL41 gene (whose product is *vhs*) by studies of mutant viruses defective in the early shut-off process (Fenwick *et al.*, 1979; Kwong *et al.*, 1988). The *vhs* protein is non-essential for replication in tissue culture and *vhs* mutants over-express viral proteins (Read and Frenkel, 1983) due to the stabilisation of viral RNA (Kwong and Frenkel, 1987; Oraskar and Read, 1987). Recent studies have shown that *vhs* forms a complex with VP16 (α-TIF) and that this blocks the ability of VP16 to form a transactivating multiprotein complex (see Section 1C1.1(ii)) with cellular factors on immediate early gene promoters (Smibert *et al*, 1994). However, host factors bind VP16 more avidly than *vhs* and it may be that early in infection, the host cell proteins Oct-1 or HCF displace *vhs* from VP16 such that transactivation of immediate-early gene promoters is concomitant with early host shut-off.

There is also evidence that another virion protein, the product of the UL13 gene, is involved in host cell shut-off since UL13 mutants display a *vhs*-type phenotype (Overton *et al.*, 1992; 1994).

1B3 The latent state

As well as productively infecting host epithelial cells *in vivo*, HSV-1 has the ability to latently infect host cell sensory neurons. Latent infection is characterised by the absence of viral antigens and infectious virus. There are three aspects of latent infection; establishment, maintenance and reactivation.

Insights into the mode of establishment of latent infection have come mainly from studies with mutant viruses capable of latent infection but which do not replicate in ganglia during productive infection at the epithelia. Viral genomes, which are probably encapsidated, pass by fast retrograde axonal transport to sensory neuron cell bodies

located in ganglia. Once in the ganglia, either viral replication, with production of progeny virus and neuronal cell death, or establishment of latent infection occurs. The precise mechanism for the latter situation is unclear. It has, however, been concluded that the decision to enter latent infection is made early in the virus:cell interaction, prior to high levels of expression of IE polypeptides. It is possible that cellular factors may be involved in repressing IE gene expression (reviewed by Stevens, 1989; Rock, 1993).

(i) Latency in mice

Early studies investigating the latency competence of mutant viruses in mouse models measured the ability of a virus to reactivate from latency. It was possible, however, that replication deficient viruses could establish latent infection which would not have been detectable in these experiments. A careful study to differentiate between the ability of mutant viruses to establish latent infection and to reactivate was carried out by Lieb *et al.* (1989). They examined the role of three IE proteins (Vmw110, Vmw175, Vmw63) in latent infection in the mouse ocular model and they also addressed the question of whether or not viral replication at the initial site of infection is necessary for the establishment of latency. It was found that viral mutants in the genes encoding Vmw175 and Vmw63 did not replicate in the eye, as detected by plating eye swab material onto complementing cell lines. In addition, these viruses could not be reactivated from explanted ganglia which were superinfected with a helper virus or ganglia which were explanted onto complementing cell lines. It was concluded that viruses which fail to replicate at the initial site of infection also fail to establish latent infection in this model. Vmw110 deficient viruses were found to replicate in the eye and also to establish reactivatable latent infection in the ganglia in these experiments, although at reduced efficiency compared with wild type virus.

The latency competence of a Vmw65 mutant virus, *in*1814, was also examined in the mouse ocular model (Steiner *et al.*, 1990). The 12bp insertion in the gene encoding Vmw65 yields a product which is functional in its structural role in the virion but is incapable of transactivating IE gene promoters (Ace *et al.*, 1989; Section 1C1.1(ii)). The virus was shown to be avirulent in mice (Ace *et al.*, 1989; Steiner *et al.*, 1990) and also to establish latency in trigeminal ganglia in spite of its inability to replicate following corneal inoculation (Steiner *et al.*, 1990). This contradiction with the results of Lieb *et al.* (1989) was explained by proposing that mutant viruses in the genes encoding Vmw175 and Vmw63 had established a low level latent infection which was not detectable by the investigative methods utilised.

(ii) Host cell factors

Even though *in*1814 can reactivate from latency in mice, it has been proposed that the failure to express IE genes, as mediated via the TAATGARAT motif in IE promoters, is

an important step in the establishment of latency. This suggestion has arisen as a result of a number of observations; Vahlne and Lycke (1977) showed that HSV-1 replication was restricted in the mouse neuroblastoma cell line C1300 and attributed this to a non-interferon factor (Vahlne and Lycke, 1978). The block was overcome by pretreatment of cells with sodium butyrate which resulted in increased transcription of IE genes (Ash, 1986; Kemp and Latchman, 1989). The repression was later found to be mediated via the TAATGARAT element (Kemp *et al.*, 1990).

The CAT gene under the control of an IE promoter in a plasmid construct was expressed weakly in the ND series of cell lines which are derived from immortalised primary rat dorsal root ganglion (DRG) neurons (Kemp *et al.*, 1990). However, cotransfection of plasmids encoding isolated TAATGARAT elements or the octamer motifs of cellular promoters led to increased CAT gene expression (Kemp *et al.*, 1990; Wheatley *et al.*, 1991). This suggested that an inhibitor of IE gene expression is present in ND cell lines which is titrated out by the addition of excess TAATGARAT or octamer motifs. In contrast to fibroblasts which express the Oct-1 factor which forms a complex with VP16 at the TAATGARAT motif (Section 1C1.1(ii)), ND cells and DRG were shown to contain significant amounts of Oct-2 mRNA (Lillycrop *et al.*, 1991) which had previously been shown to be present in the embryonic sensory ganglia and adult neuronal tissues of rats (He *et al.*, 1989) and in mouse brain extracts (Scholer *et al.*, 1989). The octamer/TAATGARAT binding protein Oct-2 was found to be absent from cells permissive for HSV-1 replication and down regulation of IE gene expression could be achieved by transfection of plasmids expressing Oct-2, thus confirming the role of Oct-2 as a repressor of IE promoters in neuronal cells (Lillycrop *et al.*, 1991). Latchman's group have hypothesised that although neuronal cells express limited amounts of Oct-1 (He *et al.*, 1989), Oct-2 binds TAATGARAT motifs preventing the formation of the Vmw65/Oct-1 mediated transactivation complex (a similar complex cannot form with Oct-2). This would lead to repression of IE gene expression and the switch from the lytic cycle to latency.

The Oct-2 repressor protein found in ND cell lines was shown to be related to that found in B-cells, in which it acts as an activator (Muller *et al.*, 1988; Lillycrop *et al.*, 1991; Latchman *et al.*, 1992). The RNA encoding Oct-2 is alternatively spliced in both B-cells (Wirth *et al.*, 1991) and neuronal cells (Lillycrop and Latchman, 1992) to yield multiple protein isoforms when translated. The predominant isoforms in neuronal cell lines, Oct 2.4 and Oct 2.5, lack a C-terminal activation domain (Wirth *et al.*, 1991) and it is feasible that they are unable to form a complex with Vmw65 due to the absence of specific contact amino acids necessary for complex formation (Section 1C1.1(ii)). The predominant B-cell isoform, Oct 2.1, which possesses the C-terminal activation domain was found to activate IE promoters (Lillycrop and Latchman, 1992; Lillycrop *et al.*, 1994). Lillycrop *et al.* (1994) have concluded that expression of Oct 2.4 and Oct 2.5

renders neuronal cells non-permissive for the HSV-1 lytic cycle. The role of these isoforms *in vivo* has yet to be established.

(iii) Latent viral genomes

The lack of detectable viral genomic termini during latent infection has led investigators to suggest that the viral genome is maintained either as a circular molecule or as a linear concatamer (Efstathiou *et al.*, 1986; Rock and Fraser, 1983;1985). Density gradient centrifugation has indicated that it is not integrated into the host cell genome (Mellerick and Fraser, 1987). Deshmane and Fraser (1989) have reported that latent HSV-1 DNA is associated with nucleosomes in a chromatin structure in latently infected neurons.

There has been much debate as to the number of viral genomes present in latently infected neurons. Early DNA hybridisation experiments suggested the number is between 0.01 and 1.0 per neuron (Puga *et al.*, 1978; Cabrera *et al.*, 1980; Rock and Fraser, 1983; Efstathiou *et al.*, 1986) but more recent *in situ* PCR experiments indicate that the number is much higher, although exact values have not been calculated (Ramakrishnan *et al.*, 1994; Mehta *et al.*, 1995). The higher values suggest either that the original multiplicity of infection for latently infected neurons is higher than previously thought or that replication of the viral genome occurs without destruction of the infected neurone. As HSV can be recovered from the central nervous system only at very low efficiency, it is possible that the majority of latent genomes are associated in a chromatin-like structure which confers on them an inability to reactivate; perhaps the low level of reactivation seen reflects viral genomes which are not associated with nucleosomes.

Viral gene expression during latent infection is restricted to the latency associated transcripts (Section 1C3).

(iv) *In vitro* latency models

Studies of reactivation have been carried out in *in vitro* latency systems as well as in animal models (reviewed in Stevens, 1989; Rock, 1993). The molecular processes are thought to involve the immediate-early protein Vmw110, as Vmw110 mutant viruses reactivate poorly from the latent state *in vivo* (Clements and Stow, 1989; Lieb *et al.*, 1989; Cai *et al.*, 1993) and Vmw110 mutant viruses are unable to stimulate the reactivation of latent virus in an *in vitro* latency system (Russell *et al.*, 1987; Harris *et al.*, 1989). Interestingly, unlike the situation observed in *in vivo* latency systems, Preston's group (Jamieson *et al.*, 1995) has shown that the *tk* gene of a mutant virus in their *in vitro* latency system is not arranged into the regular structure characteristic of cellular chromatin.

1C HSV-1 gene expression

1C1 The temporal regulation of HSV-1 gene expression

The analysis of viral mRNAs and polypeptides produced during the course of HSV-1 infection led to the identification of three groups of HSV genes characterised by their specific temporal expression and sensitivity to metabolic inhibitors (Honess and Roizman, 1974; 1975; Clements *et al.*, 1977); alpha (α) or immediate-early (IE), beta (β) or early (E) and gamma (γ) or late (L). Transcription of immediate-early (IE) genes occurs immediately upon infection, in the absence of *de novo* viral protein synthesis.

Early (E) polypeptides are detectable soon after infection and are present at their highest levels 5-7hr post infection. Many of these genes encode polypeptides involved in viral nucleic acid metabolism. Genes in this group are sometimes classified as β1 and β2, indicating that that not all E genes are transcribed simultaneously during infection (Honess and Roizman, 1974).

The late genes can also be divided into two groups, 'leaky-late' and 'true-late' or γ1 and γ2 respectively. Their products can be detected 2hr post infection and increase to a maximum at approximately 12hr post infection. 'Leaky-late' genes are expressed at low levels prior to viral DNA synthesis but require the onset of viral DNA replication for maximal expression. 'True-late' genes, require the onset of viral replication before expression occurs.

1C1.1 Activation of IE gene transcription

(i) Promoter sequences

Analysis of the four HSV-1 IE promoters led to the identification of two separable regions (Makem and Roizman, 1982a; 1982b; Cordingley *et al.*, 1983; Kristie and Roizman, 1984; Preston *et al.*, 1984; Bzik and Preston, 1986): (i) a minimal promoter region comprising a TATA box and proximal upstream sequences which direct basal transcription, and (ii) an upstream enhancer element to which host factors and a complex of viral and host factors bind. Sequence analysis identified a conserved motif, TAATGARAT, within the IE enhancer regions (Makem and Roizman, 1982b; Murchie and McGeoch, 1982; Preston *et al*, 1984).

Early experiments indicated that IE promoters are stimulated by a component of the virion (Post *et al.*, 1981). In a series of experiments in which cloned HSV-1 DNA fragments derived from various parts of the genome were cotransfected into BHK cells with chimaeric plasmids expressing the thymidine kinase (*tk*) gene under IE control, the virion component was identified as Vmw65 (also known as VP16 or α-TIF) (Campbell *et al.*, 1984), a late gene product which is found in the tegument of the virion (Batterson and Roizman, 1983; Weinheimer *et al.*, 1992). Further transfection experiments with

mutant and chimaeric forms of HSV-1 IE and E promoters showed that the conserved TAATGARAT motif present in one or more copies in IE gene promoter regions was necessary for Vmw65-mediated transactivation (Kristie and Roizman, 1984; Preston *et al.*, 1984). However, the transactivating function of Vmw65 is non-essential for lytic infection in tissue culture; a virus, *in*1814, which encodes a Vmw65 protein which is functional in its role in virion structure but cannot transactivate IE promoters, has a high particle:pfu ratio and enters the lytic cycle in tissue culture, although only at high multiplicities of infection. Virus *in*1814 is also avirulent *in vivo* (Ace *et al.*, 1989).

A GA-rich motif upstream of the the TAATGARAT motif in the IE3 promoter was shown to be necessary for optimum levels of transcription from this promoter (Bzik and Preston, 1986; Triezenberg *et al.*, 1988a; 1988b). The IE4/5 and IE2 promoters also contain similar GA-rich motifs. LaMarco and McKnight (1989) identified a cellular factor which binds to the GA-rich motif, IE facilitator or $(IEF)_{ga}$ activity, but it was found not to mediate its effect by interacting with Vmw65. Characterisation of $(IEF)_{ga}$ led to the identification of the cellular GA binding protein (GABP) which has two subunits (LaMarco *et al.*, 1991). It is thought that GABP mediates promoter induction by cooperating with the cellular factor Oct-1 to enhance the formation of a multiprotein complex, which includes Vmw65, on the TAATGARAT motif. Interestingly, GABP binds the GA repeats of interferon-inducible promoters, indicating that during activation of IE genes HSV-1 may exploit a mechanism employed by mammalian cells to combat infection.

Analysis of GC-rich sequences in IE promoters showed the presence of motifs which bind the cellular factor Sp-1 *in vitro* (Jones and Tijan, 1985). These may account for the constitutive activity of IE promoters (Kristie and Roizman, 1984; Preston *et al.*, 1984; Bzik and Preston, 1986).

(ii) Vmw65 transactivation

A number of groups showed that Vmw65 did not bind directly to DNA, thereby raising the question of the mechanism of transactivation induced through the TAATGARAT motif (Marsden *et al.*, 1987; O'Hare *et al.*, 1988; Preston *et al.*, 1988). A hint came with the identification of a cellular factor, NF-III (also called Oct-1) which binds to the octamer element in the adenovirus type 2 origin of DNA replication (the Ad2 octamer element) (Pruijn *et al.*, 1986). It was quickly found that a cellular factor bound to the HSV-1 TAATGARAT motif (Kristie and Roizman, 1987; Preston *et al.*, 1988) and this was identified as NF-III (Oct-1) (O'Hare and Goding, 1988).

In a series of binding experiments with infected cell extracts it was found that the complex formed on the TAATGARAT motif was modified by virus infection (Preston *et al.*, 1988; O'Hare *et al.*, 1988), and use of an anti-Vmw65 antibody identified Vmw65 as one of the components. Mutational analysis showed that the TAAT component of

the motif was necessary for Oct-1 binding and that mutations in the GARAT region affected complex mediated transactivation of IE genes (O'Hare *et al.*, 1988; O'Hare and Goding, 1988). Oct-1 is a ubiquitous octamer binding homeodomain protein containing a POU domain which is essential for DNA binding. The POU domain consists of two subdomains, a POU-specific domain and a POU homeo domain. Expression of these two subdomains in isolation has shown that the Ad2 octamer element can be divided into two regions; one is recognised by both the POU-specific domain and the POU homeo domain and the other only by the POU-specific domain. The differing sequence specificity of the Oct-1 POU-specific domain compared with that of the POU homeodomain adds to the overall sequence specificity of this homeodomain protein. Interestingly, it has been shown that the sequence preferences of the isolated POU-specific domain are distinct from those of the entire POU domain, suggesting that the POU domain binding site is more than a simple juxtaposition of the POU-specific and POU homeo domain target sequences (Verrijzer *et al.*, 1990; 1992).

In vitro binding experiments have shown that Oct-1 and Vmw65 interact weakly and that an additional cellular factor, host cell factor (HCF), is necessary for efficient Vmw65-mediated transactivation (Gerster and Roeder, 1988; Kristie *et al.*, 1989; Katan *et al.*, 1990; Kristie and Sharp, 1990; Xiao and Capone, 1990; Stern and Herr, 1991; Walker *et al.*, 1994). Purification of HCF showed that it consisted of six related and tightly but non-covalently linked polypeptides of 110-150kD with a minor 300kD protein (Wilson *et al.*, 1993). Two cDNAs encoding these polypeptides have been isolated; a major species encoding a 2035 residue protein (approximately 300kD) and a minor species encoding a 1966 residue protein which is derived from the major species by alternative splicing (Wilson *et al.*, 1993). The 300kD protein shows no homology with any known protein but contains eight 26 residue repeats at which proteolytic cleavage occurs to give the smaller polypeptides (Wilson *et al.*, 1995a). The role of HCF in uninfected cells is unknown; it is most abundant in foetal and placental tissues and cell lines, suggesting that it may be involved in cell proliferation (Wilson *et al.*, 1995b).

More recently, it has been shown that Vmw65 interacts with residues in the POU domain of Oct-1 to form a complex which exhibits identical functional requirements to those of the complex formed in the presence of HCF; this includes the specificity of POU domain residues contacting Vmw65 and the requirement of the GARAT component of the octamer sequence (Walker *et al.*, 1994). The fact that greater levels of the Vmw65 protein were required for the formation of the complex in the absence of HCF was interpreted as indicating that HCF stabilises the complex. In spite of the requirement of the GARAT element for Vmw65 recognition, no differences were found between the DNA contacts of the POU domain alone and the POU domain in a complex with Vmw65. Vmw65 does not seem to contact DNA in this complex; on its own it

binds DNA weakly and non-specifically (Kristie and Sharp, 1990; Walker *et al.*, 1994). Walker *et al.* suggested that the presence of the GARAT sequence in the TAATGARAT octamer induces a site specific conformational alteration in the nature of the POU domain of Oct-1 that is not seen when Oct-1 binds other octamer sites; this change enables Vmw65 to bind to Oct-1 and is the basis of selective recognition and subsequent transcriptional activation by Vmw65. The mechanism by which the GARAT element induces the conformational change in Oct-1 remains unknown.

Mutational analysis of Vmw65 has identified regions important for its transactivating capabilities (reviewed by O'Hare, 1993). Deletion of the acidic C-terminal domain (approximately 80 residues) of Vmw65 leads to loss of its transactivating function. Two important C-terminal regions (residues 412-456 and 452-490 of the 490 residue protein) have been identified as potent transcriptional activation domains during investigations using 'GAL-4-VP16' fusion proteins (these contained the GAL-4 DNA binding domain and the relevant residues of Vmw65). Residues 412-456 have been reported to interact directly with the basal transcription factor TFIID (Lin *et al.*, 1991). It has been shown that residues 457-490 are necessary and sufficient to mediate an interaction with $TAF_{II}40$, a component of the TFIID complex that also interacts with TFIIB (Goodrich *et al.*, 1993). It has been suggested that the interaction between Vmw65 and the basal transcription complex results in a change in conformation of the transcription machinery which promotes recruitment of other cellular factors to make a functional transcription complex (Hahn, 1993).

1C1.2 Negative regulation of IE genes

From the results of early experiments carried out by Honess and Roizman (1974; 1975) it was evident that treatment of cells with cycloheximide, an inhibitor of protein synthesis, led to the overproduction of IE transcripts. This implied that during the normal course of virus infection, IE gene transcription is down-regulated. Infection with the *ts*K mutant virus resulted in a similar phenotype (in addition no E or L gene expression occurred) which suggested that Vmw175 may be a negative regulator of IE gene transcription. Quantitative Northern blotting experiments have shown that throughout the course of normal virus infection Vmw110 and Vmw175 transcripts are present; however, compared with their peak levels of transcription, Vmw175 transcripts remained at low levels whereas Vmw110 transcripts were present at high levels (Harris-Hamilton and Bachenheimer, 1985). In a more extensive analysis of the rates of IE mRNA transcription using nuclear run-on assays, Weinheimer and McKnight (1987) also showed that IE gene transcription persisted throughout infection. However, whilst IE1 and IE3 mRNAs were shown to accumulate with similar kinetics (increasing from 1-6hr post infection) it was evident that the rate of transcription of the IE3 gene was reduced by 1hr post infection. This study also showed that the rate of transcription from

the IE4/5 promoter was not down-regulated during infection. The conclusion from these studies was that transcription from the IE3 promoter was reduced during viral infection by a mechanism which involves Vmw175.

The transactivating ability of Vmw175 was examined in transient transfection assays using various combinations of plasmid-borne copies of HSV-1 IE genes and their promoters (reviewed by Everett, 1987b). Due to the nature of the systems used, the results varied, but the consensus was that Vmw175 represses the IE3 promoter (DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985b; Gelman and Silverstein, 1986). It has been shown that Vmw175 binds to a consensus sequence at the mRNA capsite of the IE3 promoter and that this is necessary for Vmw175-mediated repression (DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985; Gu *et al.*, 1993; Michael and Roizman, 1993). A similar consensus sequence is present in the IE1 promoter, further upstream of the capsite; in transfection assays IE1 gene transcription was increased when this sequence was mutated (Resnik *et al.*, 1989), but in the context of the virus there was no observable effect on IE1 gene expression (Everett and Orr, 1991).

It has been suggested that Vmw175 represses IE3 gene transcription by interfering with the basal transcription machinery (Didonato and Muller, 1989), although repression has been shown not to arise as a direct result of destabilisation of the transcription complex as Vmw175, TFIIB and TFIID simultaneously (as a tripartite complex) and cooperatively occupy their binding sites on the IE promoter (Smith *et al.*, 1993). Gu *et al.* (1993) have shown that Vmw175 has little effect on basal transcription but dramatically represses Sp-1 activated transcription from the IE3 promoter. An important aspect of Vmw175 mediated repression of the IE3 promoter is the position of the Vmw175 consensus binding site; it is necessary that it is present within 45bp 3' of the TATA box such that the tripartite complex can form (Gu *et al.*, 1995). It is likely, therefore, that Vmw175 masks or alters the preinitiation complex through specific protein:protein interactions so that activators communicate less efficiently with TFIID and the initiation of transcription is inhibited. As Vmw175 is a potent transactivating protein, being essential for E and L gene expression, it is not surprising that its expression is regulated; autoregulation is an extremely efficient mechanism for ensuring that the protein is not over or under expressed.

1C1.3 IE transcripts and proteins

The designated names of the five IE genes and their protein products are summarised in Table 1C1.3. Figure 1C1.3 shows the locations of the IE genes in the HSV-1 genome (Clements *et al.*, 1979; Murchie and McGeoch, 1982; Rixon *et al.*, 1982; McGeoch *et al.*, 1985; 1986: 1988b; Perry *et al.*, 1986). They are concentrated within the repeat regions, the only exception being IE2 which is found in the long unique segment (U_L). Of the five HSV-1 primary transcripts that are spliced, three are IE transcripts. Two of these are the IE4 and IE5 transcripts which are derived from identical promoters located in the short repeat region. Their splice sites are also derived from sequences in the repeat region (Watson *et al.*, 1981; Rixon and Clements, 1982) whilst their individual coding sequences are present in the short unique segment (U_S). The third spliced primary transcript is that of the IE1 gene. This gene lies in the long repeat region and is therefore diploid (Perry *et al.*, 1986). Gene IE3 is also diploid as it lies in the short repeat sequence. IE2 and IE3 gene transcripts are not spliced.

Glasgow nomenclature			Chicago nomenclature	
Gene*	Gene†	Product	Gene	Product
IE-1	RL2	Vmw110	α0	ICP0
IE-2	UL54	Vmw63	α27	ICP27
IE-3	RS1	Vmw175	α4	ICP4
IE-4	US1	Vmw68	α22	ICP22
IE-5	US12	Vmw12	α47	ICP47

* Original nomenclature (Clements *et al.*, 1979).

† Later nomenclature (McGeoch *et al.*, 1988a).

Table 1C1.3 Table summarising the different terms used to identify the immediate-early genes and their products.

The analysis of *ts* mutants showed that ***Vmw175*** is essential for the expression of E and L gene products (Courtney *et al.*, 1976; Marsden *et al.*, 1976; Watson and Clements, 1978; 1980; Preston, 1979a; Dixon and Schaffer, 1980; Preston, 1981; DeLuca *et al.*, 1985). Moreover, its presence is required throughout the course of normal viral infection in tissue culture (Preston, 1979b; Watson and Clements, 1980). As the *ts* viruses over-expressed IE products, it was assumed that Vmw175 represses IE gene expression (Preston, 1979a; Dixon and Schaffer, 1980; Watson and Clements, 1980; Section 1C1.2).

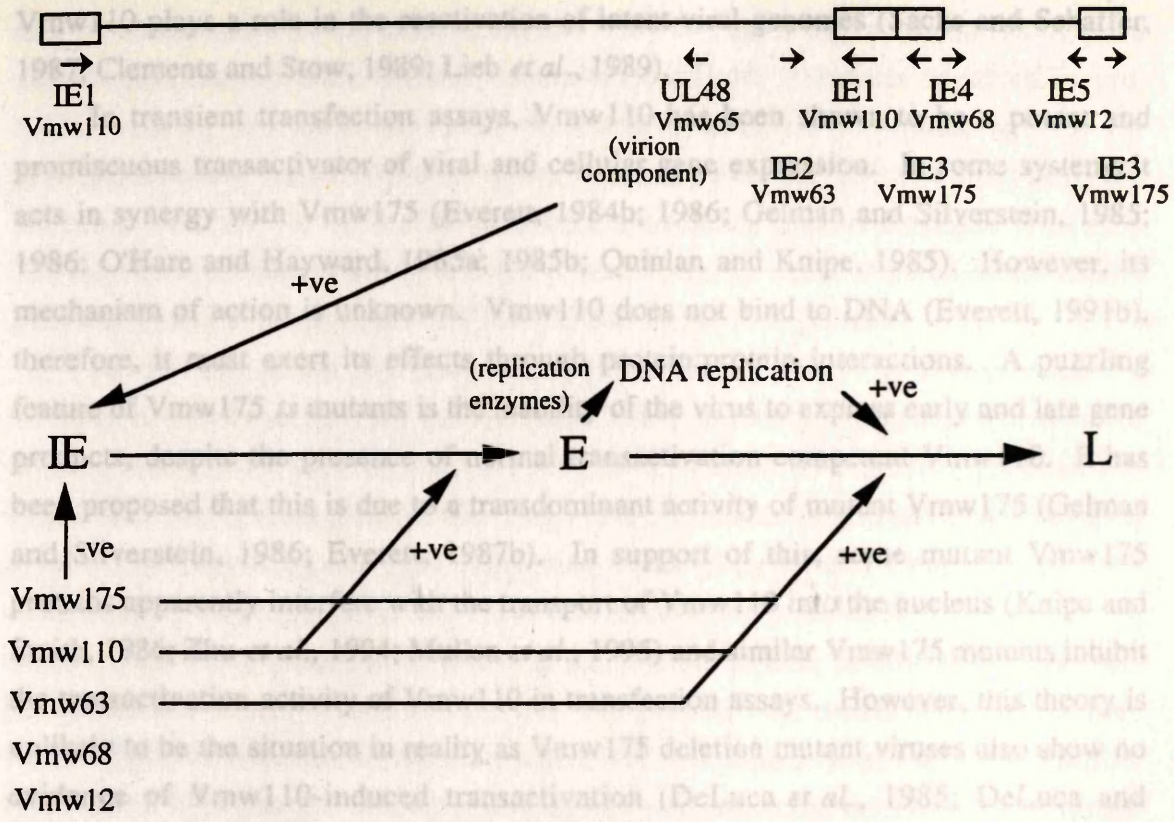

Figure 1C1.3 A schematic diagram showing the locations of the IE genes on the HSV-1 genome and the regulation of gene expression by HSV-1 gene products. The approximate relative positions of the five HSV-1 IE genes on the HSV-1 genome are shown. The IE4 and IE5 genes are transcribed from a common promoter which is in the short repeat region of the genome. The role played by some of the IE gene products in infection is indicated. Vmw68 may play a role in regulating late gene expression and Vmw12 affects MHC class I presentation of peptides on infected cell plasma membranes. Early gene expression leading to replication is required for late gene expression.

Investigations of insertion and deletion mutant viruses which either do not express ***Vmw110*** or express non-functional Vmw110, have shown that Vmw110 is non-essential in tissue culture. However, these viruses exhibit a cell type, cell cycle and multiplicity dependent defect during growth (Stow and Stow, 1986; Sacks and Schaffer, 1987; Everett, 1989; Cai and Schaffer, 1991). It is possible that HSV-1 infection *in vivo* may be at low multiplicity and Vmw110 may therefore play a role in the establishment of lytic infection *in vivo*. Vmw110 also appears to play a role in the reactivation of latent viral genomes. In an *in vitro* latency system, Vmw110 deficient viruses failed to reactivate latent virus (Russell *et al.*, 1987; Harris *et al.*, 1989). *In vivo* experiments with Vmw110 deletion mutant viruses showed that whilst these viruses replicated at the periphery and established latent infection, they reactivated poorly, indicating that

Vmw110 plays a role in the reactivation of latent viral genomes (Sacks and Schaffer, 1987; Clements and Stow, 1989; Lieb *et al.*, 1989).

In transient transfection assays, Vmw110 has been shown to be a potent and promiscuous transactivator of viral and cellular gene expression. In some systems it acts in synergy with Vmw175 (Everett, 1984b; 1986; Gelman and Silverstein, 1985; 1986; O'Hare and Hayward, 1985a; 1985b; Quinlan and Knipe, 1985). However, its mechanism of action is unknown. Vmw110 does not bind to DNA (Everett, 1991b), therefore, it must exert its effects through protein:protein interactions. A puzzling feature of Vmw175 *ts* mutants is the inability of the virus to express early and late gene products, despite the presence of normal transactivation competent Vmw110. It has been proposed that this is due to a transdominant activity of mutant Vmw175 (Gelman and Silverstein, 1986; Everett, 1987b). In support of this, some mutant Vmw175 proteins apparently interfere with the transport of Vmw110 into the nucleus (Knipe and Smith, 1986; Zhu *et al.*, 1994; Mullen *et al.*, 1995) and similar Vmw175 mutants inhibit the transactivation activity of Vmw110 in transfection assays. However, this theory is unlikely to be the situation in reality as Vmw175 deletion mutant viruses also show no evidence of Vmw110-induced transactivation (DeLuca *et al.*, 1985; DeLuca and Schaffer, 1988; Shepard and DeLuca, 1989).

Studies of infections in tissue culture with ***Vmw63*** *ts* or deletion mutant viruses have shown that Vmw63 is essential for normal virus growth (Sacks *et al.*, 1985; McCarthy *et al.*, 1989; Rice and Knipe, 1990). Vmw63 null mutant viruses over-express some E gene products and induce reduced levels of L gene expression.

The activity of the protein has been examined in transient transfection assays by a number of groups with complex and sometimes contradictary results. The results showed that Vmw63 alone could transactivate gene expression from the gB promoter (Rice and Knipe, 1988). In the presence of Vmw175 and Vmw110, Vmw63 was found to increase transactivation (late gB and VP5 promoters), decrease transactivation (early *tk* promoter) and also to have no effect on transcription levels (late gC promoter). (Everett, 1986; Rice and Knipe, 1988; Sekulovich *et al.*, 1988; Su and Knipe, 1989). Closer analysis of Vmw63 revealed the presence of both repressor and activator domains in the C-terminal end of the protein (Hardwicke *et al.*, 1989; Rice *et al.*, 1989; McMahan and Schaffer, 1990): however, the mechanisms by which Vmw63 acts are unknown.

From experiments in which the accumulation of specific transcripts and their protein products were compared during infection with a Vmw63 *ts* mutant virus, it was concluded that Vmw63 probably functions at the post-transcriptional level (Smith *et al.*, 1992). It has been shown that Vmw63 causes a reduction in host cell pre-mRNA splicing (Sandri-Goldin and Mendoza, 1992), a property of the protein which is probably in part responsible for shut-off of host cell macromolecular synthesis. This

may be connected with the observation that Vmw63 causes the redistribution of small nuclear ribonucleoprotein (snRNP) complexes, cellular structures involved in pre-mRNA splicing (Phelan *et al.*, 1993). Recent analysis of the C-terminal region of the protein has shown that the repressor region is necessary for the redistribution of snRNPs but is not sufficient for the inhibition of host cell splicing (Sandri-Goldin *et al.*, 1995). The relevance of these observations to the mechanism by which Vmw63 transactivates gene expression in the presence of Vmw175 and Vmw110 is unclear.

The product of the IE4 gene, ***Vmw68***, has been shown to be non-essential for infection in tissue culture (Post and Roizman, 1981). An early experiment with a partial deletion mutant virus showed that it did not grow efficiently on some cell types and was avirulent in mice (Sears *et al.*, 1985). Sears *et al.* (1985) also concluded that Vmw68 is involved in stimulating late gene expression. This is supported by more recent work carried out by Poffenburger *et al.* (1994) who showed that a complete Vmw68 deletion mutant virus was impaired in its ability to cause death in mice following intracerebral, intraperitoneal or intravaginal inoculation. The Vmw68 null mutant virus produced no visible signs of infection in the cornea but was reactivated from explanted ganglia; reactivation from ganglia following intravaginal inoculation did not occur, even though PCR analysis showed the presence of latent viral genomes. Vmw68 is modified by the U_L13 kinase (a structural component of the virion) and the product of the U_S3 gene (Purves *et al.*, 1993); viruses with deletions in U_L13 or the gene encoding Vmw68 express reduced levels of Vmw110 and some late viral proteins as a result of reduced levels of transcription from the genes encoding these proteins. Interestingly, Vmw68 has recently been shown to be necessary for virus induced aberrant phosphorylation of the large subunit of cellular RNA polymerase II which occurs on HSV-1 infection. It is speculated that this results in the preferential transcription of viral genes by RNA polymerase II but this has yet to be shown (Rice *et al.*, 1994; 1995).

Viruses with deletions in the IE5 gene, encoding ***Vmw12***, showed that Vmw12 is non-essential in tissue culture (Longnecker and Roizman, 1986; Mavromara-Nazos *et al.*, 1986). The protein is found in the cytoplasm, indicating that it is unlikely to play a role in the regulation of transcription. Indeed, it has recently been shown to play a role in rendering infected cells resistant to lysis by $CD8^+$ cytotoxic T lymphocytes. One property of HSV-1 is the down-regulation of MHC class I and associated peptides on the cell surface, and it has been shown that Vmw12 is both necessary and sufficient to cause the retention of MHC class I proteins in the endoplasmic reticulum and cis-Golgi (York *et al.*, 1994). More recently it has been observed that Vmw12 binds to a transporter associated with antigen processing (TAP), preventing peptide translocation into the endoplasmic reticulum (Hill *et al.*, 1995). As a result, MHC molecules do not become loaded with peptide which means that the MHC subunits are not stably associated and cannot be transported to the plasma membrane.

1C1.4 Activation of E gene expression

(i) Cis-acting elements

The discovery that Vmw175 was essential for E and L gene expression and that it transactivated a number of E gene promoters in synergy with Vmw110 in transient transfection assays (Section 1C1.3) led to investigations of the sequence requirements of E gene promoters. The *tk* and gD promoters have been investigated extensively.

Initial experiments investigating the nature of the *tk* promoter involved microinjection of plasmids containing the *tk* gene with mutated promoter sequences into *Xenopus laevis* oocytes (McKnight *et al.*, 1981). Further investigations involved a more detailed analysis using 'linker-scanning' mutations (McKnight and Kingsbury, 1982). This defined the promoter region as extending approximately 110 nucleotides upstream of the mRNA start site. It was found that a TATA box was necessary for 'accurate' initiation of transcription and that the presence of upstream elements consisting of a CCAAT motif and two GC boxes enhanced transcription. These upstream regulatory elements were found to bind the cellular transcription factors CCAAT binding protein (CBP) and Sp-1, which also binds GC motifs in IE promoters (Jones *et al.*, 1985; Jones and Tijan, 1985; Graves *et al.*, 1986). Analyses of these mutations in the viral context failed to uncover additional sequences necessary for transactivation by viral proteins (Coen *et al.*, 1986).

The role played by Vmw175 in the transactivation of the *tk* promoter was also investigated. In the viral context, it was found that deletion of the GC boxes greatly reduced transcription of the *tk* gene in the absence of Vmw175, but that in the presence of Vmw175 transcriptional reduction was minimal (Imbalzano *et al.*, 1991). This indicated that Vmw175 can functionally substitute for Sp-1 activity. Transcription levels in the absence of the CCAAT box did not differ depending upon whether or not Vmw175 was present. This group also found that, whilst only the TATA box was necessary for efficient Vmw175-induced *tk* gene transcription, Vmw175 could still induce expression when the TATA box was disrupted. Interestingly, in the absence of the GC boxes and CCAAT motif, *tk* was still expressed as an E gene in the presence of Vmw175. This raises the interesting question as to what defines an early promoter since none of the defined regulatory sequences seem to do so. Whilst it is possible that the context of the promoter in the viral genome contributes to its temporal regulation, it was found that replacement of gC promoter sequences with *tk* promoter sequences did not result in this L gene being expressed as an E gene (Homa *et al.*, 1988).

The sequence requirements for transactivation of the gD promoter were investigated by transfecting gD reporter constructs into cells in tissue culture followed by superinfection with HSV-1 (Everett, 1983). These experiments defined the minimal promoter region as lying within 83bp upstream of the mRNA start site. Linkage of the SV40 enhancer region upstream of various gD promoter mutants showed that there were

no differences required in promoter sequence for cellular-mediated transactivation compared with viral-mediated transactivation (Everett, 1984a). As with the *tk* promoter, sequence requirements were found to be a TATA box and a CCAAT motif (inverted). The upstream GC-rich sequences were also important, the distal most one of which binds the cellular transcription factor YY1 (Seto *et al.*, 1991; Chen *et al.*, 1992a; Mills *et al.*, 1994).

(ii) Transacting factors - Vmw175 mechanism of action

When it was observed that Vmw175 binds to DNA (Freeman and Powell, 1982; Faber and Wilcox, 1986), consensus sequences were identified and the ability of Vmw175 to bind to various promoter sequences was investigated. It was found that the gD promoter contained three Vmw175 binding sites, two upstream of the CAP site and one downstream (Faber and Wilcox, 1986; Tedder and Pizer, 1988; Tedder *et al.*, 1989). In an *in vitro* transcription assay, Tedder *et al.* (1989) showed that deletion of the Vmw175 binding sites in the gD promoter led to reduced levels of Vmw175-stimulated transcription. However, removal of all three Vmw175 binding sites from the gD promoter in the viral context had no effect on gD expression during HSV-1 infection in cell culture (Smiley *et al.*, 1992). Similarly, the *tk* promoter was found to contain Vmw175 binding sites although a virus carrying a mutant IE3 gene encoding a Vmw175 protein which was unable to bind to these sites *in vitro* did not show reduced levels of *tk* expression during infection (Kristie and Roizman, 1986a; Imbalzano *et al.*, 1990). The importance of these Vmw175 binding sites and their precise function and relevance to Vmw175-mediated transactivation in the viral context remains unclear. However, these experiments showed that the TATA box is required for efficient transactivation of these promoters, indicating that Vmw175 may mediate transactivation by interacting with a cellular transcription factor such as TFIID.

More is known of the interactions of Vmw175 with cellular transcription factors in the repression of the IE3 promoter (Section 1C1.2). Further hints that Vmw175 may interact with components of the basal transcription machinery came with the work of Abmayr *et al.* on the related IE protein of Pseudorabies virus (PRV). This group initially showed that the PRV IE protein was responsible for the non-specific increase in transcription seen in infection from RNA polymerase II genes in an *in vitro* system using infected nuclear extracts (Abmayr *et al.*, 1985). They then went on to show that in their *in vitro* assay, transcription levels were increased by the addition of non-promoter containing DNA which titrated non-specific DNA binding proteins, or by the preincubation of template DNA either with an unfractionated nuclear extract or with purified TFIID. They concluded that the IE protein acts by increasing the rate or extent of formation of a preinitiation complex rather than by acting at the initiation or elongation steps of transcription (Abmayr *et al.*, 1988). It has also been shown in an *in*

vitro assembly system that the PRV IE protein stimulates TFIID binding to promoter sequences, resulting in effective competition with nucleosomes during chromatin folding (Workman *et al.*, 1988). DeLuca's group have shown that Vmw175 mediates its repression of the IE3 promoter by repressing Sp-1 activated transcription, as a result of the formation of a tripartite complex with TBP and TFIID (Gu *et al.*, 1993; 1995; Smith *et al.*, 1993; see also Section 1C1.2).

1C1.5 Activation of L gene expression

Early investigations of the requirements of late gene promoters involved studies of the promoters of the 'true late' ($\gamma 2$) genes US11 and gC. In the context of the replicating viral genome, it was found that gC sequences spanning the mRNA start site and TATA box were sufficient for late promoter activity (Homa *et al.*, 1986). In transient transfection assays, Johnson and Everett (1986a; 1986b) showed that an active origin of DNA replication was required for efficient transactivation of the US11 promoter in the plasmid context, and Shapira *et al.* (1987) showed that this was also necessary for transactivation of the gC promoter. The conclusion from these experiments was that a TATA box/cap site region was sufficient for L gene transcription in a replication competent template. In support of this model, deletion of the gD upstream regulatory sequences and linkage of the remainder of the promoter to a functional origin of replication conferred late kinetics on the gD gene (Johnson and Everett, 1986a). The situation may be different for 'leaky-late' ($\gamma 1$) genes as binding sites for the cellular transcription factor YY1 have been identified in the promoters of $\gamma 1$ genes UL19, UL37 and gB. In the case of UL19, deletion of this binding site in the promoter leads to a reduced level of transcription of the UL19 gene (Seto *et al.*, 1991; Chen *et al.*, 1992a; Mills *et al.*, 1994).

A number of groups have provided experimental evidence which implies that the type of TATA box in the promoter contributes to the characteristics of late gene expression (Homa *et al.*, 1988; Flanagan *et al.*, 1991). This has been supported in the viral context in an experiment in which the *tk* gene was expressed as a late gene when its TATA box was replaced with the gC promoter region (Levine *et al.*, 1991). However, conflicting results arose in an experiment carried out by Imbalzano and DeLuca (1992) which showed that substitution of the *tk* TATA box with that of the gC promoter failed to alter the temporal expression of the *tk* gene.

Sequences at or near the mRNA start site may contribute to L promoter activity and even play a role in the requirement of a replication origin for L gene expression (Blair *et al.*, 1987; Mavromara-Nazos and Roizman, 1989; Kibler *et al.*, 1991; Steffy and Weir, 1991). It may be that cellular factors bind these sequences, contributing to gene expression or repressing expression, with the latter effect removed by replication of the viral genome. However, such cellular factors have not yet been identified. As a

consensus sequence is absent, the requirement would be for promiscuous DNA binding proteins. As with E gene promoters, it is likely that Vmw175-mediated transactivation of L genes occurs through Vmw175 interacting with components of the basal transcription machinery.

1C2 HSV infection and cellular gene expression

Whilst the majority of cellular genes are shut-off during HSV infection, a subset are expressed (Patel *et al.*, 1986; Kemp and Latchman, 1988; Section 1B2.5). A number of groups showed that when viral genes were inserted into the cellular genome, they were transactivated by HSV-1 superinfection. This led to the conclusion that there were specific sequences in viral gene promoters that distiguished them from cellular promoters (Sandri-Goldin *et al.*, 1983; Dennis and Smiley, 1984; Silver and Roizman, 1985). However, Everett (1985) showed that infection with HSV-1 could stimulate non-viral promoters (ε-globin and β-globin) which were stably integrated in the cellular genome and that this effect was mediated by Vmw175 and Vmw110. As these cellular promoters are not activated on HSV infection in their normal locations, it was concluded that chromatin structure must play a role in the expression of cellular genes. For example, it is possible that the integrated cellular genes were present at sites of open nucleosomal structure and as such were accessible by the viral transactivators. The down-regulation of cellular gene expression during infection may also be a result of Vmw63-mediated repression of transcript splicing (Hardy and Sandri-Goldin, 1994).

Experiments in which the β-globin gene was inserted into the viral genome showed that it was expressed as an E gene, whether the gene was under the control of its own promoter or the gD promoter (Smiley *et al.*, 1987). This supports conclusions concerning the mode of action of Vmw175, namely that Vmw175-mediated transactivation requires no virus-specific regulatory sequences. The α-globin gene is expressed constitutively from transfected plasmids during transient expression assays in the absence of viral proteins but is expressed as an E gene in the context of the viral genome (Smiley *et al.*, 1991). The adenine phosphoribosyl transferase (*aprt*) gene, which lacks a functional TATA box, was not expressed in the context of the viral genome, which supports the requirement of interactions of Vmw175 with the basal transcription machinery for its transactivation ability (Tackney *et al.*, 1984). The conclusions drawn from this work were that viral transactivators act on all polymerase II promoters provided they are accessible and TATA-dependent.

1C3 Gene expression during latent infection

(i) Latency associated transcripts

Transcription during latent infection of HSV-1 in neurons is restricted mainly to the latency associated transcripts (LATs). Transcription occurs from a promoter situated in the repeat sequence bounding the long unique region of the genome, and the stable transcripts overlap approximately a third of the IE1 gene coding sequence (Figure 1D2). Two major and partially co-linear polyA$^-$ RNAs of approximately 1.2kb and 2.0kb have been found (Rock *et al.*, 1987; Spivak and Fraser, 1987; Stevens *et al.*, 1987); the 1.2kb LAT is thought to be derived from the 2.0kb LAT by splicing in a process which probably involves neuron-specific factors (Spivak *et al.*, 1991). There are also less abundant polyA$^+$ LATs (the minor LATs), and it has been suggested that all latent phase transcripts arise from a single 8.3kb transcript which is subsequently processed to give the abundant polyA$^-$ LATs (Dobson *et al.*, 1989). There have also been suggestions that the polyA$^-$ LATs are stable introns (Devi-Rao *et al.*, 1991; Farrell *et al.*, 1991).

Due to their unusual structure, it is unlikely that any of the major LATs encode protein. However, one group detected a latency associated protein in primary neurons using an antiserum raised against a bacterially expressed fusion protein from a plasmid construct containing the 2.0kb LAT sequences (Doerig *et al.*, 1991). The putative 80kD latency associated protein has not been detected in infected cells in culture or in latently infected mice. It is not known whether the minor polyA$^+$ transcripts encode latency associated proteins.

It was initially thought that as LAT transcripts are antisense in part to the IE1 transcript, they could regulate latency by inhibiting translation of IE1 mRNA (Stevens *et al.*, 1987). In view of the fact that Vmw110 mutant viruses reactivate poorly from latency (Clements and Stow, 1989; Lieb *et al.*, 1989; Cai *et al.*, 1993) and that Vmw110 mutant viruses are unable to reactivate latent virus in an *in vitro* latency system (Russell *et al.*, 1987; Harris *et al.*, 1989) this was an attractive suggestion. However, the situation is more complicated; experiments with LAT mutant viruses have shown that LAT expression is non-essential for the establishment and maintenance of latent infection in mouse and rabbit models (Javier *et al.*, 1988; Ho and Mocarski, 1989; Lieb *et al.*, 1989; Sedarati *et al.*, 1989; Steiner *et al.*, 1989; Hill *et al.*, 1990; Block *et al.*, 1990). Some mutants, but not all, showed defects in the efficiency of reactivation compared with wild type virus. However, Sawtell and Thompson (1992) have suggested that LAT expression promotes the establishment of latency in mouse trigeminal ganglia since a LAT deletion mutant that they constructed established latent infection in 80% fewer ganglia than the parent virus. This difference was not seen when latency was established in the lumbosacral ganglia, which implies that the establishment of latent infection is, to an extent, dependent upon the host cell. It is generally held that between 1-5% of neurons in latently infected mouse ganglia harbour LATs (Speck and

Simmons, 1991; Rock *et al.*, 1992); these experiments were carried out using *in situ* hybridisation techniques following isolation of ganglia which were fixed, sectioned and mounted on slides. *In situ* hybridisation has also shown that the minor LATs are localised to sharply defined intranuclear foci of 1-3μm in diameter in latently infected primary sensory neurons of mice and humans (Arthur *et al.*, 1993). The major LATs were found to be distributed throughout the nucleoplasm, with the exclusion of the nucleolus. An investigation of the nature of the foci may uncover cellular or viral proteins with which the minor LATs interact which could lead to an understanding of their function.

(ii) Are LATs a suitable marker for latency?

When mice were infected with equal amounts of plaque forming units of wild type virus or *in*1814 via the right rear footpad, it was found that the dorsal root ganglia of *in*1814 infected mice contained many more LAT$^+$ neurons compared with ganglia of mice harbouring wild type virus (Ecob-Prince *et al.*, 1993a). Virus *in*1814 encodes a mutant Vmw65 protein which is incapable of transactivating IE gene expression but is functional in its role in virion structure (Section 1C1.1). LAT$^+$ neurons were also found in the dorsal root ganglia of *in*1814 mice which did not innervate the original site of infection. It was concluded that the large number of LAT$^+$ neurons and the spread of this virus was related to the high particle numbers in the inoculum and that spread of the virus was a consequence of limited replication as well as the low neurovirulence of *in*1814. Ecob-Prince and co-workers also investigated the possibility that a greater population of LAT$^+$ neurons led to a more extensive reactivation upon administration of a reactivation stimulus (Ecob-Prince *et al.*, 1993b). Mice were infected with wild type virus or with *in*1814 via the right rear footpad and reactivation was induced by neurectomy of the sciatic nerve. Ganglia were removed and fixed at various times post neurectomy or were explanted into tissue culture. Reactivation was detected in fixed cells by *in situ* hybridisation with an IE1 mRNA probe and in tissue culture by removing the medium from explanted ganglia and plating dilutions on BHK cell monolayers under conditions which would complement the *in*1814 mutation. It was found that the frequency of reactivation did not correlate with the number of LAT$^+$ neurons, as the levels of reactivation corresponded to 0.25% of neurons in ganglia regardless of the input virus.

The examination of a ribonucleotide reductase-deleted HSV-1 mutant in rat trigeminal ganglia following corneal inoculation indicated that a population of LAT$^-$ latent genomes may exist (Ramakrishnan *et al.*, 1994). *In situ* hybridisation with a digoxigenin-labelled riboprobe specific for LATs detected an average of 1-2 LAT$^+$ cells in 6μm ganglion sections. In contrast, *in situ* PCR using digoxigenin-labelled nucleotides with primers specific for HSV-1 gB DNA identified up to 120 genome

positive cells per section. These results suggest that cells with detectable LATs represent only a small proportion of those ganglionic neurons containing HSV-1.

(iii) Do LAT$^+$ neurons reactivate?

Ecob-Prince and Hassan (1994) have found evidence of reactivation occurring primarily in neurons with do not express LATs. Ganglia were isolated, explanted into tissue culture for various lengths of time (2-3 days), fixed, sectioned and examined using *in situ* hybridisation and immunocytochemistry. Reactivation was detected by ascertaining the presence of the virus specific mRNAs and antigens associated with lytic infection. Infrequently, IE2 and IE4/5 mRNAs were found in LAT$^+$ neurons. On the whole, viral mRNA and antigens were found in neurons which did not contain LATs. It is possible that the abundant LATs were lost quickly following the onset of replication but there was little evidence of this because, as the number of reactivating neurons increased following explantation, the number of LAT$^+$ neurons was not seen to decrease. It is therefore possible, although it has not been conclusively shown in these experiments, that there is a population of neurons in latently infected ganglia which harbour the latent HSV genome but do not express LATs.

An attempt was made to identify a population of latently infected neurons which do not express LATs in experiments carried out with *in*1853, a derivative of *in*1814 with the β-galactosidase gene inserted at the TK locus under the control of the HCMV enhancer (Ecob-Prince *et al.*, 1995). The reporter gene was found to be weakly expressed in the dorsal root ganglia of mice infected via the rear footpad for up to five months post infection. In ganglia which were explanted, fixed and sectioned at the appropriate times, about 30% of β-galactosidase positive neurons did not contain LATs as detected by *in situ* hybridisation. However, the detergents used to enable penetration of the β-galactosidase substrate also reduced the levels of LATs; in neurons with low levels of LATs this may have reduced levels to below those detectable by *in situ* hybridisation. Consequently, the evidence from this experiment for the existence of a population of latently infected LAT$^-$ neurons was weak. However, an *in situ* PCR based method for detecting viral genomes in latently infected neurons has been developed (Mehta *et al.*, 1995). This method has been used to show that 4.8% of neuronal cell bodies contain latent viral genomes, as detected by amplification of the viral DNA polymerase gene, compared with 1.6% of cell bodies which were LAT positive, as detected by RNA hybridisation. Amplification of a single copy mouse gene, *all-1*, in a control experiment gave a positive signal in 48% of cells, indicating that up to 9.6% of neurons could contain latent viral genomes. However, it is possible that cells which appeared to be LAT$^-$ may have expressed low levels of LATs which were not detected in the RNA hybridisation experiment. Consequently, whilst it appears that some latently infected neurons may harbour viral genomes which do not express LATs, it has

yet to be conclusively proven. The implications of the existence of such a population of neurons are intriguing; in particular, if the LAT expressing viral genomes do not reactivate, this could be applicable for gene therapy if the mechanism of activation of expression was understood.

(iv) Expression of other viral genes

A lack of assay techniques sufficiently sensitive to detect very low levels of viral gene expression has led, in the past, to the assumptions that LATs are the only transcripts produced during latent infection. Kramer and Coen (1995) have developed a sensitive and quantitative PCR assay which showed that one to seven IE3 gene transcripts per viral genome were present in about 20% of ganglia examined. In order to determine whether or not Vmw175 was present, they looked at the levels of *tk* gene transcription, as this is dependent on the presence of Vmw175 in lytic infection, and found a corresponding number of transcripts (about 500 per latent viral genome). This observation has interesting implications for the mechanism of reactivation from latency (as defined by the detection of infectious virus). It has been proposed in the past that reactivation is the result of a single step which produces a switch to the lytic cycle (Stevens *et al.*, 1987; Sawtell and Thompson, 1992). The recent results of Kramer and Coen (1995) imply that the switch could be much more gradual, depending upon threshold levels of accumulation of certain transcripts and their proteins.

1D Vmw110

1D1 History

Vmw110 was first classified as an IE gene product by Honess and Roizman (1974). Using HSV-1 and HSV-2 intertypic recombinants, the IE1 gene encoding Vmw110 was initially mapped to the repeat sequences bounding the long unique region of the genome (Preston *et al.*, 1978). The 5' and 3' ends of the mRNA were subsequently mapped more precisely and correlation between the map sites for the ends of the transcript and its actual size suggested that the primary transcript is spliced (Clements *et al.*, 1979; Watson *et al.*, 1979; Anderson *et al.*, 1980; Mackem and Roizman, 1980; Rixon *et al.*, 1984). This was later confirmed by sequence analysis (Perry *et al.*, 1986; McGeoch *et al.*, 1988a).

The failure to isolate Vmw110 *ts* mutants meant that its properties could not be extensively investigated until a number of groups almost simultaneously discovered that it is a potent transactivator of gene expression in transient transfection assays (Everett, 1984a; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a; Quinlan and Knipe, 1985). The isolation of Vmw110 deletion mutant viruses showed that Vmw110 is non-essential for lytic infection in tissue culture although virus growth is impaired at low multiplicities of infection (Stow and Stow, 1986; Sacks and Schaffer, 1987). However, there has been much interest in the potential role that Vmw110 plays in latent infection. Vmw110 mutant viruses are unable to induce the reactivation of latent virus in an *in vitro* latency system (Russell *et al.*, 1987; Harris *et al.*, 1989) and they reactivate poorly from latency in mouse models (Clements and Stow, 1989; Lieb *et al.*, 1989; Cai *et al.*, 1993).

It has long been known that Vmw110 is located in the nucleus of infected cells (Pereira *et al.*, 1977; Wilcox *et al.*, 1980; Ackerman *et al.*, 1984) and later immunofluorescence experiments confirmed this and showed that Vmw110 was localised to specific domains within the nucleus (Gelman and Silverstein, 1986; Everett, 1988a). More recently, the identification of these domains as novel nuclear structures, whose disruption during HSV-1 infection has been attributed to the presence of Vmw110 in the cell, has sparked an interest in the potential interactions of Vmw110 with cellular proteins (Sections 1D9.2 and 1D9.3). These interactions are likely to be crucial to the understanding of both lytic and latent infection.

1D2 IE1 gene and transcript structure

Gene IE1 lies entirely in the long repeat region of the HSV-1 genome and encodes a predicted protein product of 775 amino acids. In the interior repeat, gene IE1 is transcribed from right to left with the transcriptional start site at position 124,256 and the polyadenylation signals (preceeding the 3' terminus of the primary transcript) at

120,693 and 120,734 (Mackem and Roizman, 1982b; Perry *et al.*, 1986; McGeoch *et al.*, 1988a). There are two introns, the first between coordinates 122,484-122,621 (after codon 19) and the other between 123,287-124,053 (within codon 242). The predicted translational stop and start sites are at positions 124,108 and 120,882 respectively. The gene is partially overlapped in the same orientation by one of the ICP34.5 transcripts and in the opposite orientation in part by the latency associated transcripts (LATs) (Figure 1D2).

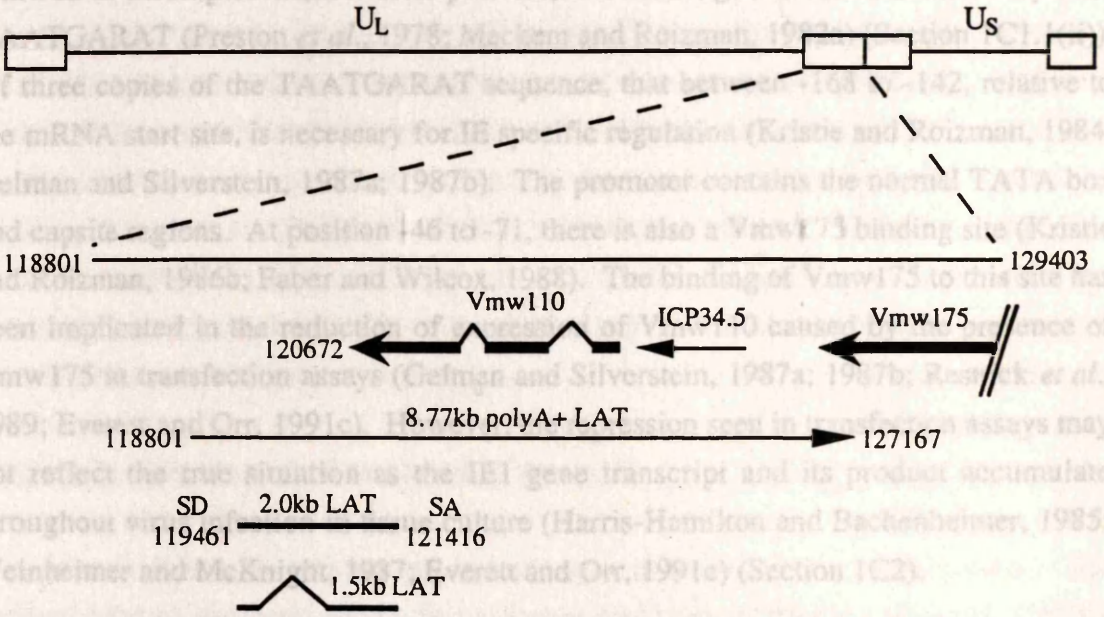

Figure 1D2 Diagram showing the relative positions of the Vmw110, ICP34.5 and LAT transcripts. The Vmw110/LAT region is in the repeat region bounding U_L. The Vmw110 and ICP34.5 genes overlap in the same orientation but the transcripts do not. The 8.77kb minor LAT overlaps the Vmw110, ICP34.5 and part of the Vmw175 encoding genes; it is present during productive infection, as is the stable 2kb LAT RNA, which is the major latency transcript. The 2kb LAT is derived from the 8.77kb LAT by splicing; the splice donor (SD) and splice acceptor (SA) sites are shown. The smaller 1.5kb LAT represents a spliced variant found only in latent infection.

As very few HSV-1 transcripts are spliced, it was of interest to investigate the importance of the introns in the Vmw110 primary transcript. In transfection assays, loss of the first intron reduced the transactivation of a reporter construct by 60%. This figure was 40% with loss of the second intron and deletion of both led to abolition of transactivation (Everett, 1991a). Surprisingly, removal of the introns in the viral context had little effect on the behaviour of the virus. The growth rate, particle:pfu ratio and viral polypeptide expression was similar to that of the parent virus (Everett, 1991a). In contrast, removal of the introns from the SV40 late transcription unit resulted in little or no accumulation of SV40 late transcripts (Ryu and Mertz, 1989). It is possible that

the differences between the results of the transfection assays and the properties of the virus mutants is due to a defect in IE1 RNA processing in transfected cells which leads to low levels of Vmw110 production. The defect may be overcome in the presence of other viral proteins. Interestingly, an HSV-1 virus mutant with the IE1 gene introns deleted establishes and reactivates from latency in mice with the efficiency of a wild type virus (Natarajan *et al.*, 1991).

The IE1 promoter, like all IE promoters, is responsive to transactivation by Vmw65 in a complex with cellular proteins, which recognises the consensus sequence TAATGARAT (Preston *et al.*, 1978; Mackem and Roizman, 1982a) (Section 1C1.1(ii)). Of three copies of the TAATGARAT sequence, that between -168 to -142, relative to the mRNA start site, is necessary for IE specific regulation (Kristie and Roizman, 1984; Gelman and Silverstein, 1987a; 1987b). The promoter contains the normal TATA box and capsite regions. At position -46 to -71, there is also a Vmw175 binding site (Kristie and Roizman, 1986b; Faber and Wilcox, 1988). The binding of Vmw175 to this site has been implicated in the reduction of expression of Vmw110 caused by the presence of Vmw175 in transfection assays (Gelman and Silverstein, 1987a; 1987b; Resnick *et al.*, 1989; Everett and Orr, 1991c). However, the repression seen in transfection assays may not reflect the true situation as the IE1 gene transcript and its product accumulate throughout virus infection in tissue culture (Harris-Hamilton and Bachenheimer, 1985; Weinheimer and McKnight, 1987; Everett and Orr, 1991c) (Section 1C2).

1D3 Vmw110 polypeptide structure and physical properties

The 775 amino acid IE1 gene product (Perry *et al.*, 1986) has a predicted molecular weight of 78,452D but the protein migrates on SDS polyacrylamide gels with an apparent molecular weight of approximately 110kD (Honess and Roizman, 1974). The protein contains an unusually high proportion of alanine, proline, glycine and arginine residues, which is probably a reflection of the high GC content of the gene. Phosphorylation of a serine-rich region between residues 554-591 may be responsible for the increased molecular weight of the protein seen on polyacrylamide gels (Ackerman *et al.*, 1984). There are small clusters of acidic and basic residues throughout the protein, but mutational analysis of these and of the serine-rich region indicates that they are not essential for the function of the protein (Everett, 1987a; 1988a; Section 1D5). Figure 1D3 shows the amino acid sequence of Vmw110.

A cysteine-rich region between residues 106 and 150 has been shown, in transfection assays and in the context of the viral genome, to be essential for Vmw110 function (Everett, 1987a; 1988a; 1989; Section 1D5). It is now known that these sequences comprise a zinc binding domain, a RING finger, whose structure has been elucidated by nuclear magnetic resonance spectroscopy (Everett *et al.*, 1993b; Barlow *et al.*, 1994; Section1D8.2).

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MEPRPGASTRRPEGRPQREPAPDVWVFPCDRDLPDSSDSEAETEVGGRGD  50
ADHHDDDSASEADSTDTELFETGLLGPQGVDGGAVSGGSPPREEDPGSCG 100
GAPPREDGGSDEGDVCAVCTDEIAPHLRCDTFPCMHRFCIPCMKTWMQLR 150
NTCPLCNAKLVYLIVGVTPSGSFSTIPIVNDPQTRMEAEEAVRAGTAVDF 200
IWTGNQRFAPRYLTLGGHTVRALSPTHPEPTTDEDDDDLDDADYVPPAPR 250
RTPRAPPRRGAAAPPVTGGASHAAPQPAAARTAPPSAPIGPHGSSNTNTT 300
TNSSGGGGSRQSRAAAPRGASGPSGGVGVGVGVVEAEAGRPRGRTGPLVN 350
RPAPLANNRDPIVISDSPPASPHRPPAAPMPGSAPRPGPPASAAASGPAR 400
PRAAVAPCVRAPPPGPGPRAPAPGAEPAARPADARRVPQSHSSLAQAANQ 450
EQSLCRARATVARGSGGPGVEGGHGPSRGAAPSGAAPLPSAASVEQEAAV 500
RPRKRRGSGQENPSPQSTRPPLAPAGAKRAATHPPSDSGPGGRGQGGPGT 550
PLTSSAASASSSSASSSSAPTPAGAASSAAGAASSSASASSGGAVGALGG 600
RQEETSLGPRAASGPRGPRKCARKTRHAETSGAVPAGGLTRYLPISGVSS 650
VVALSPYVNKTITGDCLPILDMETGNIGAYVVLVDQTGNMATRLRAAVPG 700
WSRRTLLPETAGNHVMPPEYPTAPASEWNSLWMTPVGNMLFDQGTLVGAL 750
DFRSLRSRHPWSGEQGASTRDEGKQ                           775
```

Figure 1D3 The amino acid sequence of Vmw110.

Vmw110 has been expressed in large quantities in a baculovirus system. The purified protein has been shown to exist as a multimer in solution (Everett, 1991b). This result was confirmed by Silverstein's group who expressed sufficient quantities of Vmw110 in HeLa cells infected with a recombinant adenovirus expressing Vmw110 (Chen *et al.*, 1992b). This property is one of the functions of the C-terminal end of Vmw110 (Ciufo *et al.*, 1994; Sections 1D11 and 3B). Everett (1991b) also showed that Vmw110 bound to single and double stranded DNA cellulose matrices, confirming earlier findings in which passage of crude nuclear extracts of infected cells down calf thymus DNA-cellulose columns resulted in retention of Vmw110 on the columns (Hay and Hay, 1980). However, it was not possible to detect a stable interaction between Vmw110 and DNA in filter binding assays (Everett, 1991b) which suggests that Vmw110 does not exert its effects through a direct interaction with DNA.

Analysis of the protein content of nuclear and cytoplasmic fractions of infected, radiolabelled tissue culture cells showed that Vmw110 was localised primarily in the nucleus (Pereira *et al.*, 1977; Wilcox *et al.*, 1980; Ackerman *et al.*, 1984). It was later shown in immunofluorescence experiments that Vmw110 expressed from a plasmid construct localises to discrete domains in the nucleus of host cells (Gelman and Silverstein, 1986; Everett, 1988a). In the viral context, Vmw110 is localised to the punctate domains at 2hr post infection but by 8hr post infection, it is present in both nucleus and cytoplasm and the punctate domains are no longer visible (Everett and

Maul, 1994). These domains have since been identified as novel nuclear structures of unknown function called ND10, Kr bodies or PODs (Ascoli and Maul, 1991; Xie *et al.*, 1993; Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994; Section 1D9.1). They are known to contain a number of cellular proteins, one of which is PML, a RING finger oncoprotein implicated in the pathogenesis of acute promyelocytic leukaemia (de Thé *et al.*, 1991; Goddard *et al.*, 1991; Kakizuka *et al.*, 1991; Kastner *et al.*, 1992; Section 1D9.2). The disruption of ND10 during the course of infection is known to be a property of Vmw110 (Maul *et al.*, 1993). It is evident from the analysis of mutant Vmw110 proteins that the RING finger and C-terminal regions of Vmw110 are important for the normal interaction of Vmw110 with ND10 during infection with wild type virus (Maul and Everett, 1994; See Section 1D9.1).

1D4 Activation of gene expression in transfection assays

The use of transient transfection assays has proved to be invaluable in the study of the regulation of gene expression and it was in using such assays that Vmw110 was found to be a potent transactivator of gene expression (Everett, 1984a; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a; Quinlan and Knipe, 1985). These assays involved transfecting a plasmid encoding Vmw110 into cells together with a plasmid construct in which the marker gene was under the control of a promoter of choice. The detection of increased promoter activity was carried out either by direct quantification of correctly initiated RNA or by the analysis of chloramphenicol acetyltransferase (CAT) enzyme activity, when this was expressed from the reporter gene construct.

It was found that the transactivating ability of Vmw110 could be divided into three categories: the transactivation of viral promoters, the transactivation of heterologous promoters and transactivation in synergy with the IE polypeptide Vmw175.

A number of groups have shown that Vmw110 transactivates promoters of all three classes of HSV-1 genes (Everett, 1984; 1986; O'Hare and Hayward, 1985a; 1985b;1987; Quinlan and Knipe, 1985; Gelman and Silverstein, 1985; Mavromara-Nazos *et al.*, 1986; Shapira *et al.*, 1987; Sekulovich *et al.*, 1988b). In the category of heterologous promoters, Vmw110 transactivates the SV40 early promoter (O'Hare *et al.*, 1985a; Everett, 1988) as well as the HIV LTR (Mosca *et al.*, 1987). Vmw110 can also transactivate the rabbit β-globin and the human ε-globin promoters (Everett, 1985). The only promoter tested which has been found to be unresponsive to Vmw110 is the VZV *tk* promoter (Inchauspe and Ostrove, 1989). Examination of the promoters which respond to transactivation by Vmw110 shows that apart from normal RNA polymerase II promoter motifs, there are no consensus sequences which could explain Vmw110 induced activation by a direct DNA binding mechanism. This makes sense given that Vmw110 has not been found to form a complex with DNA in solution (Everett, 1991b).

However, Blair and Wagner (1986) reported that sequences between -168 and -75 in the HSV-1 major capsid protein (VP5, 'leaky-late' gene) were necessary for Vmw110 to transactivate gene expression, but this observation was not pursued. Groups which found Vmw110 to be a potent transactivator also noted that Vmw110 acted in synergy with the IE protein Vmw175. However, in one system, the levels of transactivation seen were approximately 20 times greater than with either Vmw110 or Vmw175 alone (Everett, 1984). The levels of transactivation varied widely between different experimental systems. It is likely that one reason for this was the amounts of plasmid DNA transfected. This would affect the levels of Vmw110 and Vmw175 present both directly and indirectly as Vmw175 represses transfected IE1 and IE3 promoters (Section 1C1.2). Although there are many variations in the levels of activation seen by Vmw110 both alone and in synergy with Vmw175, there is agreement that Vmw110 is a potent and promiscous transactivator of gene expression.

1D5 Functional regions of Vmw110

The relative ease of setting up transfection assay systems has made it possible to analyse closely the regions of Vmw110 which are important for its ability to transactivate gene expression, both alone and in synergy with Vmw175. It has been found that the results from various laboratories regarding levels of activation of gene expression were inconsistent, and that the details of the experimental system utilised affected the results. Thus the target promoter, amounts of plasmid DNA transfected, the cell type, and the cell growth conditions all affected the outcome of the experiments (Everett, 1988b). However, the results in any one transfection system were consistent, so that it was possible to examine the effects of mutations in Vmw110 provided the assay conditions remained constant.

Everett (1987a; 1988a) constructed a family of plasmid-borne IE1 genes containing 12bp insertions throughout the Vmw110 coding sequence such that four amino acid insertion mutants of Vmw110 were expressed. From these mutants, a family of in-frame deletion mutants were derived. The ability of the various mutant Vmw110 proteins to transactivate gene expression was tested by cotransfection with a reporter gene construct expressing the CAT gene under the control of the viral gD promoter and a construct expressing Vmw175 if appropriate.

1D5.1 Transactivation in the presence of Vmw175

The transactivating abilities of the various mutant Vmw110 proteins in the presence of Vmw175 showed that there were five mutation sensitive regions. These are numbered from the N- to the C-terminus of the protein as 1 to 5 (Figure 1D5.1). The region most affected was the C-terminal end, region 5; all insertions between residues 633-723 led to a significant loss of activity. Insertions between residues 150-222 in the second exon of

the gene, region 1, also substantially reduced the ability of Vmw110 to transactivate gene expression in synergy with Vmw175. This region encompasses the RING finger (Everett, 1988a). Region 2, at residue 278, and region 3, at residue 406, are represented by single insertion mutants. Insertions at residues 575-592 (region 4) showed that disruption of the serine-rich tract led to a moderate reduction in reporter gene expression.

These results were substantiated by experiments with the in-frame deletion mutants (Everett, 1987a; 1988a). The deletion mutant Vmw110 protein expressed from p110FXE, missing residues 106-150, was the most severely impaired. Substantial reductions in reporter gene expression were also seen with the C-terminal deletion mutants expressed from constructs p110D12, p110D13, p110D14 and p110D15. The expressed proteins were missing residues 594-633, 633-680, 680-720 and 723-767 respectively. Deletion mutant proteins expressed from p110D8 and p110D9, missing residues 475-548 and 424-549 respectively, were also significantly affected. Immunofluorescence experiments have shown that these latter two proteins do not enter the nucleus so that loss of residues in the region between 424-549 appears to define a nuclear localisation signal. Further mutational analysis has narrowed down the nuclear localisation signal to between residues 474-509.

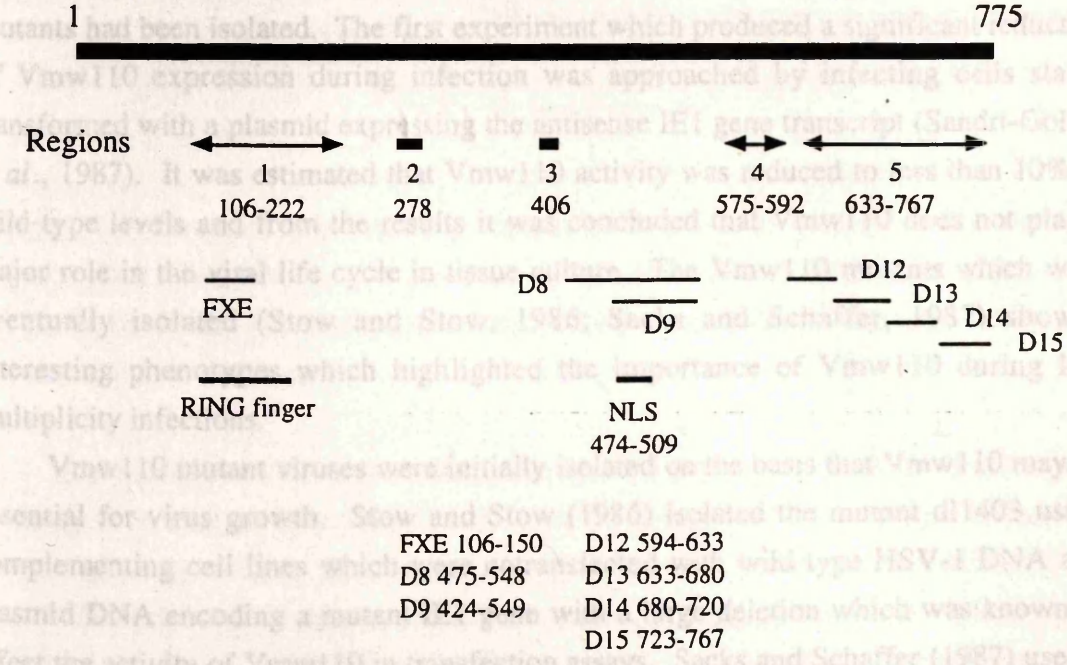


Figure 1D5.1 Diagram showing the five functional regions of Vmw110, as determined by linker insertion mutagenesis. The coordinates of the functional regions of Vmw110, regions 1,2,3,4 and 5, as determined by the abilities of various insertion and deletion mutant proteins to activate gene expression in transfection assays, are shown. The positions of a selection of the deletion mutants are shown.

1D5.2 Transactivation in the absence of Vmw175

Transactivation levels resulting from Vmw110 activity in the absence of Vmw175 have been more difficult to assess in some systems as Vmw110 gives only a 3-5 fold activation of the gD promoter compared with the 20 fold increase seen in synergy with Vmw175. However, the results showed two significant differences between the activity of Vmw110 alone compared with its activity in synergy with Vmw175. Firstly, insertions in regions 3 and 5 had little effect on the activity of Vmw110 alone, indicating that these regions may be important in the synergistic activity of Vmw110 with Vmw175. It has been suggested that these proteins physically as well as functionally interact (Gelman and Silverstein, 1986; Yao and Schaffer, 1994; Mullen *et al.*, 1995) but this is not necessarily the case (Section 1D10). The second difference is that insertions in region 1 completely abolish Vmw110 activity. This comparison of the effects of the same mutations on the activation of the gD promoter in the presence and absence of Vmw175 suggests that Vmw110 may act through two different but possibly related mechanisms. Analysis of experiments with the deletion mutants yielded similar results.

1D6 Analysis of Vmw110 mutants in the viral context

The role of Vmw110 during viral infection was difficult to assess before deletion mutants had been isolated. The first experiment which produced a significant reduction of Vmw110 expression during infection was approached by infecting cells stably transformed with a plasmid expressing the antisense IE1 gene transcript (Sandri-Goldin *et al.*, 1987). It was estimated that Vmw110 activity was reduced to less than 10% of wild type levels and from the results it was concluded that Vmw110 does not play a major role in the viral life cycle in tissue culture. The Vmw110 mutants which were eventually isolated (Stow and Stow, 1986; Sacks and Schaffer, 1987) showed interesting phenotypes which highlighted the importance of Vmw110 during low multiplicity infections.

Vmw110 mutant viruses were initially isolated on the basis that Vmw110 may be essential for virus growth. Stow and Stow (1986) isolated the mutant dl1403 using complementing cell lines which were cotransfected with wild type HSV-1 DNA and plasmid DNA encoding a mutant IE1 gene with a large deletion which was known to affect the activity of Vmw110 in transfection assays. Sacks and Schaffer (1987) used a similar approach to isolate mutants dlX0.7 and dlX3.1. They cotransfected the DNA of a Vmw175 null mutant virus into a Vmw110 complementing cell line with plasmid DNA encoding Vmw175 and a mutant IE1 gene.

Virus dl1403 contains a 2kb deletion within both IE1 genes such that the gene encodes a polypeptide consisting of the N-terminal 105 amino acids of the protein fused to 56 amino acids encoded by a new reading frame (Figure 1D6). This mutant showed

no differences in polypeptide profile, DNA replication and encapsidation when compared with wild type virus at a multiplicity of infection of 5pfu/cell in BHK cells. However, although similar numbers of particles were produced, the dl1403 progeny showed a significantly higher particle/pfu ratio than wild type virus. It was found that at low multiplicities of infection the plaquing efficiency of dl1403 was much less in Vero and HFL cells than in BHK cells (Stow and Stow, 1986). The effects of the deletion are therefore only seen at low multiplicities of infection (the probable *in vivo* situation) and are overcome at high multiplicities of infection.

Mutant dlX3.1 contains a 3.1kb deletion in both IE1 genes which removes the transcriptional regulatory region and most of the 5' coding sequences, whilst dlX0.7 contains a 700bp deletion (Figure 1D6). The viruses were grown in Nero cells (Vero cells with G418 resistance) and it was found that they produced similar protein profiles to wild type virus although slightly lower levels of late gene products were seen. It was also observed that the levels of DNA replication were reduced in these mutants. The conclusions reached were that Vmw110 is non-essential for viral growth in tissue culture, an observation which has simplified the isolation of further Vmw110 mutants.

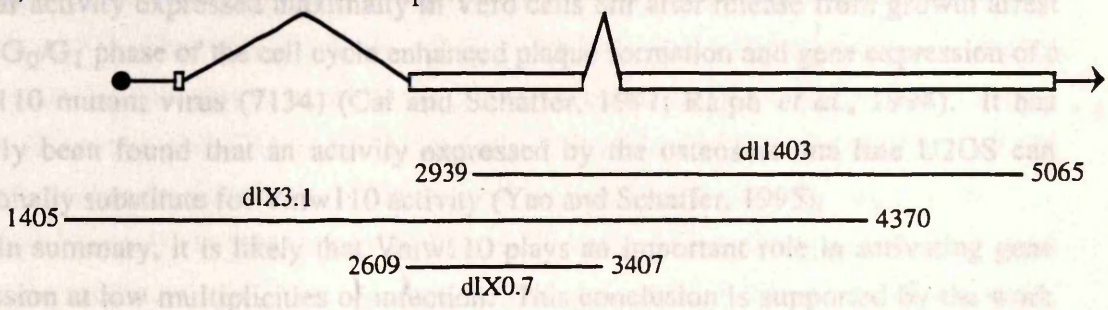


Figure 1D6 Diagram showing the postitions of deletions in Vmw110 mutant viruses dl1403, dlX3.1 and dlX0.7. The positions of the deletions in the IE1 gene of IR_L in the HSV-1 genome are indicated. The details of the mutant Vmw110 proteins produced are discussed in the text.

A series of ten viruses with lesions in the IE1 gene were constructed in order to investigate more closely the regions of Vmw110 important for its activity in the viral context (Everett, 1989). The viruses were made by cotransfecting virus dl1403 DNA into BHK cells with linearised plasmids encoding IE1 genes containing insertions or deletions known to affect the transactivating ability of Vmw110. The viruses were analysed for defects in single-step growth curve experiments and by analysis of viral polypeptide synthesis at low and high multiplicities of infection. Just as the activity of Vmw110 in transfection assays varied in different experimental systems, it was found that mutant virus growth also varied, depending upon the cell type used and even on the culture medium utilised. However, it was generally found that mutations in the functional regions of Vmw110, as defined by the transfection assays, were reflected in

the growth properties of viruses carrying mutations in these regions. This meant that the viruses with mutations in region 1, encoding the RING finger, and region 5, the C-terminal end, were most affected. Virus FXE, missing residues 106-150 which encode the RING finger, grew almost as poorly as dl1403 at low multiplicities of infection. The same was true of the C-terminal deletion mutants, D14 and D15, missing residues 680-720 and 723-767 respectively. The growth reduction seen in D13, with Vmw110 residues 633-680 deleted, was also significant.

From the studies of Vmw110 deletion mutant viruses, attempts have been made to explain the role of Vmw110 in the viral life cycle. The high particle/pfu ratio of dl1403, dlX0.7 and dlX3.1 has been explained by the suggestion that Vmw110 mutants have a reduced ability to initiate plaque formation at low multiplicities of infection. Also, mutants dlX3.1 and dlX0.7 plaque 15-50 times more efficiently on complementary cell lines which express Vmw110.

The differences between mutant plaquing efficiencies on different cell types led to the suggestion that a cellular function could substitute for Vmw110. It was found that a cellular activity expressed maximally in Vero cells 8hr after release from growth arrest in the G_0/G_1 phase of the cell cycle enhanced plaque formation and gene expression of a Vmw110 mutant virus (7134) (Cai and Schaffer, 1991; Ralph *et al.*, 1994). It has recently been found that an activity expressed by the osteosarcoma line U2OS can functionally substitute for Vmw110 activity (Yao and Schaffer, 1995).

In summary, it is likely that Vmw110 plays an important role in activating gene expression at low multiplicities of infection. This conclusion is supported by the work of Everett (1989), as viruses with mutations in Vmw110 which affected their ability to transactivate gene expression in transfection assays grew poorly in tissue culture. It is evident that in low multiplicity infections, viral gene expression is not sufficiently high for the virus to enter the lytic cycle.

1D7 The role played by Vmw110 in latent infection

(i) *In vitro* latency

The potential involvement of Vmw110 in the establishment or reactivation of HSV-1 from latency was indicated initially by the failure of the Vmw110 deletion mutant virus, dl1403, to reactivate latent virus in an *in vitro* latency system (Russell *et al.*, 1987). The *in vitro* latency system was set up with HFL cells by infection with HSV-2 at the supra-optimal temperature of 42°C (Russell and Preston, 1986). Infectious virus was not detected in the medium when the cells were subsequently maintained at 37°C for a number of days, indicating that a latent or quiescent infection had been established. Superinfection with HSV-1 or HCMV stimulated reactivation of HSV-2 latent viral genomes. Although the failure of dl1403 to reactivate HSV-2 in this system indicated that Vmw110 was involved in the process, it was possible that the LATs were

important, as the deletion in dl1403 also affects these transcripts. However, adenovirus recombinants expressing Vmw110 were also able to reactivate latent HSV-2 in the *in vitro* latency system, indicating that disruption of the IE1 coding sequences rather than the LAT coding sequences led to the failure of dl1403 to reactivate latent viral genomes (Harris *et al.*, 1989). In the *in vitro* latency system, it was found that the HSV-1 mutant virus, FXE (Vmw110 Δ106-150), was incapable of reactivating HSV-2 genomes, indicating that the RING finger region which is important in transfection assays (Section 1D5) is important in reactivation in this system (Harris *et al.*, 1989). The Vmw110 C-terminal deletion mutant virus, D14 (Vmw110 Δ680-720), was able to reactivate HSV-2, indicating that the deleted residues are not important for this function in this system. This suggests that C-terminal residues of Vmw110 may not be important for the role of Vmw110 in *in vitro* latency, although it would be of value to examine more mutants in the light of the findings presented in this Thesis.

(ii) Latency in mouse models

Virus dl1403 was used to investigate the role of Vmw110 in latency in the mouse footpad model (Clements and Stow, 1989). The Vmw110 deletion reduced virulence and the mutant was slower to reactivate from explanted ganglia compared to wild type virus. Clements and Stow (1989) concluded that Vmw110 is dispensable for the establishment and maintenance of latency and for reactivation from the latent state.

The Vmw110 mutant viruses dl1403, dlX3.1 and dlX0.7 (Section 1D6) were used to investigate the role of Vmw110 in latency in the mouse ocular model (Lieb *et al.*, 1989). Viruses dlX3.1 and dlX0.7 were replication competent in the eye and in ganglia, although levels were approximately 10-100 fold lower than those of wild type virus in the eye and approximately 100 fold lower than wild type virus in ganglia. These figures were obtained by plating eye swab material and homogenated ganglia on Vero cell monolayers in standard plaque assays. It was found that virus did not reactivate from the explanted trigeminal ganglia of mice infected with dlX3.1 and dlX0.7. Further investigations by slot blot hybridisation and superinfection of dissociated ganglionic neurons with a replication incompetent virus indicated that this was not due to the absence of latent viral genomes. Interestingly, it was evident that although the levels of replicating dlX0.7 virus in ganglia were the same as those of dlX3.1 in the initial stages of infection, there was much less viral DNA in the ganglia of mice infected with dlX0.7 compared with those infected with dlX3.1. These dlX0.7 genomes were resistant to reactivation in the superinfection assay. It was found in these assays that whilst dl1403 was less latency competent than its parent virus, HSV-1 strain 17$^+$, it was significantly more competent than dlX3.1 or dlX0.7 which were derived from the wild type KOS strain. It was concluded from these results that whilst Vmw110 is not essential for the

establishment, maintenance or reactivation of the virus from latency, it plays a role in the efficiency of the process.

Schaffer's group have also examined whether or not mutations in the IE1 gene or mutations in the LATs are responsible for the reduced efficiencies of Vmw110 mutant viruses in establishing latency and in reactivating from the latent state (Cai *et al.*, 1993). They constructed a series of Vmw110 nonsense, insertion and deletion mutant viruses which expressed Vmw110 polypeptides with C-terminal truncations and all of which expressed near wild type levels of the 2kb LAT. The mutant Vmw110 polypeptides exhibited graded levels of Vmw110 specific transactivation in transient transfection assays. On testing the mutant viruses for their ability to establish and reactivate from latency, it was found that all of them entered and reactivated from latent infection less efficiently than wild type virus but to an extent which reflected the ability of the encoded mutant Vmw110 protein to transactivate gene expression. Quantitative PCR analysis carried out using DNA isolated from ganglia showed that whilst the amounts of viral DNA in latently infected ganglia varied, there was no correlation between virus reactivation frequencies and the levels of viral DNA present.

In another investigation of the particular roles of Vmw110 and LATs in latent infection, Schaffer's group inserted a copy of the IE1 gene between the UL26 and UL27 genes of the IE1 and LAT deletion mutant virus 7134. The 7134 virus exhibits a marked impairment in its ability to replicate in the mouse eye and to reactivate from latency. The presence of the inserted IE1 gene restored the ability of the mutant to replicate in the eye and to reactivate from latency to approximately half of wild type levels. Taken together with the results from the insertion mutants this indicates that, although Vmw110 is not essential for establishment, maintenance or reactivation from latency, it plays a role in latent infection which is distinct from that of the LATs.

1D8 The RING finger

1D8.1 RING finger proteins

Vmw110 is a member of a growing family of proteins containing the C_3HC_4 or RING finger motif. Named after the RING-1 gene product, the first protein identified as containing this sequence (Freemont, 1991; 1993; Hanson *et al.*, 1991; Lovering *et al.*, 1993), the motif is represented more fully as C-X_2-C-X_{11-30}-C-X-H-X_2-C-X_2-C-X_{11-19}-C-X_2-C. The function of the RING-1 gene product is unknown but, on the basis of other RING finger proteins apparently being involved in the regulation of gene expression, it was initially proposed that the RING-1 gene product regulated gene expression by binding to DNA. Members of the family have since been found in a variety of organisms and appear to be involved in diverse cellular metabolic pathways. Some appear to be involved in the regulation of gene expression but others play a role in

nucleic acid metabolism, cell cycle regulation and in cytoplasmic signal transduction pathways. Many of these proteins also have a variety of motifs, other than the RING finger, such as SH3 domains or coiled-coil motifs, suggestive of their involvement in protein:protein interactions.

The variety of functions in which RING finger proteins are involved is not surprising given the lack of homology between many of them outside the RING domain, and even within the variable parts of the RING finger itself (Barlow *et al.*, 1994). It is apparent that the domain is unlikely to be involved in DNA interactions *per se* as some RING finger proteins are components of multiprotein complexes and others are located in the cytoplasm.

(i) Regulation of gene expression

Examples of proteins involved in the regulation of gene expression include *rpt-1*, a protein involved in the regulation of the interleukin 2 receptor gene in resting T-lymphocytes (Patacara *et al.*, 1988), and the *Drosophila* protein *male specific lethal 2*, which also contains a metallothionein-like cysteine cluster and is involved in the equalisation of X-linked gene products in male flies (Zhou *et al.*, 1995). A number of RING finger proteins are involved in development and therefore, by inference, in the regulation of gene expression; these include *COP-1*, an *Arabidopsis* regulatory gene involved in seedling morphogenesis (Deng *et al.*, 1992), *neuralised*, a *Drosophila* protein involved in the decision-making process that determines whether a primary cell will have a neuronal or epidermal fate (Price *et al.*, 1993) and A33, a newt protein which is associated with lampbrush chromosomes and may influence development by acting at the post-transcriptional level (Bellini *et al.*, 1993). Also included in this group are Vmw110 and the related proteins of other α-herpesviruses such as the VZV gene 61 protein and the EHV-1 gene 63 protein.

(ii) Recombination and repair

RAG-1 is a RING finger protein involved in V(D)J recombination in maturing B lymphocytes (Schatz *et al.*, 1989). The yeast proteins *rad8* and *RAD18*, from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* respectively, are involved in DNA repair following damage by UV radiation (Jones *et al.*, 1988; Doe *et al.*, 1993).

(iii) Replication

Some viral expressed RING finger proteins may be involved in replication; the pox viruses Shope fibroma virus and Ectromelia virus both express proteins which localise to sites of viral DNA replication (Upton *et al.*, 1994; Senkevich *et al.*, 1995). It is interesting to note that the nuclear domains to which Vmw110 localises early in infection are adjacent to or overlap viral replication compartments (Maul *et al.*, 1996).

(iv) Oncogene translocations

The RING finger protein PML is fused to the RARα protein as a consequence of a chromosomal translocation in the immature granulocytes of patients with acute promyelocytic leukaemia (Section 1D9.1). Like PML, the RING finger proteins *rfp* and T18 are implicated in cell transformation as a result of chromosomal translocations (Takahashi *et al.*, 1988; Miki *et al.*, 1991; Isomura *et al.*, 1992). The *rfp* gene is fused with the *ret* proto-oncogene and the mouse T18 gene to the B-*raf* proto-oncogene.

(v) Protein:protein interactions

Recently, a number of RING finger proteins have been implicated in protein:protein interactions. The *Caenorhabditis elegans* protein, *Sli-1*, is a developmental regulatory protein which acts on an epidermal growth factor receptor-like tyrosine kinase (Yoon *et al.*, 1995). It has been proposed that *Sli-1* and its mammalian counterpart, *c-cbl*, a proto-oncogene, belong to a new class of proteins that modify receptor tyrosine kinase-mediated signal transduction. The human protein, HT2A, mediates the interaction between the HIV-1 Tat protein and its cellular cofactor Tat binding protein, enabling the complex to interact with the TAR element (Fridell *et al.*, 1995). TRAF-2 is a protein which binds to the cytoplasmic tails of members of the tumour necrosis factor (TNF) receptor subfamily (Rothe *et al.*, 1994; 1995): the RING finger domain of this protein has been shown to be necessary in a signal transduction pathway which leads to the activation of the transcription factor NF-κB. The related protein, CRAF1, interacts with the cytoplasmic tail of CD40, another member of the TNF receptor subfamily which is found in B-cell membranes (Cheng *et al.*, 1995). Like TRAF-2 and CRAF1, PAF-1, a peroxisome associated protein, is cytoplasmic in its location (Tsukamoto *et al.*, 1994). Further examples of the diversity of function exhibited by RING finger containing proteins are *iap*, a baculovirus protein involved in preventing apoptosis (Clem and Miller, 1994) and p36, a protein which forms a multiprotein complex in a cell cycle dependent fashion (Fisher *et al.*, 1995).

1D8.2 Structure and functional analysis of the EHV-1 gene 63 RING finger

As the RING finger is present in such a large variety of proteins, it was of interest to determine the its structure. The Vmw110 RING finger region is important for its function in transfection assays (Section 1D5) and also for the normal disruption of nuclear structures called ND10 (Everett and Maul, 1994; Section 1D9.2), so attempts were made to obtain structural information from this protein as well as from the equivalent RING finger proteins of two other alpha herpesviruses.

The regions encompassing the Vmw110 RING finger and also those of the related genes in VZV (gene 61) and EHV-1 (gene 63) were cloned and expressed in bacteria, and the expressed proteins were purified by gel filtration chromatography under non-

denaturing conditions (Everett *et al.*, 1993b). The purified proteins were analysed for their zinc content by atomic absorption spectroscopy and their concentrations were determined by amino acid analysis. The results showed that the Vmw110 and equivalent VZV peptides contained 3-6 atoms of zinc per protein monomer and that the EHV-1 protein contained between 1.4(±0.4)-3.7(±0.4) atoms of zinc per molecule. Analysis of the *COP-1* RING finger and that of the RING-1 gene product showed that these molecules coordinate approximately 1.7 and 1.8 zinc atoms respectively (Lovering *et al.*, 1993; von Arnim and Deng, 1993). The purified VZV gene 61 and Vmw110 RING finger peptides aggregated, making them unsuitable for structural analysis by nuclear magnetic resonance (nmr) spectroscopy.

The structural analysis revealed that the EHV-1 RING finger motif adopts a ββαβ fold around two zinc atoms (Everett *et al.*, 1993b; Barlow *et al.*, 1994). Cysteines 1, 2, 4 and 5 coordinate one zinc atom, with cysteines 3, 6 and 7 and the histidine residue coordinating the other. The structure folds such that an α-helix lies along the surface of a triple-stranded anti-parallel β-sheet. The sequences involved in forming one of the β-strands are C-terminal to the final cysteine residue. A phenylalanine residue prior to the fourth cysteine lies at the centre of the structure; it is worth noting that all RING finger proteins contain a residue with a bulky, hydrophobic side-chain at this position. Another interesting structural feature is that cysteine 3 and the histidine residue, which are separated by only one amino acid, coordinate the same zinc atom. In all other known zinc binding proteins, the separation between residues coordinating the same metal ion is at least two amino acids.

Although part of the RING finger showed limited structural similarity to the TFIIIA type of zinc finger, the purified viral RING finger domains did not bind DNA or RNA in gel retardation assays and they also did not bind to DNA cellulose columns (Everett *et al.*, 1993b). In contrast, the RING finger domain of the RING-1 gene product was shown to weakly bind DNA in solution (Lovering *et al.*, 1993), but this may be a reflection of the overall positive charge of this domain. Consideration of the EHV-1 RING finger structure suggested that exposed α-helical polar residues may be important for its functions. To test this possibility, equivalent residues in the predicted Vmw110 α-helix were mutated and it was shown that some point mutations, as well as a number of double and triple point mutations, abolished Vmw110 function in transient transfection assays (Barlow *et al.*, 1994). This region of the Vmw110 RING finger domain has also been shown to be important in the context of the virus (Everett *et al.*, 1995c).

The structure of the PML RING finger has also been determined by nmr analysis (Borden *et al.*, 1995). The overall structure is similar to that of the EHV-1 RING finger; the positions of the central β-strands and the residues coordinating zinc atoms, as well as the packing of hydrophobic residues, are identical. However, there are some

differences in the structure which is not surprising as outside of the zinc coordinating residues the overall homology of the domains is less than 15%. The main structural difference is that in place of the EHV-1 α-helix, there is a β-strand. This is suggestive of the RING finger structure acting as a scaffold for protein domains involved in very different functions.

The structural differences between the PML and Vmw110 RING finger proteins were highlighted in domain-swap experiments (Everett *et al.*, 1995b). Replacing the Vmw110 RING finger with that of PML resulted in a virus which grew no better than the RING finger deletion mutant virus FXE. Complete replacement of the IE1 gene with the PML gene also produced a virus with a Vmw110-null phenotype, indicating that PML is not a functional counterpart of Vmw110. However, it is now known that the Vmw110-PML RING finger domain-swap did not encompass the entire PML RING finger domain as sequences C-terminal of the final cysteine residue which are known to form a β-strand were missing.

Another series of experiments were carried out in which the Vmw110 RING finger domain was replaced with the entire EHV-1 gene 63 RING finger (Everett *et al.*, 1995b). The hybrid protein did not transactivate gene expression in transient transfection assays. However, the growth properties of an HSV-1 virus expressing the entire EHV-1 gene 63 protein in place of Vmw110 were intermediate to those of the wild type virus and virus FXE. These experiments show that the RING finger domain is not a simple moveable element and support the conjecture that the RING finger structure is a scaffold around which a variety of sequences involved in different functions can fold. However, a larger domain swap has been successful (Moriuchi *et al.*, 1994). VZV gene 61 residues 1-105 fused to Vmw110 residues 246-775 and the reciprocal domain-swap protein (Vmw110 residues 1-245 and VZV gene 63 residues 106-467) partially maintained the transactivating functions of both proteins in transient transfection assays. It could be that the RING finger domain needs to be in the context of a larger portion of the parent protein to be active.

1D9 The nuclear localisation of Vmw110

1D9.1 ND10

(i) Identification

The nucleus is the site of a wide variety of cellular processes, including DNA replication, RNA transcription and processing, and ribosome biogenesis (Hancock and Boulikas, 1982; Newport and Forbes, 1987). DNA replication occurs at specific sites (Gerace and Burke, 1988; Hozák *et al.*, 1994) and ribosome biogenesis occurs within the nucleolus (Finley *et al.*, 1989). Factors involved in RNA processing, hnRNPs and snRNPs, are organised in 20-50 speckled domains within the nucleus (Spector *et al.*,

1983; Fakan *et al.*, 1984; Hamm *et al.*, 1988; Fu and Manniatis, 1990). Autoimmune sera often stain nuclear components such as kinetochores, telomeres and coiled bodies (Moroi *et al.*, 1981; Earnshaw *et al.*, 1984; Gottschling and Zakian, 1986; Cech, 1988; Morin, 1989; Andrade *et al.*, 1991). A number of nuclear domains including perichromatin fibrils and interchromatin granules have also been identified (Fakan *et al.*, 1984; Puvion *et al.*, 1984; Visa *et al.*, 1993).

On screening over 1,700 human autoimmune sera in immunofluorescence studies, a novel nuclear domain of 0.2-0.3μm diameter was identified by a small proportion of the sera (Ascoli and Maul, 1991). The sera, as well as two monoclonal antibodies which were isolated, stained approximately 10 nuclear dots per cell, hence the name nuclear dot 10 (ND10). Double label immunofluorescence experiments showed that ND10 were separate from chromosomes, kinetochores and centromeres (in spite of their frequent paired appearance), and were not sites of abundant RNA synthesis and mRNA processing (Ascoli and Maul, 1991; Kastner *et al.*, 1992; Xie *et al.*, 1993; Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994). Their relatively large size indicated that ND10 are probably multiprotein complex structures and to date they are known to contain at least five proteins, three of which have been cloned.

(ii) Protein constituents

Ascoli and Maul (1991) investigated methods of enriching cell extracts for ND10 in a series of *in situ* biochemical fractionation experiments combined with immunofluorescence assays. These experiments revealed that ND10 were highly hydrophobic and were stably attached to a nuclear matrix component. The *in situ* procedure was scaled up and residual proteins separated by polyacrylamide gel electrophoresis, Western blotted and probed with sera that detected ND10 in immunofluorescence experiments. Proteins such as the centromere proteins CENP-A, -B and -C were detectable, indicating that proteolysis was minimal. The anti-ND10 autoimmune sera recognised a band of approximately 55kD, and isoelectric focusing and size separation followed by immunoblotting with one of the monoclonal antibodies showed that there were three charge isomers of the 55kD protein.

A second ND10 protein, Sp100, was identified earlier than the 55kD protein but was not recognised as localising to ND10 until double label immunofluorescence experiments were carried out with the anti-55kD antisera and Sp100 antisera (Stuurman *et al.*, 1992). The Sp100 autoantigen was initially identified as a DNase and RNase insensitive, readily extractable nuclear protein with a molecular weight of approximately 100kD, as determined by polyacrylamide gel electrophoresis (Szostecki *et al.*, 1987). The gene encoding Sp100 was cloned following the screening of a λgt11 cDNA expression library (Szostecki *et al.*, 1990). Xie *et al.* (1993) screened 892 autoimmune sera and used one which detected ND10 in immunofluorescence

experiments (as shown by double labelling experiments with Ascoli and Maul's anti-55kD antiserum) to screen a λgt11 cDNA expression library. They also identified a clone encoding the Sp100 protein although this group observed that the protein migrated with an apparent molecular weight of 72kD on polyacrylamide gels. In contradiction to Ascoli and Maul's *in situ* biochemical fractionation experiments, Xie *et al.* (1993) reported that ND10 were associated with DNA. They suggested that in Ascoli and Maul's experiment DNase treatment may have been incomplete. However, this observation has not been supported by other groups and it is generally held that ND10 are attached to a component of the nuclear matrix. The gene encoding the Sp100 protein encodes a protein of a predicted molecular weight of 53kD. Analysis of a number of library clones indicated that the primary transcript was alternatively spliced. The anomalous molecular weights seen in polyacrylamide gel electrophoresis probably reflect the different gel systems used and it is also possible that Sp100 is modified. It has been suggested that residues 360-409 of the 480 residue protein are weakly homologous to some transcription factors (Szostecki *et al.*, 1990) and Xie *et al.* (1993) showed experimentally that fusion of Sp100 residues 333-407 to the lexA DNA binding domain resulted in transcriptional activation of a reporter gene construct in *Saccharomyces cerevisiae*. It was concluded from these experiments that Sp100 is a transcription factor. However, these results should be interpreted with caution as the homology with transcription factors is very weak and transcriptional activation experiments are better approached using whole proteins in transfection assays rather than just isolated domains.

A third component of ND10 was identified as PML (Section 1D9.2) after the isolation and characterisation of the monoclonal antibody 5E10 (Stuurman *et al.*, 1992). This group showed that the antigen recognised by 5E10, as well as being in the same domains as the 55kD protein and Sp100, was associated with the nuclear matrix (in agreement with Ascoli and Maul, 1991). It was found by Western blot analysis that 5E10 recognised four protein bands which were of 149kD, 126kD, 95kD and 63kD, with the 126kD band being most prominent. Dyck *et al.* (1994) showed, using a rabbit antiserum raised against a bacterially expressed PML-GST fusion protein, that PML localised to nuclear dots which they called PML oncogenic domains (PODs). Double label immunofluorescence experiments led to PODs being excluded as previously recognised nuclear structures. Western blot analysis of bacterially expressed PML showed that 5E10 recognised an epitope in the PML protein. Analysis using anti-55kD antisera and Sp100 antisera confirmed that PML colocalised to ND10 (or PODs). It was not possible to demonstrate an interaction between PML and Sp100 in an *in vitro* translation assay in which PML and PML-RARα formed heterodimers, indicating that another protein may bind both PML and Sp100 and confirming further the multiprotein nature of ND10 (Koken *et al.*, 1994).

Dyck *et al.* (1994) also carried out immunofluorescence experiments with a monoclonal antibody derived from sera originally raised against EBV infected cells (Epstein, 1984) and showed that the antibody cross-reacted with ND10 with a 65kD protein being the target. This protein has yet to be further characterised.

More recently, another ND10 protein has been cloned (Korioth *et al.*, 1995). A monoclonal antibody was isolated which detected nuclear dots in immunofluorescence assays, following immunisation of mice with crude nuclei from human osteosarcoma cells and the generation of hybridomas. In combination with immunofluorescence assays, *in situ* cell fractionation showed that this antigen was also associated with the nuclear matrix. Western blotting of electrophoresed proteins from crude nuclear fractions led to the detection of a 55kD protein using the antibody. NEPHGE (near equilibrium pH gradient electrophoresis) analysis showed that the protein is acidic and has no charge isomers, making it distinct from the 55kD antigen previously identified (Ascoli and Maul, 1991). The gene encoding the protein was isolated by screening a λgt11 cDNA expression library and sequence analysis showed that the clone encoded a 52kD protein which was consequently named nuclear dot protein 52 (NDP52). The protein contains a predicted coiled-coil motif which is followed by a leucine zipper motif. At the C-terminal end, there are seven cysteine residues spaced such that there is sequence homology with the LIM domain which has the consensus sequence $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-21}CX_{1-3}$(C,H,D). These domains, which coordinate zinc, have been found in proteins that appear to be involved in the control of gene expression and differentiation (Freyd *et al.*, 1990). However, one of the cysteine residues and the histidine residue is not present indicating that NDP52 must coordinate zinc in a different manner to LIM domains, or possibly not at all. Therefore, as with Sp100, caution should be exercised in ascribing a transcriptional activation function to NDP52.

Whilst more ND10 proteins are being discovered and their genes cloned, the function of these domains remains obscure. That they are potentially important for normal cell function has been shown in cells expressing the PML-RARα fusion protein; treatment with RA causes the reassembly of disrupted ND10 and concomitant regression of the associated leukaemia (Section 1D9.2). It has been shown that interferon treatment increases the number of ND10 as shown by immunofluorescence detecting Sp100 and NDP52 (Guldner *et al.*, 1992; Korioth *et al.*, 1995). This arises as a result of increased mRNA levels as determined by Northern blot analysis. In addition, interferon treatment of Cos-7 cells, which do not contain NDP52 in ND10, caused NDP52 to be localised to nuclear dots in approximately 10% of cells (Korioth *et al.*, 1995). ND10 were also dramatically increased in number during heat stress (Ascoli and Maul, 1991, unpublished data). Histological analysis has shown that ND10 are upregulated in the hepatocytes of patients with inflammatory liver disease such as chronic hepatitis or primary biliary cirrhosis and in proliferating epithelia as well as in

epithelial tumours (Terris *et al.*, 1995). Whether or not the upregulation of ND10 in these latter instances is a cause or effect of the associated diseases and cellular processes is not known.

(iii) The effects of viral infection

Perhaps more of an idea as to the function of ND10 has come with studying the effects of viral infection on the known protein components. It has been shown that by 5hr post infection with HSV-1, ND10 staining, as detected by an anti-55kD protein antibody, disappears (Maul *et al.*, 1993). This has been shown to be a direct result of Vmw110 expression (Section 1D9.3). The PML protein is also redistributed during HSV-1 infection (Maul and Everett, 1994). Early experiments suggested that ND10 did not coincide with viral replication compartments, as indicated in immunofluorescence experiments using an antibody directed against the major viral DNA binding protein, ICP8; it was found that loss of the anti-55kD protein staining occurred 2hr before ICP8 aggregation in replication compartments (Maul *et al.*, 1993). More recently, however, it has been shown by *in situ* DNA hybridisation and immunofluorescence analysis that viral DNA is preferentially localised to sites which are adjacent to or overlap ND10 by 3hr post infection. As ND10 were disrupted at later times in infection with wild type virus, infection with a Vmw110 deletion mutant virus was carried out. It was found that 5hr post infection with dl1403, the Sp100 and ICP8 staining patterns were also closely adjacent or overlapping, indicating that replication compartments are closely associated with ND10 (Maul *et al.*, 1996).

HCMV infection also causes disruption of ND10 and this has been shown, as with HSV-1, to be an immediate-early protein function (Kelly *et al.*, 1995). Experiments carried out to observe the effect of adenovirus infection on ND10 showed that PML was apparently redistributed into a meshwork of fibrous structures which also contain the gene product of the adenovirus E4-ORF3 gene (Carvalho *et al.*, 1995). The E4-ORF3 gene product is a non-essential viral protein thought to be involved in the regulation of alternative splicing of viral mRNAs and also in the regulation of viral DNA synthesis (Carvalho *et al.*, 1995 and references therein). Transfection assays have shown that the adenovirus E1A protein localises to ND10 and that the (D)LXCXE sequence, which is necessary for binding pRb and the related p107 oncoproteins, is required for this localisation. The SV40 large T antigen has been shown to locate in close proximity to ND10 in similar assays. Consistent with these findings are observations made by G. Maul which show that the adenovirus and SV40 DNA genomes, like the HSV-1 DNA genome, are associated with ND10 early in infection (personal communication).

Whilst infection with different viruses results in different effects on ND10 protein relocalisation, it is evident that disruption of the normal pattern is a common consequence of nuclear DNA virus infection. In view of the fact that ND10 are

increased upon treatment with interferon (Guldner *et al.*, 1992), it is possible that their disruption during virus infection circumvents a cellular defence mechanism. As Vmw110 and the adenovirus E4-ORF3 protein are involved in the reorganisation of ND10, yet neither are essential viral proteins, it is possible that disruption of ND10 is only essential in low multiplicity infections when viral gene expression is too low to overcome cellular defence mechanisms. It is likely that the cloning of more ND10 proteins and discovery of their functions in the cell will give a clearer understanding of the role of ND10.

1D9.2 PML

(i) APL

PML is a RING finger protein which is of interest in the role that Vmw110 plays in the HSV-1 infected cell as both proteins localise to the same nuclear domains at the onset of infection (Section 1D9.3). However, PML initially became a focus of interest with the observation that its gene was involved in a chromosomal translocation (t(15;17)) which was associated with acute promyelocytic leukaemia (APL). This reciprocal translocation between the long arms of chromosomes 15 and 17, links the C-terminal portion of the retinoic acid receptor (RAR) α to the N-terminal portion of PML, which includes its RING finger domain (de Thé *et al.*, 1990; Borrow *et al.*, 1990; Kakizuka *et al.*, 1991; Goddard *et al.*, 1991). The disease is characterised by the clonal expansion of myeloid cells that are unable to differentiate into mature granulocytes. Treatment with retinoic acid (RA), a vitamin D derivative that is known to influence differentiation in a number of experimental systems (Strickland and Mahdari, 1978; Roberts and Sporn, 1984; Thalle and Eichele, 1987), stimulates differentiation of these cells leading to a complete remission (Huang *et al.*, 1988; Castaigne *et al.*, 1990).

(ii) Gene structures

RARα belongs to a superfamily of nuclear steriod/thyroid hormone receptors. There are a number of retinoic acid receptors, arising from the differential use of two promoters and alternative splicing (Petkovich *et al.*, 1987; Brand *et al.*, 1988; Evans, 1988; Green and Chambon, 1988). The proteins all have DNA binding and retinoic acid recognition domains, as well as a dimerisation interface. These domains are all retained in the fusion protein, PML-RARα, which arises as the result of the t(15;17) translocation in APL patients (de Thé *et al.*, 1991). Transcriptional transactivation of retinoic acid responsive gene promoters occurs in the presence of RA when RAR proteins form heterodimers with RXR, an auxiliary nuclear protein of similar structure. The heterodimers bind to specific target DNA sequences in responsive promoters (reviewed by Green, 1993). RXR can also heterodimerise with a number of other

nuclear receptor proteins such as the thyroid hormone receptor and the vitamin D receptor. RARα does not bind efficiently to DNA as a homodimer (Leid *et al.*, 1992).

Analysis of the translocation breakpoint on chromosome 17 led to the cloning and characterisation of the PML gene and protein (Goddard *et al.*, 1991; Kakizuka *et al.*, 1991). The gene encodes at least three isoforms arising from alternatively spliced transcripts; the largest of these is 633 residues and the isoforms have residues 27-46 or 419-466 missing. The distinctive features of the protein which are retained in PML-RARα fusion proteins are a cysteine-rich region and a potential coiled-coil α-helical region. Analysis of the sequence of the cysteine-rich region revealed that it is composed of three elements, one of which forms a RING finger, while the other two are more similar to previously characterised zinc finger DNA binding domains. This latter observation led to the suggestion that PML may be a transcription factor. Interestingly, although later immunofluorescence experiments showed that PML localises to the nucleus (Section 1D9.1(iv)), the protein does not contain a recognised nuclear localisation signal.

Translocation breakpoints in APL patients cluster in two regions of the gene, either side of an alternatively spliced exon. This is reflected in the sizes of PML-RARα fusion proteins in the immature myeloid cells of APL patients (Kastner *et al.*, 1992; Pandolfi *et al.*, 1992). Transcripts also arise from the reciprocal translocation, encoding RARα-PML products but these are not always translated (Alcalay *et al.*, 1992; Kastner *et al.*, 1992). It has therefore been concluded that that the PML-RARα fusion protein rather than the RARα-PML fusion protein is responsible for the block in differentiation of myeloid cells in APL. It has also been suggested that disruption of the RARα gene is responsible for the pathogenesis of APL rather than disruption of the PML locus. This is based on the ability of RA to induce remission in APL patients, as well as the observation that APL has been associated with a t(11;17) translocation involving the RARα receptor and a Krüppel-like zinc finger encoding gene (Chen *et al.*, 1993). However, it has more recently been shown that PML may be a growth supressor; transfection of APL-derived cells in tissue culture with a construct expressing PML resulted in supression of anchorage independent growth in soft agar and reduced tumorigenicity of the cells in nude mice (Mu *et al.*, 1994). PML was also found to supress the transformation of rat embryo fibroblasts by the cooperative oncogenes *neu* and Ha-*ras*. Cotransfection of PML and PML-RARα expression plasmids resulted in a significant reduction of the suppressor function of PML in these experiments, indicating that the fusion protein can act as a dominant negative inhibitor of PML function (Kastner *et al.*, 1992; Mu *et al.*, 1994). This means that disruption of both RARα and PML may contribute to APL pathogenesis.

(iii) Functional studies

Transient transfection assays have shown that the transactivating properties of PML-RARα fusion proteins differ from those of RARα. Transfection assays carried out in HepG2 cells showed that, in the presence of RA, RARα is a more efficient transactivator of the retinoic acid response element (RARE) β than a PML-RARα fusion protein, as indicated by the level of luciferase reporter gene expression (de Thé *et al.*, 1991). It was also shown that cotransfection of PML-RARα and RARα led to reduced luciferase expression in the presence of RA, indicating that PML-RARα interferes with the normal function of RARα. Experiments carried out in Cos-1 and HeLa cells showed that, in the presence of RA, PML-RARα could transactivate gene expression from RAREs as efficiently, or less or more efficiently than RARα depending on the nature of the target RARE (Perez *et al.*, 1993). As with the transient transfection assays carried out with Vmw110 and Vmw175 (Section 1D5), it was evident that the levels of gene expression induced could vary widely in different experimental systems and caution was needed in interpreting the results. Generally, it was found that PML-RARα repressed the basal activity of RAREs in the absence of RA, an activity which is not a property of RARα.

Chambon's group showed that PML-RARα forms homodimers via the coiled-coil region in the PML protein *in vitro* (Perez *et al.*, 1993). This region of PML is also the interface through which PML-RARα heterodimerises with PML (Kastner *et al.*, 1992). The PML-RARα protein interacts with RXR via RARα sequences and contacts DNA in a similar manner to RAR-RXR heterodimers, with one RARα DNA binding domain and one RXR DNA binding domain in contact with DNA. Immunofluorescence studies showed that the PML-RARα protein localised mainly to the cytoplasm and that it sequestered RXR (Perez *et al.*, 1993; Weis *et al.*, 1994). This observation has implications for the disruption of more than one hormonal stimulatory pathway, as RXR interacts with other hormone responsive nuclear receptors (reviewed by Green, 1993).

(iv) Nuclear localisation

The earliest immunofluorescence experiments with PML were carried out with transfected cells; PML was seen to localise to domains within the nucleus unless the RING finger was disrupted, in which case PML staining was diffuse within the nucleus (Kastner *et al.*, 1992). The PML-RARα fusion protein was found in both the nucleus and cytoplasm but was located in the PML domains in the presence of RA. Kastner *et al.* suggested that PML might be colocalising with ribonucleoprotein particles, based on its homology with Ro/SSA, the 52kD RING finger component of ribonucleoprotein particles which localises in a speckled pattern in the nucleus (Ben-Chetrit *et al.*, 1988). It has since been found, however, that PML localises to distinct nuclear structures called ND10, Kr bodies or PODs which contain a number of cellular proteins (Ascoli and

Maul, 1991; Kastner *et al.*, 1992; Xie *et al.*, 1993; Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994) (Section 1D9.2). Two groups have since shown that in APL derived cell lines, treatment with RA causes the cytoplasmic PML-RARα fusion protein to be located to ND10 in the nucleus, while RXR returns to its normal distribution (Koken *et al.*, 1994; Weis *et al.*, 1994). Weis *et al.* (1994) have also shown that in APL cells, one of the ND10 antigens, Sp100, is located in abnormal protein complexes with the PML-RARα fusion protein and that treatment with RA relocated this protein to the nucleus also.

(v) How does APL develop?

In spite of much investigation into APL, the role played by the PML-RARα fusion protein is far from being completely understood, and even less so is the mechanism by which RA induces differentiation of diseased myeloid cells. However, it is likely that the reassembly of ND10 may play a role in the remission seen in APL patients treated with RA (Weis *et al.*, 1994). A number of different mechanisms could account for the block in differentiation of APL cells. Firstly, the ND10 antigens that are mislocated may be prevented from acting in the normal way, or their activity may be redirected. There is evidence for the former scenario regarding PML; the binding of PML to PML-RARα disrupts the supressive activity that PML has on cell growth (Kastner *et al.*, 1992; Mu *et al.*, 1994). Secondly, sequestration of RXR in the cytoplasm of APL cells is likely to have a number of knock-on effects. In connection with this, it has been shown that vitamin D_3 can induce differentiation of APL cells (reviewed by DeLuca, 1988; Reichel and Norman, 1989). Chambon's group have shown that large amounts of PML-RARα in transfected cells inhibits the transcriptional activation mediated by vitamin D_3 (Perez *et al.*, 1993). Thirdly, PML could act on its own, as it has been demonstrated that it represses some RAREs (Kastner *et al.*, 1992). The location of the PML-RARα protein to ND10 in the presence of RA could remove this activity. Possibly, the key to understanding the pathogenesis of APL will come with a clearer knowledge of the function of ND10 in the cell.

1D9.3 Vmw110 and ND10

(i) Infected cells

An investigation of IE1 deletion mutant viruses determined that Vmw110 was responsible for the apparent disappearance of ND10 by 5hr post infection in all cell types tested; ND10 remain undisturbed until later in infection in cells infected with the Vmw110 deletion mutant virus dl1403 (Maul *et al.*, 1993). ND10 are still present at 6hr post infection with dl1403 and by 16hr post infection ND10 proteins form hollow spheres and nuclear tracks of unknown significance (Maul and Everett, 1994). Immunofluorescence experiments also showed that Vmw110 localised to ND10 early in

infection (before 4hr), prior to ND10 disappearance. That Vmw110 was sufficient for this process was shown in an experiment with an adenovirus recombinant where the IE1 gene was placed under the control of the adenovirus major late promoter. It is known that ND10 proteins are redistributed and not degraded from Western blot analyses of infected cell extracts (Maul *et al.*, 1993). This is supported by immunofluorescence experiments which have shown that PML and other ND10 antigens are relocated in part to the nuclear membrane (Everett and Maul, 1994; Maul and Everett, 1994). During wild type HSV-1 infection, Vmw110 becomes increasingly diffusely spread through both the nucleus and cytoplasm following its transient localisation to ND10. It is possible that other ND10 proteins are similarly re-distributed into both nucleus and cytoplasm (Maul and Everett, 1994).

It has been shown that the regions of Vmw110 responsible for the redistribution of ND10 proteins involve the RING finger and C-terminal residues (Everett and Maul, 1994; Maul and Everett, 1994). Experiments with the RING finger deletion mutant viruses FXE (Δ106-150) and D22 (Δ162-188) showed that the mutant Vmw110 proteins localised to ND10 for a longer period than wild type Vmw110 protein; late in infection there is a general disruption of nuclear architecture and the RING finger deficient Vmw110 proteins were sometimes seen in long fibrous structures, which may be related to the PML tracks seen in dl1403 infected cells. By 16hr post infection with D22, Vmw110 was found in precise spherical structures suggestive of membranous origin. Under the electron microscope membranous whorls were seen, containing cytoplasmic material such as rough endoplasmic reticulum and it was suggested that they arose as a result of major invagination of the nuclear envelope. Direct evidence that these structures contain Vmw110 or ND10 antigens was not obtained but confocal microscopy of sections through the nucleus showed that ND10 antigens were present at the nuclear membrane.

Experiments with the C-terminal deletion mutant viruses D13 (Δ633-680) and D14 (Δ680-720) showed that, at early times in infection, the mutant Vmw110 proteins were diffusely spread through the nucleus, supporting the results of transfection assays (Everett, 1988a; Chen *et al.*, 1991). The D13 and D14 deletions cause only moderate reductions in Vmw110 function in transfection assays except in synergistic activation with Vmw175 (Section 1D5), and deletion mutant virus D14 is able to reactivate latent virus in an *in vitro* latency system (Harris *et al.*, 1989). The apparent non-essential nature of the C-terminal region in transfection assays may be explained by the eventual loss of ND10 in cells infected with the D13 and D14 viruses. It is evident that the RING finger domain of Vmw110 is necessary for ND10 disruption but that residues at the C-terminal end are required for the initial interaction of Vmw110 with ND10.

(ii) Transfected cells

Expression of Vmw110 in transfected cells resulted in weaker PML staining with 5E10 antibody and in some instances PML staining disappeared altogether (Everett and Maul, 1994). This indicated that PML was redistributed or that a conformational change occurred which resulted in occlusion of the 5E10 epitope. It was evident also from these assays that C-terminal residues of Vmw110 are necessary for the localisation of Vmw110 to ND10 and that the RING finger is needed for their disruption. Interestingly, confocal lasar scanning microscopy showed that Vmw110 missing the C-terminal 180 residues was strongly associated with the nuclear membrane. Experiments were also carried out to investigate the nature of the C-terminal domain of Vmw110 by generating fusion proteins with β-galactosidase. Transfection with a plasmid construct expressing the C-terminal 300 residues of Vmw110 fused to β-galactosidase showed that this protein was distributed in a micropunctate pattern within the cell, but it was difficult to see whether or not colocalisation with PML occurred, as indicated by the use of monoclonal antibody 5E10. A related fusion protein with the C-terminal 228 residues of Vmw110 was diffusely distributed throughout the cell and, in a significant number of cells expressing this fusion protein, PML was also found in the cytoplasm. This indicates that by itself, the C-terminal end of Vmw110 can have a dramatic effect on PML and ND10 structures. PML was also seen in the cytoplasm of cells transfected with a plasmid expressing a mutant Vmw110 protein lacking the nuclear localisation signal (Δ424-549). The significance of these observations is unknown but Vmw110 and PML may both be involved in nuclear and cytoplasmic functions, given that Vmw110 is found in the cytoplasm as infection proceeds.

The results of Hayward's group are, to an extent, contrary to those of Everett and Maul (Everett and Maul, 1994; Maul and Everett, 1994; Mullen *et al.*, 1995). In agreement with other groups, they found Vmw110 proteins truncated at residues 519 and 712 gave predominantly diffuse nuclear staining in immunofluorescence experiments but truncations at residues 312 and 364 gave mixed cytoplasmic and nuclear staining which was more punctate in character. They found that shorter N-terminal versions of Vmw110 were unstable and so, to further define the N-terminal sequences of Vmw110 responsible for the punctate property, fusions with Vmw175 were made. In their immunofluorescence assays, Hayward's group found that the diffuse staining pattern of Vmw175 was altered to a punctate nuclear staining in cells transfected with a construct expressing Vmw110 residues 1-244 fused to Vmw175 residues 84-1298. They suggested that the punctate staining of Vmw110 is a property of the N-terminal domain but that C-terminal residues contribute. However, Everett and Maul (1994) have expressed Vmw110 residues 1-262 stably in transfection assays and immunofluorescence experiments have shown that this protein is diffusely spread throughout the cytoplasm. It is likely, therefore, that fusion of Vmw110 and Vmw175

sequences gives rise to proteins which exhibit characteristics that are not representative of the individual proteins.

1D10 Do Vmw110 and Vmw175 interact physically?

The question as to whether or not Vmw110 and Vmw175 interact physically is pertinent given that these proteins activate gene expression synergistically. The experimental approaches taken to investigate their potential interaction have included immunofluorescence assays as well as more direct molecular biological approaches. The results of immunofluorescence assays have often differed between different groups and have, in some cases, yielded contradictory results. This is reminiscent of the transient transfection assay systems (Section 1D5) and may only be a reflection of the various experimental conditions used, indicating that the results from any one group should be interpreted with care.

Gelman and Silverstein (1986) showed that Vmw110 was located in the nucleus in punctate domains in transfected cells and that Vmw175 was diffusely spread throughout the nucleus. Transfection with constructs expressing Vmw110 and a *ts* Vmw175 protein at the permissive temperature showed that both Vmw110 and *ts*Vmw175 were located throughout the nucleoplasm. At the non-permissive temperature both proteins were located in nuclear granules. The conclusion drawn from this was that Vmw175 is necessary for the diffuse distribution of Vmw110 within the nucleus, but that it has no effect on the nuclear localisation of Vmw110. In contrast, Knipe and Smith (1986) observed that in cells infected with a different Vmw175 *ts* mutant at the non-permissive temperature, Vmw110 and Vmw175 were located in the cytoplasm. As Vmw110 can localise to the nucleus in the absence of Vmw175 (Gelman and Silverstein, 1986), it was concluded that Vmw175 had a negative effect on the nuclear localisation of Vmw110. Knipe and Smith (1986) also observed that in the presence of *ts*Vmw175, the nuclear localisation of ICP8 was affected. It was concluded from this either that Vmw175 interacts with Vmw110 and also with ICP8 (for which there is very little evidence) or that Vmw175 interacts with a transport protein that is involved in the nuclear localisation of both Vmw110 and ICP8. The evidence for a direct interaction between Vmw110 and Vmw175 in these experiments is weak; even though double label immunofluorescence experiments were not carried out, it can be seen that when Vmw110 and Vmw175 are present in the cytoplasm, Vmw110 is present in perinuclear granules whereas Vmw175 is in diffusely spread punctate granules.

In direct contrast to the observations of Knipe and Smith (1986), Hayward's group have shown in transfection assays that Vmw110 can relocate a cytoplasmic form of Vmw175 to punctate granules in the nucleus (whether or not these were ND10 was not investigated) (Mullen *et al.*, 1995). Additionally, a mutant Vmw110 protein located in punctate domains in the cytoplasm was shown to locate Vmw175 to the same domains,

although some remained diffusely distributed in the nucleus. From these experiments it was concluded that Vmw110 is the dominant protein affecting the localisation of Vmw175. Interestingly, it was also noted that Vmw110 and Vmw175 colocalised to punctate domains in the nucleus of Vero cells and not in 293 cells. Thus, the results of these experiments may be cell type dependent. However, Knipe and Smith's experiments were also carried out in Vero cells so the different results cannot be explained by differences in the cell types used in this case. It is, however, possible that these proteins behave differently depending on whether or not they are in the viral context. In addition, results obtained from transfection assays can be unrealistic in that the amounts of viral protein present in cells can be far higher than in infected cells.

Schaffer's group has also examined the localisation of Vmw110 in the cell, with particular reference to the effects of Vmw175 and Vmw63 on its distribution. In contrast to the results of Hayward's group, which inferred that Vmw110 was the more dominant of the two proteins in affecting the cellular localisation patterns, Schaffer's group suggested that Vmw175 was the more dominant of the two (Zhu *et al.*, 1994). They attributed the presence of Vmw110 in the cytoplasm by 5hr post infection with a Vmw175 null mutant as a result of the absence of the nuclear localising activity of Vmw175. However, the presence of Vmw110 in the cytoplasm of infected cells by approximately 5hr post infection is observed in infections with wild type virus (the exact time-course is cell type dependent) (Everett and Maul, 1994). Vmw63 appeared to have a negative effect on the nuclear localisation of Vmw110 as the latter was found localised more strongly to the nucleus in infection with a Vmw63 mutant virus than in wild type infection. These effects could be indirect and do not necessarily mean that Vmw110 is interacting with Vmw175 or Vmw63. To an extent, these results were also seen in transfection assays, particularly when large amounts of DNA (5-10μg per 60mm cell culture dish) were used in the assays. Based partly on these results, and also on the observations of the ability of Vmw110 to transactivate gene expression in transient transfection assays, it was concluded that Vmw110 and Vmw175 may interact physically.

Schaffer's group have attempted a more rigorous investigation of the potential physical interaction between Vmw110 and Vmw175 using molecular biological techniques (Yao and Schaffer, 1994). In Far Western blotting experiments carried out on infected cell extracts using [^{35}S]methionine-labelled *in vitro* translated Vmw110 or [^{35}S]methionine-labelled *in vitro* translated Vmw175 as probes, they showed that Vmw110 interacted with Vmw175 and with itself. Vmw110 residues 1-244, 1-394, 395-616 and 395-775 were expressed as GST fusion proteins and it was found that residues 395-775 interacted with [^{35}S]methionine-labelled *in vitro* translated Vmw175. All of the GST-Vmw110 fusion proteins bound [^{35}S]methionine-labelled *in vitro* translated Vmw110. This latter observation throws doubt regarding the specificity of

binding in the experiments with the GST fusion proteins. That Vmw110 exists as a dimer or higher order multimer has been established (Everett, 1991b; Chen *et al.*, 1992), but it is unlikely that all of the Vmw110 domains expressed as fusion proteins in these experiments would contain dimerisation interfaces. Additionally, in order to avoid the danger of non-specific protein:protein interactions arising, it would have been of greater value to have carried out the assay in the presence of other proteins. In this context, it has not yet been shown that Vmw175 is immuneprecipitated with Vmw110 and *vice versa*. Whilst this does not necessarily mean that these proteins do not interact, as the evidence from immunofluorescence studies is also not conclusive, caution should be taken in ascribing that these two proteins bind specifically to each other. It is equally possible that the synergistic transactivation properties of Vmw110 and Vmw175 arise as a result of Vmw110 displacing a cellular protein from ND10 which interacts with Vmw175 in such a way as to enhance its transactivating potential.

1D11 Mapping the Vmw110 multimerisation domain

Various groups have attempted to map a domain within Vmw110 through which self interactions occur to form dimers or higher order multimers. The multimeric nature of Vmw110 was shown initially by the sedimentation rate of purified Vmw110 on glycerol gradients compared to that of known standards (Everett *et al.*, 1991b). Vmw110 isolated from mammalian cells infected with an adenovirus recombinant was also shown to exist as a multimer in solution based on its elution profile from a Sephacryl-S400 column (Chen *et al.*, 1992b). This group also found that some mutant forms of Vmw110 could negatively dominate the transactivating ability of Vmw110 in transient transfection assays. They proposed that this was likely to be due to the formation of hetero-multimers containing both mutant and wild type Vmw110, with the mutant protein inhibiting the normal activity of the wild type protein. On this basis they mapped a multimerisation domain to the N-terminal end of Vmw110. However, such indirect assays cannot be taken as strong evidence for a multimerisation domain within this part of Vmw110.

The existence of dominant negative mutant Vmw110 proteins was also observed by Weber and Wigdahl (1992). Rather than suggesting that this was a result of Vmw110 multimerisation, they proposed that Vmw110 may mediate its promiscuous transactivation property by interacting with some very general component of the host cell transcription machinery, so competing with the mutant protein. It is also possible that the mutant Vmw110 protein occludes the Vmw110 binding sites in the cell. Interestingly, it has been shown that a dominant negative mutant Vmw110 protein is expressed in infected cells when the second intron, which contains an in frame stop codon, is not excised (Everett *et al.*, 1993a). The 262 residue product contains residues 1-241 of Vmw110 plus additional intron coded residues. It is possible that expression

of this product is dependent upon cellular factors and that production of large amounts of the 262 residue protein could lead to a generalised suppression of viral gene expression and aid the establishment of latent infection.

Hayward's group have identified a multimerisation domain in the C-terminal end of Vmw110 (Ciufo *et al.*, 1994). They constructed a plasmid which expressed a Vmw175 epitope tagged C-terminal domain of Vmw110 (Vmw175 residues 1-383/Vmw110 residues 313-775) and found that the hybrid protein was located diffusely in the nucleus, but in the presence of wild type Vmw110 it localised to punctate granules. That Vmw110 contains a multimerisation domain cannot be concluded on the strength of this experiment alone as hybrid proteins may have properties which do not reflect those of the individual proteins (Section 1D9). However, Hayward's group have shown the formation of stable mixed-subunit oligomers by coimmune precipitation of different sized, *in vitro* translated Vmw110 polypeptides which were haemagglutinin epitope tagged. It was found that the oligomers formed only when the subunits were cotranslated and not when they were simply mixed together, which indicates the involvement of a high affinity subunit interaction mechanism with a low dissociation rate. The presence of oligomers of higher order than dimers was ascertained in glutaraldehyde cross-linking experiments. Using the coimmune precipitation assay, the N-terminal boundary of the multimerisation domain was mapped to between residues 555-617 and the C-terminal boundary to between residues 712-767. Experiments in this Thesis have also mapped a multimerisation domain to the C-terminal end of Vmw110 (Section 1B). Although the evidence for the presence of a multimerisation domain in the C-terminal end of Vmw110 is stronger than that for a domain in the N-terminal end, the presence of a N-terminal multimerisation domain cannot be excluded. It is possible that there is more than one multimerisation interface on the surface of the intact and correctly folded protein.

1D12 Aims of the work presented in this Thesis

The main aim of the work presented in this Thesis was to identify and characterise cellular proteins which interact with the HSV-1 IE protein Vmw110. At the start of the project there was evidence that Vmw110 is implicated in latency (Section 1D7), a process which almost certainly involves some kind of virus:cell interaction, and immunofluorescence experiments had shown that some C-terminal mutant Vmw110 proteins were unable to interact with ND10 in the nucleus. These observations indicated that Vmw110 may interact with one or more cellular proteins. It was therefore an appropriate time in the history of Vmw110 investigations to attempt to uncover its mode of action in the viral life cycle by looking at cellular proteins with which it interacts.

Initial experimental work involved the expression of C-terminal residues of Vmw110 as GST fusion proteins from GEX plasmids, and their use in 'pull-down' assays with whole cell extracts, which would minimise the observance of non-specific interactions. It was proposed that any cellular protein which strongly and specifically interacted with Vmw110 residues would be purified, subjected to amino acid analysis and the encoding cDNA cloned by a PCR method.

An additional aim of the project was to show biochemically the multimeric nature of the C-terminal domain of Vmw110. The relevant Vmw110 sequences were removed from the GEX plasmids, expressed in a T7 system and purified by ion exchange chromatography. These investigations served as a means of partially separating sequences involved in interacting with a cellular protein of 135kD from those involved in multimerisation.

CHAPTER 2 MATERIALS AND METHODS

2A Materials

2A1 Plasmids

The following plasmids were provided by the acknowledged authors:

p111: (Everett, 1987a) This encodes the entire Vmw110 protein and was derived from plasmid pJR3 through an intermediate p110del7 (Perry *et al.*, 1986).

p110A8: (Everett, 1987a) This encodes the entire Vmw110 protein with an 8bp HindIII linker inserted at the AvaI site at position 4699 (residue 647).

p110E2: (Everett, 1987a) This encodes the entire Vmw110 protein with a 12bp EcoRI linker inserted at the HaeIII site at position 4930 (residue 723).

p110E35: (Everett, 1987a) As p110E2 but with the linker inserted at position 4849 (residue 696).

p110R5: (Everett, 1987a) As p110E2 but with the linker inserted at the RsaI site at position 4801 (residue 680).

p110E52: (Everett, 1987a) As p110E2 but with the linker inserted at the HaeIII site at position 4543 (residue 594).

p110E58: (Everett, 1987a) As p110E2 but with the linker inserted at the HaeIII site at position 4660 (residue 633).

p110D12: (Everett, 1988a) This encodes the Vmw110 protein with residues 594-633 deleted.

p110D14: (Everett, 1988a) This encodes the Vmw110 protein with residues 680-720 deleted.

p110FXE: (Everett, 1988a) This encodes the Vmw110 protein with residues 106-150, spanning the zinc binding domain, deleted.

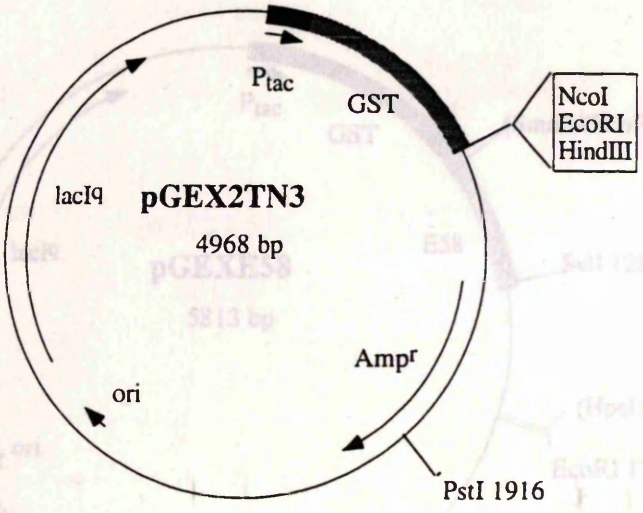
p110E52X: (R. Everett, unpublished) This encodes a Vmw110 protein truncated at residue 594.

The pGEX series of plasmids were designed for inducible expression in bacteria of fusion proteins containing a polypeptide of interest linked to glutathione-S-transferase.

pGEX2TN3: (M.Hughes; Meredith *et al.*, 1994) This was derived from the commercial vector pGEX2T (Pharmacia) by inserting a DNA fragment containing NcoI-EcoRI-HindIII sites between the BamHI and EcoRI sites downstream of the GST coding sequence, thus removing the original BamHI, SmaI and EcoRI sites (Figure 2A1(i)).

The p110 series of plasmids were derived from p111. They contain the IE1 gene region including the IE1 promoter, intron and 3' processing signals in a pUC9 background (Perry et al., 1986; Everett, 1987a; 1988a).

Figure 2A1(i)



pGEX2T

```
P  R  G  S  P  G  I  H  R  D STOP
CCGCGTGGATCCCCGGGAATTCATCGTGACTGACTG
      BamHI  SmaI  EcoRI
```

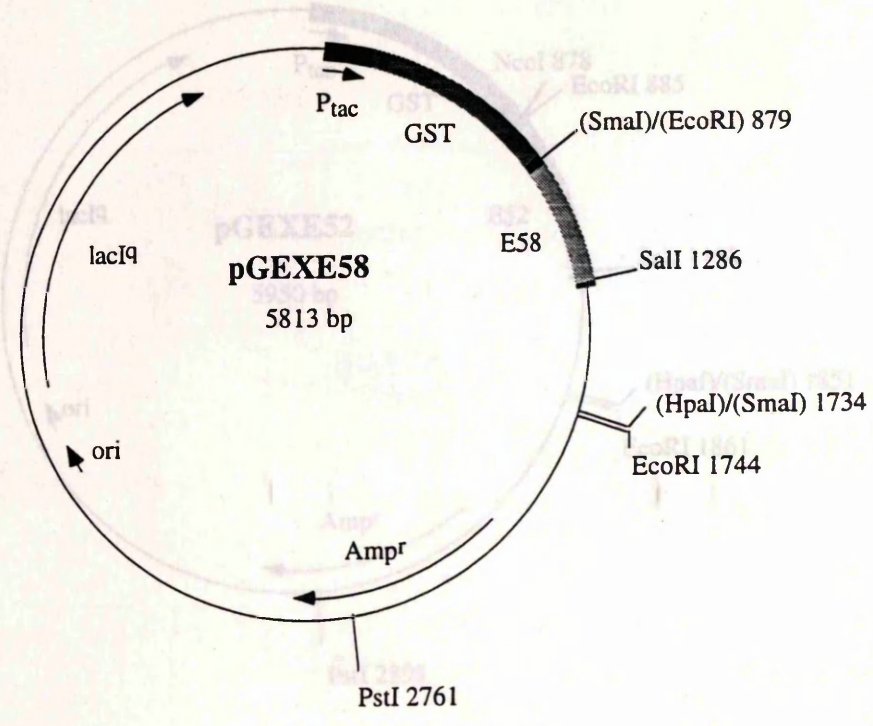
Linkers

```
5'                                  3'
GATCGACCATGGCTAGAATTCGGAAGCTT
    CTGGTACCGATCTTAAGCCTTCGAATTAA
      NcoI      EcoRI     HindIII
```

pGEX2TN3

```
P  R  G  S  T  M  A  R  I  R  K  L  N  S  S STOP
CCGCGTGGATCGACCATGGCTAGAATTCGGAAGCTTAATTCATCGTGACTGA
        NcoI      EcoRI     HindIII
```

Figure 2A1(ii)



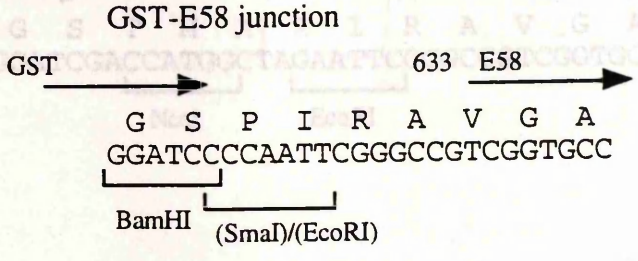
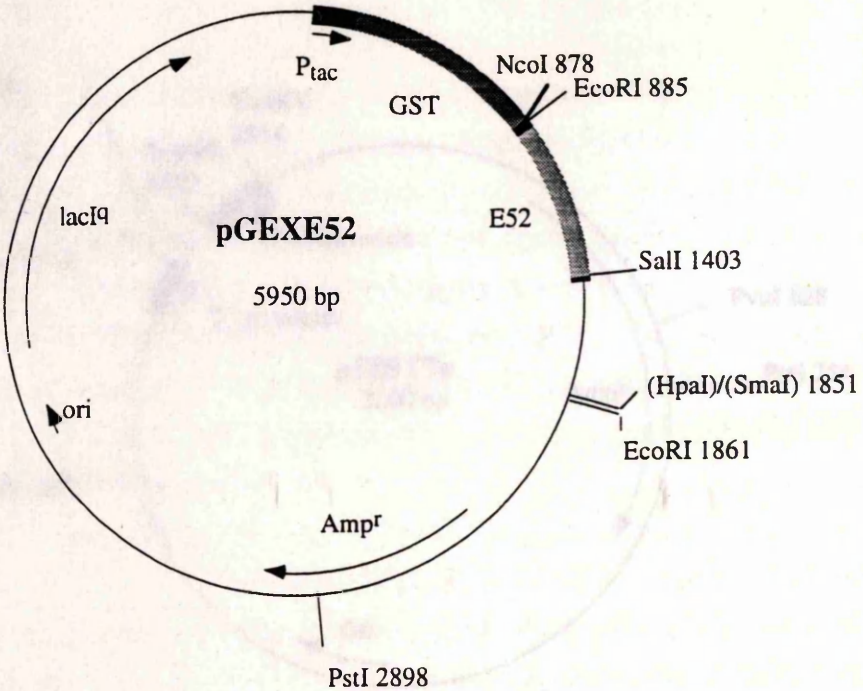


Figure 2A1(iii)



GST-E52 junction

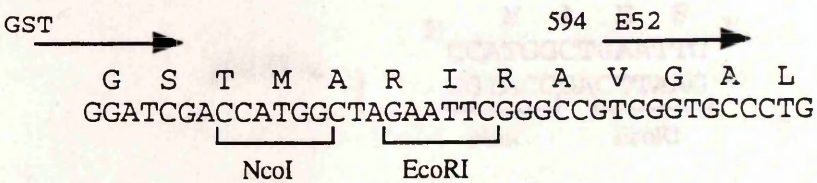
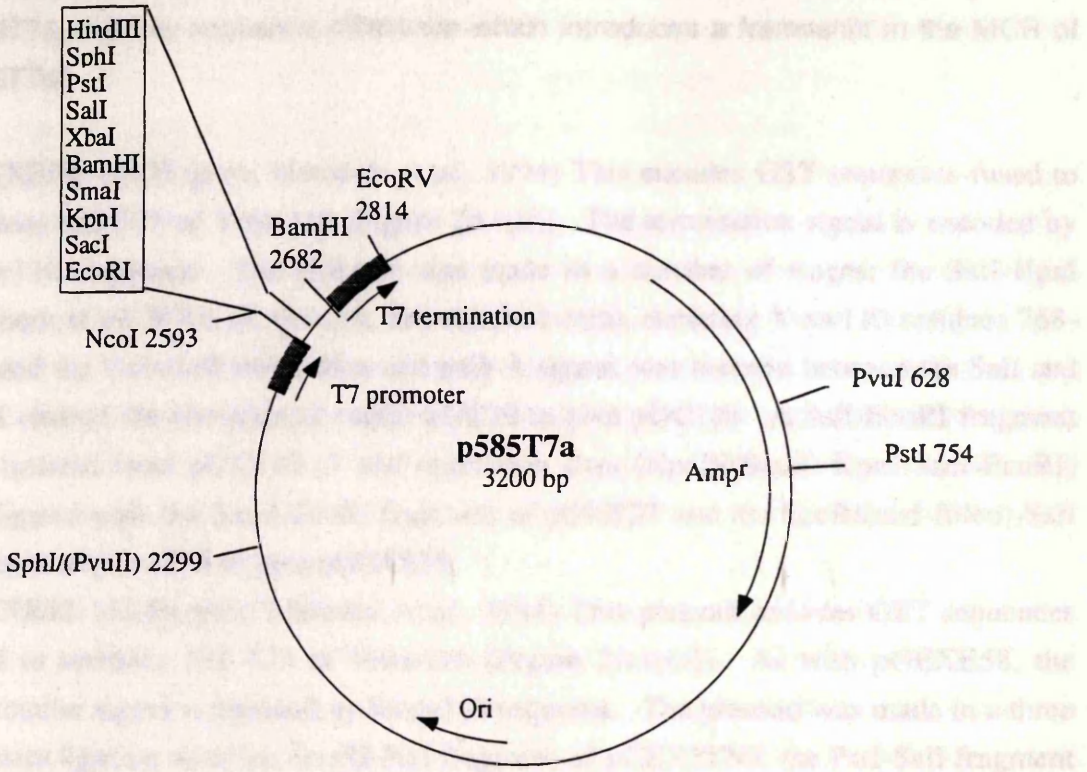


Figure 2A1(iv)



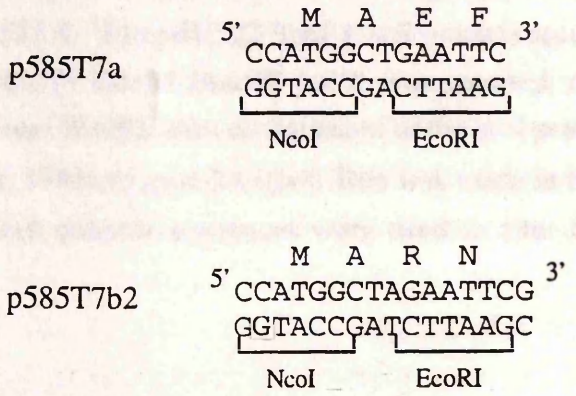


Figure 2A1 Structure of plasmids pGEX2TN3, pGEXE58, pGEXE52, p585T7a and p585T7b2. In all cases, restriction sites relevant to the construction or further use of the plasmids are shown; (i) Plasmid pGEX2TN3 was constructed from the commercial vector pGEX2T (Pharmacia) by digestion with BamHI and EcoRI and insertion of the linker sequences shown. (ii) Plasmid pGEXE58 was constructed as described in the text, such that translational initiation occurs at the GST ATG downstream of the P_{tac} promoter, and the termination signal is encoded by Vmw110 sequences. The sequence at the GST-E58 junction is shown. The SalI-HpaI segment is composed of IE1 sequences including the Vmw110 termination codon and 3' flanking sequences. (iii) Plasmid pGEXE52 was constructed as described in the text. The GST-E52

junction sequence is shown. (iv) This shows the structure of the cloning vector p585T7a and the sequence difference which introduces a frameshift in the MCR of p585T7b2.

pGEXE58; (M.Hughes; Meredith *et al.*, 1994) This encodes GST sequences fused to residues 633-775 of Vmw110 (Figure 2A1(ii)). The termination signal is encoded by Vmw110 sequence. The plasmid was made in a number of stages: the SalI-HpaI fragment of pT7FXE (R. Everett, unpublished data), encoding Vmw110 residues 768-775 and the Vmw110 stop codon and poly A signal, was inserted between the SalI and SmaI sites of the commercial vector pUC19 to give pUC110. A SalI-EcoRI fragment was isolated from pUC110 (3' end restriction sites (HpaI)/(SmaI)-KpnI-SacI-EcoRI) and ligated with the SmaI-EcoRI fragment of pGEX2T and the EcoRI(end-filled)-SalI fragment of p110E58 to give pGEXE58.

pGEXE52: (M.Hughes; Meredith *et al.*, 1994) This plasmid encodes GST sequences fused to residues 594-775 of Vmw110 (Figure 2A1(iii)). As with pGEXE58, the termination signal is encoded by Vmw110 sequence. The plasmid was made in a three fragment ligation with the EcoRI-PstI fragment of pGEX2TN3, the PstI-SalI fragment of pGEXE58 and the EcoRI-SalI fragment of p110E52.

p585T7a: (Tyler, 1994) (Figure 2A1(iv)) This is a cloning vector used for expression of peptide sequences from a T7 promoter. It was originally derived from pET8c (Studier *et al.*, 1990) via p585.4. The pBR322 SphI-PvuII vector sequences were removed from p585.4 and the pUC19 EcoRI-HindIII MCR was inserted, with the aid of adaptors, between the NcoI and HindIII sites downstream of the ϕ10 promoter.

p585T7b2: (Tyler, 1994) (Figure 2A1(iv)) This was made in the same way as p585T7a except that different adaptor sequences were used to alter the reading frame of the MCR.

2A2 Enzymes

Restriction enzymes were obtained from Boehringer Mannheim or New England Biolabs. DNaseI, RNase A and lysozyme were purchased from Sigma and T4 polynucleotide kinase, T4 DNA ligase, calf intestinal phosphatase, *E.coli* DNA polymerase I Klenow fragment and proteinase K from Boehringer Mannheim. PCR was carried out using cloned *Pyrococcus furiosus* DNA polymerase obtained from Stratagene or *Thermus aquaticus* DNA polymerase obtained from Boehringer Mannheim. Reverse Transcriptase Moloney-Murine Leukaemia Virus was also obtained from Boehringer Mannheim.

2A3 Synthetic oligonucleotides

Oligonucleotides were synthesised on site using a Biosearch model 8600 DNA synthesiser or a Cruachem PS250 automated synthesiser by Dr. J. McLauchlan, Mr. F. Van Deursen, Miss S. Fitzpatrick, Mr. J. McGeehan or Mr. R. Reid.

2A4 Bacteriophage λ library

The bacteriophage λ expression library screened in the studies described in this Thesis was obtained from Professor P. Chambon of the Institut de Chimie Biologique, Strasbourg. It was derived by random priming of HeLa cell polyA+ RNA and the products were cloned into the λ ZAPII vector (Stratagene) using EcoRI oligonucleotide linkers.

2A5 Bacteria (*E.coli* strains)

The *E.coli* strain DH5α (F'/*endA1 hsdR17* ($r_k^-m_k^+$) *supE44 thi1 recA1 gyrA* (Nal^r) *relA1 Δ(lacZYA-argF)U169 (Φ80dlacΔ(lacZ)M15)* was used for maintenance and propagation of plasmid DNA. Strain BL21(DE3)pLysS (F^- *ompT* $r^-_Bm^-_B$) (Studier *et al*, 1990) was used for expression of proteins using pT7 vectors: the integrated lysogenic λ bacteriophage DE3 has an IPTG inducible promoter from which T7 RNA polymerase is expressed and the plasmid pLysS encodes T7 lysozyme and also a gene conferring chloramphenicol resistance. Protein expression from pGEX plasmids utilised strain BL21. Strain TG1 (*supE hsdΔ5 thi Δ(lac-proAB) F'(traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*) was used for blue-white screening of some cloned PCR products. The *E.coli* strain provided by the Invitrogen TA Cloning Kit was INVαF' (endA1 recA1 hsdR17(r^km^{+k}) supE44 λ^- thi-1 gyrA rel a1 Φ80lacZαΔm15Δ(lacZYA-argF) deoR+ F'). The bacteriophage λ library (Section 2A4) was plated out and amplified in the strain XL-1 Blue MRF' (*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA gyr96 relA1 lac λ-*): the F' episome encodes pili which allows infection by filamentous bacteriophage and the *laq*I^qZΔM15 mutation which enables blue/white selection of recombinant colonies/plaques on LB agar plates supplemented with IPTG and X-gal. It also carries a tetracycline resistance gene.

2A6 Bacterial culture media

The DH5α strain was grown in L-broth (10g NaCl, 10g Bactopeptone, 5g yeast extract in 1l water, pH 7.5) and all other strains in 2YT broth (5g NaCl, 16g Bactotryptone, 10g yeast extract in 1l water). Agar plates were made with 1.5% (w/v) agar in L-broth. Top agar contained 10g Bactotryptone, 8g NaCl and 8g agar in 1l water. Where necessary, media and LB agar plates were supplemented with antibiotics: 100μg/ml ampicillin for bacteria harbouring pUC and pBR322 derived plasmids (including pT7 and pGEX

series), 25µg/ml chloramphenicol for strains harbouring the pLysS plasmid and 15µg/ml tetracycline for the *E.coli* XL-1 Blue MRF' strain.

2A7 Cells and tissue culture media

All cell culture media were obtained from Gibco.

BHK-21 C13 cells, a fibroblastic line derived from baby hamster kidney cells (MacPherson and Stoker, 1962), were grown in Glasgow Modified Eagle's Medium (GMEM) supplemented with 10% newborn calf serum, 10% tryptose phosphate broth, 100 units/ml penicillin and 100µg/ml streptomycin.

WS HeLa cells (obtained from Dr. W. Schaffner, Zürich), an epithelial cell line, were grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 2.5% foetal calf serum, 2.5% newborn calf serum and antibiotics as above.

HFL cells (obtained from Flow Laboratories) are a fibroblastic line derived from human foetal lung tissue and were grown in DMEM supplemented with 10% foetal calf serum supplemented with antibiotics as above.

U-2 OS cells (obtained from American Type Culture Collection), a human osteogenic sarcoma line, were grown in McCoy's 5a medium supplemented with 15% foetal calf serum and antibiotics as above.

ND7 cells were obtained from Dr. S.M. Brown by permission of Professor D. Latchman (Department of Molecular Pathology, University College London): they are a neuronal line derived from fusion of neuroblastoma cells with primary rat sensory neurons (Wood *et al*, 1990). They were grown in Leibovitz medium supplemented with 10% foetal calf serum, 0.35% glucose, 7.5% sodium bicarbonate and antibiotics as above. The cells were differentiated by seeding in 50% DMEM, 50% Ham's nutrient mix F12, 250ng/ml bovine insulin, 5µg/ml human transferrin, 30nM sodium selenite and antibiotics as above for 3-4 days.

2A8 Antisera and monoclonal antibodies

The anti-Vmw110 mouse monoclonal antibodies 11060, 10810 and 10503 were obtained from Dr. A. Cross. MAb 11060 recognises an epitope between amino acids 20-105, 10810 an epitope between residues 594-633, and 10503 and epitope between residues 633-775 (Everett *et al*, 1993a).

The monoclonal antibodies, MAb 1150 and MAb 5E10, recognising 55kD and 126kD ND10 antigens respectively, were obtained from Professor G. Maul (Wistar Institute, Philadelphia).

Protein A horse radish peroxidase (HRP) conjugate, anti-mouse IgG whole molecule peroxidase conjugate, TRITC conjugated goat anti-rabbit and FITC conjugated goat anti-mouse immunoglobulin were supplied by Sigma.

2A9 Radiochemicals

All radiochemicals were purchased from Amersham at the following specific activities:

α^{32}P dATP	3,000Ci/mmol (10μCi/μl)
γ^{32}P ATP	5000Ci/mmol (10μCi/μl)
^{35}S L-methionine	~800Ci/mmol (15μCi/μl)

2A10 Solutions

10X agarose gel loading buffer: 1 X TBE, 1% SDS, 50% glycerol, 1mg/ml bromophenol blue

10X CAPS: 100mM CAPS, pH to 11 with NaOH

Coomassie stain: 0.2% Coomassie Brilliant Blue in methanol:water:acetic acid in a 50:50:7 ratio

50X Denhardt's solution: 1% polyvinylpyrrolidone, 1% BSA, 1% ficol

20X dNTPs: 1mM of each of dATP, dCTP, dGTP, dTTP

Electroblotting Buffer: 1X CAPS, 10% methanol

Extract Buffer: 50mM HEPES (pH 7.5), 100mM NaCl, 0.4% CHAPS, 1mM PMSF, 0.1mM DTT

Formamide dyes: 10mM EDTA, 1mg/ml xylene cyanol FF, 1mg/ml bromophenol blue in formamide

Gel soak I: 1.5M NaCl, 0.5M NaOH

Gel soak II: 1.5M NaCl, 0.5M Tris.Cl, 1mM EDTA, pH 7.2

Glycine SDS PAGE buffers:

(i) 3X gel loading buffer: 29% SGB, 6% SDS, 2M 2-ME, 29% glycerol, 1mg/ml bromophenol blue

(ii) RGB (resolving gel buffer): 1.5M Tris.HCl, 0.4% SDS, pH 8.9

(iii) SGB (stacking gel buffer): 0.5M Tris.HCl, 0.4% SDS, pH 6.7

(iv) Tank buffer: 0.05M Tris, 0.05M glycine, 0.1% SDS

Guanidine Thiocyanate solution: 4M guanidine thiocyanate, 0.02M N-lauryl-sarcosinate (sodium salt), 0.024M Na citrate, 0.7% 2-ME

2X HBS: 280mM NaCl, 50mM HEPES, 1.5mM Na_2HPO_4, pH to 7.12 with NaOH

10X hybridisation buffer: 1.5M KCl, 0.1M Tris.HCl (pH 8.3), 10mM EDTA

5X kinase buffer: 350mM Tris.HCl (pH7.5), 50mM $mgCl_2$, 25mM DTT

5X ligase buffer: 250mM Tris.HCl (pH 7.6), 50mM $MgCl_2$, 5mM DTT, 5mM ATP, 25% PEG 8000

0.5M NaCl wash buffer:	0.5M NaCl, 50mM Tris.HCl (pH 8), 1mM EDTA, 0.5% NP40, 1mM PMSF, 0.5µg/ml leupeptin, 40µg/ml bestatin
PBS(A):	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.2
PBS-complete:	PBS(A) plus $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$ at 1g/l
Protein gel destain:	5% methanol, 7% acetic acid in water
Protein gel fix:	12.5% acetic acid, 3% ethanol in water
RNase reaction mix:	100µg/ml salmon sperm DNA, 20µg/ml RNase A in TEN 100 buffer
10X seq buffer:	100mM Tris.HCl, 100mM $MgCl_2$, pH 8.5
20X SSC:	3M NaCl, 0.3M trisodium citrate
STET:	8% sucrose, 5% triton X-100, 50mM EDTA (pH 8.0), 50mM Tris.HCl (pH 8.0)
50X TAE:	0.2M Tris, 0.05M EDTA (pH 8.0), pH to 8.0 with acetic acid
TEN 100 buffer:	100mM Nacl in TE (pH 7.5)
20X TBE:	2.5M Tris, 800mM boric acid, 54mM EDTA
TBS:	0.02M Tris, 0.5M NaCl, pH 7.5 with HCl
TE:	10mM Tris.HCl, 1mM EDTA, pH 8.0
TE(P):	1mM Tris.HCl (pH 7.5), 0.05mM EDTA
Towbin (blotting buffer):	25mM Tris.HCl, 192mM glycine, 20% methanol, pH 8.3
Tricine SDS PAGE buffers:	
(i) 3X gel loading buffer:	150mM Tris.HCl, 12% SDS, 36% glycerol, 6% BME, 1mg/ml bromophenol blue, pH 6.8
(ii) Gel buffer:	3.0M Tris.HCl, 0.3% SDS, pH 8.45
(iii) Lower tank buffer:	200mM Tris.HCl, pH 8.9
(iv) Upper tank buffer:	0.1M Tris, 0.1M Tricine, 0.1% SDS
TTBS:	TBS plus 0.05% TWEEN-20
Versene:	0.6mM EDTA in PBS(A), 0.002% phenol red

2A11 Chemicals and reagents

All chemicals and reagents were purchased from BDH Chemicals UK or from Sigma Chemical Co. unless otherwise stated in this Section or in the Methods Section:

Beecham Research: Ampicillin

Bio-Rad: TEMED, ammonium persulphate, Coomassie brilliant blue, gelatin, tricine

Boehringer Mannheim: protease inhibitors, RNase Inhibitor
Dupont: En^{3}hance
Fisons: Ammonium hydroxide
Fluka: Formamide, formaldehyde, guanidine hydrochloride
Melford Labs Ltd.: Caesium chloride
Pharmacia: 7-deaza-dGTP, dNTPs, ddNTPs
Prolabo: Boric acid, butanol, chloroform, ethanol, glacial acetic acid, glycerol, hydrochloric acid, isopropanol, methanol

2B Methods

2B1. Nucleic acid manipulation and cloning procedures

2B1.1 Large scale plasmid DNA preparation

E.coli harbouring the plasmid were streaked (from an older agar plate) or spread (newly transformed bacteria - Section 2B1.6) on an agar plate containing appropriate antibiotics (see Section 2A6) and incubated overnight at 37°C. Colonies were inoculated into 300ml L-broth with antibiotics as appropriate (Section 2A6) and shaken for 16-18hr at 37°C.

Plasmid DNA was prepared by the 'maxi-boiling' technique as described by Holmes and Quigley (1981) and supercoiled plasmid DNA was purified on caesium chloride gradients. Bacterial cultures were centrifuged at 5krpm for 5min at 4°C (Sorvall GS3 rotor), the pellets were resuspended in 20ml STET and 2.5ml fresh 10mg/ml lysozyme solution in STET were added. The mixture was brought to the boil over a bunsen flame and placed in a boiling water bath for 50sec. Cell debris were pelleted by centrifugation at 18krpm for 45min at 4°C (Sorvall SS34 rotor). DNA was precipitated from the supernatant by the addition of 0.9 volumes of isopropanol and pelleted by centrifugation at 3krpm for 5min at RT (Beckman GPR centrifuge). The pellet was resuspended in 5ml TE (to give a volume Xml) and X+0.7g CsCl and 0.2ml 10mg/ml ethidium bromide added. The CsCl was dissolved over a 10-15min period on ice and the samples were centrifuged at 3krpm for 10min at RT to pellet debris. The supernatant was transferred to a crimp-seal centrifuge tube (Dupont 03945) using a syringe and centrifuged at 40 krpm (Sorvall TV865 vertical rotor) for 16hr at 15°C. The plasmid DNA band was removed using a syringe, taking care to avoid contamination with the upper chromosomal DNA band. The EtBr was removed from the plasmid solution by 2-3 extractions with TE-saturated butan-1-ol and the plasmid solution was transferred to dialysis tubing. Dialysis against TE was carried out for 2-3hr to remove CsCl. Following this, the solution was treated with 100µg/ml RNaseA at 65°C for 1hr and then with 100µg/ml proteinase K and 0.1%SDS at 37°C for 1hr. The plasmid DNA

was purified by extraction firstly with TE-saturated phenol and then chloroform and pelleted following an ethanol precipitation by centrifugation at 3krpm for 15min at RT. The pellet was resuspended in 400µl 0.3M NaAc and ethanol precipitated again. The final pellet was washed in 75% ethanol, resuspended in TE and stored at 4°C. The plasmid concentration was determined by measuring the absorbance at 260nm, assuming $1A_{260}$=50µg/ml.

2B1.2 Miniprep plasmid DNA preparation

Plasmid DNA was prepared on a small scale as follows: single colonies of transformed bacteria were picked and inoculated into 3ml aliquots of L-broth and the cultures grown overnight at 37°C in a shaking incubator. Aliquots (1.5ml) of the cultures were centrifuged for 20sec in a microfuge and the pelleted cells were resuspended in 200µl STET. Lysozyme (5µl fresh 10mg/ml solution in STET) was added to each tube which was then vortexed and placed in a boiling water bath for 50sec. Cell debris were pelleted by centrifugation for 10min and the supernatant was removed and made up to 200µl with STET. Plasmid DNA was precipitated by the addition of 180µl isopropanol (0.9 volumes) and pelleted by centrifugation for 5min. The pellets were washed with 75% ethanol, lyophilised, resuspended in 20µl water and stored at -20°C.

2B1.3 Restriction enzyme digestion of DNA

Restriction enzyme digestion of DNA was carried out at 37°C (or the temperature specified by the supplier) in 20µl volumes using 1 unit of enzyme per 0.5µg DNA per hour in the buffer supplied with the enzyme. The buffer system used most often was the Boehringer Mannheim A, B, M, L, H system and less fequently the New England Biolabs NEBuffer 1, 2, 3, 4 system. Usually, 0.5µg DNA was digested for diagnostic analysis and 2-10µg for isolation of specific restriction fragments. Samples (5µl) of plasmid DNA prepared by the miniprep method (see Section 2.2) were digested in the presence of RNase A at 50µg/ml.

2B1.4 Electrophoretic separation and purification of DNA fragments

(i) non-denaturing agarose gels

DNA fragments produced by restriction enzyme digestion or PCR were resolved by non-denaturing agarose gel electrophoresis. Horizontal slab gels approximately 0.5cm thick were utilised and samples were loaded following addition of the relevant amount of agarose gel loading buffer. For separation of fragments over 150bp, 0.5-1.5% agarose gels made up in 1XTBE or 1XTAE (for resolution of DNA for use in PCR) were used and run at 12V/cm in the appropriate buffer. For separation of small PCR products, 2-4% NuSieve GTG Agarose gels (FMC Bioproducts) made up in 1XTAE were used and run at 12V/cm in 1XTAE. Appropriate size standard markers were

always run on the gels: HpaII digested pBR322 gave fragments in the range 27-622bp, while λ DNA digested with HindIII gave fragments of 0.55-23.1kb. Following electrophoresis, DNA was visualised under short-wave UV light (long-wave for preparative gels) after staining the gel in a 1μg/ml EtBr solution followed by a thorough rinse in water. Photography was carried out using The Imager (Appligene) or a polaroid camera.

(ii) DNA purification from agarose gels

Agarose blocks containing appropriate DNA fragments were excised from gels under long-wave UV transillumination and the DNA recovered using a commercial kit, GENECLEANII (BIO101 Inc., La Jolla, CA). The kit contains a silica matrix which binds DNA in the presence of high concentrations of sodium iodide (Vogelstein and Gillespie, 1979). 1X TBE agarose blocks were mixed with 4.5 volumes NaI solution and 0.5 volumes TBE modifier and incubated at 55°C until the gel slice had dissolved. The silica matrix was then added (5μl for up to 5μg DNA) and the mixture vortexed and left at RT for 5-10min. Following a 5-10sec centrifugation, the silica matrix pellet was washed 3 times in 200μl 'NEW' wash and the DNA eluted into 20μl water by mixing the washed pellet with water and incubating at 55°C for 5min. The matrix was again pelleted and the 20μl supernatant containing the DNA removed and stored at -20°C. The GENECLEANII kit was also used in situations where electrophoresis was not required, particularly for separating DNA from nucleotides and PCR primers. In these cases, 3 volumes NaI were used and the TBE modifier omitted.

PCR products were run on TAE gels and excised bands mixed with 200-500μl water and placed in a boiling waterbath for 5min to melt the agarose: the DNA was then available for use in further PCR experiments.

(iii) Denaturing polyacrylamide gels

Vertical denaturing polyacrylamide (acrylamide:bisacrylamide 19:1) gels, containing 8M urea and 1X TBE, were used to resolve the products of DNA sequencing reactions and to purify synthetic oligonucleotides. Polymerisation was instigated by adding 0.001vol TEMED and 0.01vol 10% APS. DNA sequencing gels (0.35mm thick and 35cm long) contained 8% acrylamide. The samples were boiled with formamide dyes prior to loading, and electrophoresis was in 1XTBE running buffer at 40W for 2-5hr. 12 % acrylamide gels (1.5mm thick X 25cm long) were used for purifying oligonucleotides: the samples, again boiled with formamide dyes, were electrophoresed at 250V for 4-5hr in 1X TBE running buffer.

2B1.5 End repair and DNA ligation

It was often necessary to modify the ends of DNA fragments to enable efficient ligation. This was carried out as detailed below and, after each step, enzymes were heat inactivated by incubation of the reaction mixes in a 70°C water bath for 10min. DNA

was purified by extraction with TE-saturated phenol, followed by extraction with chloroform and an ethanol precipitation. The DNA pellets were washed in 75% ethanol and resuspended in water before use in ligation reactions or storage at -20°C.

(i) Filling-in of 5' overhangs

Restriction fragments generated by enzymes which produce 5' extensions were made blunt-ended as follows. DNA in 1X M buffer containing 50µM of all four dNTPs was incubated with 2 units of Klenow fragment for 20-30min at 37°C. The reaction could be performed either immediately following digestion with the appropriate restriction enzymes or following purification of the fragment by agarose gel electrophoresis. If a single blunt end was required, end filling was carried out after digestion with the first enzyme and prior to digestion with the second.

(ii) Removal of 3' overhangs

The removal of 3' extensions produced after restriction enzyme digestion was carried out by the addition of 8 units of T4 DNA polymerase followed by incubation for 20-30 min at 37°C.

(iii) Phosphate removal from 5' ends

Removal of the 5' phosphates of vector fragments with complementary ends produced by digestion with a single restriction enzyme was done by incubation with 1 unit of calf intestinal alkaline phosphatase in the appropriate buffer for 30min at 37°C. The reaction was carried out after heat inactivation of the restriction enzymes or following purification of digested DNA by phenol and chloroform extractions and ethanol precipitation.

(iv) Phosphate addition to 5' ends

It was necessary to add phosphate to the 5' ends of PCR products produced by Pfu polymerase to facilitate blunt-end ligation reactions with a plasmid vector. The reactions were carried out in 20µl of 1X kinase buffer containing 1mM ATP with 10units T4 polynucleotide kinase for 30min at 37°C.

(v) Ligation reactions

'Gene-cleaned' vector and insert fragments prepared by restriction enzyme digestion (Section 2B1.3 and 2B1.4(ii)) with ends suitably repaired were ligated in a 1:5-10 ratio in 20µl reaction volumes of 1X ligase buffer with 2units T4 DNA ligase overnight at RT. The efficiency of blunt-ended ligations was increased by incubating overnight at 13-15°C. The DNA was purified by a phenol and chloroform extraction and subsequent ethanol precipitation prior to transformation into competent *E.coli* (Section 2B1.6). PCR products produced with Taq polymerase were cloned into a commercial vector, pCR™II, provided in the Invitrogen TA Cloning Kit (version 2.2): Taq polymerase leaves 3' A overhangs which are complemented by 5' T overhangs in the pCR™II vector. The ligation reactions were carried out overnight at 14°C in 10µl volumes using fresh PCR products and the ligase buffer and enzyme provided in the kit. 1µl samples

of the ligation reactions were transformed into competent *E.coli* provided in the kit (strain INVαF').

2B1.6 Preparation and transformation of competent *E.coli* cells for plasmid growth and maintenance

Plasmids were grown and maintained in the *E.coli* strain DH5α. A 1ml overnight culture grown up in L-broth from a single colony was inoculated into 100ml L-broth and grown for about 3hr at 37°C in a shaking incubator until the OD_{450} was approximately 0.3. The culture was then cooled on ice and the cells pelleted by centrifugation at 5krpm for 10min at 4°C. The cells were resuspended in 25ml cold 0.1M $MgCl_2$, repelleted by centrifugation at 5krpm for 10min at 4°C and resuspended in 5ml cold 0.1M $CaCl_2$. After a 45min incubation on ice the cells were pelleted as previously and resuspended in 5ml cold 0.1M MOPS, 50mM $CaCl_2$ and 20% glycerol and incubated on ice for 20min. 100µl and 500µl aliquots were flash frozen and stored at -70°C.

About 10ng plasmid DNA (or a quarter of a ligation reaction) were transformed into a 100µl aliquot of competent DH5α bacteria which were thawed on ice. The DNA was added to the cells and the mixture incubated on ice for 20-30min prior to heat shock in a 42°C water bath for 45-50 sec followed by a 2min incubation on ice. 0.5ml 2YT broth or 0.5ml SOC broth supplemented with 20mM $MgCl_2$, 20mM $MgSO_4$ and 20mM glucose was added to the cells which were shaken for 30-60min at 37°C before plating onto LB agar plates containing the appropriate antibiotics. The plates were incubated overnight at 37°C.

2B1.7 Preparation and transformation of competent E.coli cells for protein expression

Plasmids incorporating the GST (pGEX series) or T7 (pT7 series) expression systems were transfected into the appropriate *E.coli* host immediately before use. For pGEX plasmids strain BL21 was used, while pT7 plasmids were transfected into BL21(DE3)pLysS. Bacteria were streaked onto LB or LB/chloramphenicol agar as appropriate, and 3-5 fresh colonies were inoculated into 10ml 2YT and grown in a shaking incubator at 37°C for 2-3hr until the OD_{450} reached about 0.3. The cells were pelleted by centrifugation at 3krpm for 5min at 4°C, resuspended in 0.5ml cold 0.1M $MgCl_2$ and repelleted by centrifugation under the same conditions for 2min. The pellet was resuspended in 0.5ml cold 0.1M $CaCl_2$ and incubated on ice for at least 30min prior to use. About 10ng of the relevant plasmid was transformed into a 100µl aliquot of the competent cells as described in Section 2B1.6.

2B1.8 Purification of synthetic oligonucleotides

Synthetic oligonucleotides were produced by the phosphoramidite method (Section 2A3) using 200µl synthesis columns. To remove the oligonucleotide from the column, 5ml disposable syringes were attached to both ends of the column with one containing 1.5ml ammonium hydroxide (0.88 specific gravity). The solution was pushed through the column in 200µl aliquots with a 20min incubation between each fresh addition. After incubation with the final aliquot, the 1.5ml solution was pushed backwards and forwards through the column 4-5 times to mix the aliquots thoroughly. The oligonucleotide solution was incubated in a tightly sealed tube for 5hr at 55°C to remove protecting groups. Following lyophilisation, the oligonucleotide was dissolved in 200µl water and boiled in an equal volume of formamide plus 10-15µl formamide dyes prior to electrophoresis on a 12% denaturing polyacrylamide gel (Section 2B1.4(iii)). Oligonucleotides were purified by passive elution from gel slices excised from the gel following visualisation of the DNA by UV transillumination: bands were visible as dark shadows against a fluorescing TLC plate. Gel slices were crushed and the DNA eluted overnight at 37°C in a shaking incubator. The liquid phase, containing the DNA, was separated from the gel fragments by filtering the mixture through siliconised glass wool. The DNA was purified by an ethanol precipitation and pelleted by centrifugation for 10min (microfuge). The pellet was resuspended in 100µl water, ethanol precipitated again and the final DNA pellet was washed in 75% ethanol, lyophilised and resuspended in water. The DNA concentration was determined by measuring the absorbance at 260nm, assuming that for short oligonucleotides (<30 nucleotides) $1A_{260}$=20µg/ml.

2B1.9 Amplification of λZAPII library DNA

A sample of DNA from a λZAPII cDNA library (Section 2A4) was prepared for use as a template in PCR experiments by amplification from the stock (titre $1.9X10^9$pfu/ml: see Section 2B5.1). Approximately $8.5X10^7$pfu of the λZAPII library stock was mixed with 0.3ml of an overnight culture of *E.coli* cells (strain XL-1 Blue, Section 2A5) and the mixture shaken for 20min at 37°C to allow the bacteriophage to adsorb to the bacteria. Following addition of 12ml of 2YT broth, incubation was continued for a further 4hr until bacterial lysis occurred, then chloroform was added to a concentration of 1% and incubation continued for another 15min to ensure complete lysis. Cell debris were pelleted by centrifugation at 4krpm for 10min at RT. Bacteriophage were pelleted from the supernatant by centrifugation at 40krpm for 1hr at 4°C (in Sorvall Oak Ridge Polycarbonate tubes, Sorvall T1270 rotor) and resuspended in 400µl TE. The DNA was then purified by 2 phenol extractions (mixing for 15min) and a chloroform extraction (mixing for 5min) followed by an ethanol precipitation. The DNA was pelleted by centrifugation for 10min at RT (microfuge), washed in 75% ethanol, lyophilised and

dissolved in 100µl water. The quality of the DNA was determined by digestion of a 2µl sample with EcoRI (Section 2B1.3) followed by electrophoresis on a non-denaturing agarose gel (Section 2B1.4(i)). The concentration was determined by measuring the absorbance at 260nm, assuming that $1A_{260}$=50µg/ml.

2B1.10 Preparation of RNA from tissue culture cells

All aqueous solutions used in RNA work were treated overnight with DEPC added to a final concentration of 0.01% to inactivate RNases. The DEPC was inactivated by autoclaving.

RNA was prepared from roller bottles of confluent WS HeLa cells (see Section 2B2 for growth and maintenance of tissue culture cells) by the 'single-step' method of Chomczynski and Sacchi (1987). The medium was removed from the roller bottle and 12ml guanidine thiocyanate solution added and spread over the cell sheet by rolling the bottle on its side for 10min. After standing the bottle upright for a further 10min, the cell lysate was removed into a sterile Sorvall polycarbonate tube to which was added 1.2ml 2M NaAc (pH 4.0). The mixture was thoroughly vortexed and 8ml water saturated phenol added followed by 2.4ml chloroform/isoamylalcohol (49:1), with thorough vortexing after each addition, and placed on ice for at least 15min. Centrifugation of this mixture at 10krpm for 30min at 4°C (Sorvall SS34 rotor) yielded an upper aqueous phase containing RNA and a lower organic phase containing DNA and cell debris. About 80% of the aqueous phase was removed and an equal volume of isopropanol was added to precipitate the RNA (overnight at -20°C). The RNA was pelleted by centrifugation at 10krpm for 30min at 4°C and the pellet dissolved in 300µl guanidine thiocyanate solution and reprecipitated over a period of at least 1hr at -20°C after the addition of 100µl water and 800µl ethanol. The RNA was again pelleted, washed twice in 75% ethanol and resuspended in 500µl water and stored at -70°C (for long term storage (≥2-3 weeks), the RNA was kept as an ethanol precipitate at -20°C). The concentration was determined by measuring the absorbance at 260nm, assuming $1OD_{260}$=40µg/ml.

For some experiments it was necessary to use polyA$^+$ RNA and this was separated from the total RNA mix isolated in the above protocol using the PolyATract mRNA Isolation System (Promega). The system uses a biotinylated dT oligo primer which hybridises to the 3' polyA region of mRNAs. The hybrids are then 'captured' in an interaction with streptavidin which is coupled to paramagnetic particles, using a magnetic separation stand. Total RNA (0.1-1mg) in a final volume of 500µl water was heated at 65°C for 10min in a water bath. The solution was then placed at room temperature and 3µl of the biotinylated-oligo(dT) probe added with 13µl of 20X SSC and the mixture allowed to cool to RT. Meanwhile, an aliquot of streptavidin-paramagnetic particles were resuspended in their storage buffer and captured on the side

of the tube by placing the tube in the magnetic stand. The storage buffer was removed and the particles were resuspended in 0.3ml 0.5X SSC, recaptured and washed twice more in a similar manner. The cooled RNA mix was added to the washed magnetic particles and the mixture was incubated at RT for 10min. The tube was then placed on the magnetic stand to capture the particles. The particles were washed 3 times in 0.3ml 0.1X SSC and the polyA+ RNA eluted by incubating the beads twice in water, 0.1ml the first time and 0.15 the second to give a total volume of 0.25ml. Due to the small quantities of polyA+ RNA present, the concentration was estimated by assuming that 1% of total RNA is polyA+ RNA and 50% of this was recovered. Thus approximately 5µg polyA+ RNA was recovered from 1mg of total RNA.

2B1.11 Reverse transcription of RNA

RNA was reverse transcribed to make a cDNA template for use in PCR experiments. PolyA$^+$ RNA (2µg) or 25-30µg total RNA were reverse transcribed at 42°C for 1hr in a 20µl reaction volume containing 5-10pmol primer (poly(dT), random hexamers, or reverse PCR primer), 20nmol each of dATP, dCTP, dGTP and dTTP, 25units RNase inhibitor and 40units M-MuLV reverse transcriptase. The mix was stored at -20°C and typically 0.5µl was used in a 20µl PCR amplification.

2B1.12 5' RNA extension assay

5' RNA extension assays were carried out to try to order redundant primers designed from 135kD peptide sequence information on the mRNA. This would subsequently reduce the number of primer combinations in the PCR amplifications. The method involved hybridising reverse PCR primers to RNA and extending in a mix including α^{32}PdATP. PCR primers (25ng) were hybridised to 25µg RNA in a total volume of 15µl containing hybridisation buffer at 65°C for 90min. To each hybridisation mix the following reagents were added: 0.9µl 1M Tris.HCl (pH 8.3), 0.9µl 0.5M $MgCl_2$, 0.25µl 1M DTT, 6.75µl 1mg/ml actinomycin D (Cosmogen), 1µl α^{32}PdATP, 3.3µl each of 0.5mM solutions dCTP, dGTPand dGTP, 10µl water and 5units M-MuLV reverse transcriptase. This latter mixture was incubated at 42°C for 1hr and the reaction chased by the addition of 0.5µl 5mM dATP with further incubation for 30min. Extension was stopped by addition of 105µl RNase reaction mix and incubation at 37°C for 15min. cDNA was purified by a phenol and chloroform extraction, ethanol precipitated and pelleted by centrifugation for 10min (microfuge). The pellet was washed in 75% ethanol and dissolved in 5µl formamide dyes and placed in a boiling water bath for 2min. The samples were electrophoresed on an 8% denaturing polyacrylamide gel (Section 2B1.4(iii)) and the gel dried and exposed to Kodak X-OMAT S film.

2B1.13 Polymerase chain reaction (PCR) amplification of DNA

PCR amplification was carried out using redundant primers designed from 135kD peptide sequence information in an attempt to clone a cDNA encoding the 135kD protein.

(i) Amplification with Pfu DNA polymerase

This enzyme was used in early experiments until it was discovered that the 3' ends of primers were often missing from cloned PCR products (see 3E2). Typically, 30µl reaction volumes contained 0.1-250ng template DNA (cDNA made by reverse transcription from polyA+ or total RNA (Section 2B1.11)), 1-5µM primers, 100µM dNTPs, 3µl 10X reaction buffer (supplied with enzyme) and 1-2units Pfu DNA polymerase. The mixtures were overlayed with light mineral oil to prevent evaporation during cycling. Cycling conditions were varied in an attempt to minimise non-specific priming events, which gave rise to single primer artifacts and other non-specific products, and to obtain a single product from any one reaction. Initial conditions were: 1 cycle at 95°C for 5min (denaturing), 42°C for 5min (annealing),74°C for 5min (elongation), followed by 38 cycles at 95°C 50sec, 42°C 1min, 74°C 2min 40sec. Annealing conditions were then altered by increasing the temperature to 65°C and reducing the time to 20sec in stages. Further optimisation involved altering the duration of the denaturing and extension stages. Reaction products were purified by an ethanol precipitation and pelleted by centrifugation, washed in 75% ethanol and dissolved in water. They were treated with polynucleotide kinase (Section 2B1.5(iv)) or digested with the relevant restriction enzymes if the primers were designed with restriction enzyme sites (Section 2B1.3) and ligated into pUC9 (Section 2B1.5(v)) prior to transformation into competent *E.coli* TG1. Addition of 20µl 100mM IPTG and 40µl 20mg/ml X-gal to the 600µl transformed cell mix, prior to spreading on LB agar plates containing ampicillin (Section 2A6), enabled blue-white selection of colonies containing vector with inserted PCR products.

(ii) Amplification with Taq DNA polymerase

This enzyme was used for the majority of PCR amplifications. A wide variety of reagent concentrations were used in an attempt to minimise non-specific primer annealing. Generally, 10-30µl reaction volumes were made up containing 0.1-250ng template DNA (cDNA made by reverse transcription from polyA+ or total RNA (Section 2B1.11), or λZAPII library DNA (Section2B1.9) with parallel control reactions using bacteriophage λ DNA), 1-5µM primers, 100µM dNTPs, 0.5-3µM $MgCl_2$, 1-3µl 10X reaction buffer (supplied with the enzyme) and 0.1-2.5units Taq DNA polymerase. Cycling conditions were varied widely: initial conditions were similar to those used with Pfu DNA polymerase but with an extension temperature of 72°C. Subsequently, annealing temperatures and times as well as extension times were altered. For a description of other strategies which were employed in an attempt to

clone a cDNA using a PCR method see Section 3E2. PCR products were cloned by ligation into the pCR™II vector (Section 2B1.5(v)).

2B1.14 Dideoxy sequencing of DNA

Plasmid DNA was sequenced by the dideoxynucleotide chain termination method based on that of Sanger *et al* (1977). Purified plasmid DNA (about 2µg) was denatured at RT for 10min in a 20µl reaction volume containing NaOH at a final concentration of 0.4M. The denatured DNA was precipitated by addition of 6µl 3M Na acetate, 14µl water and 120µl ethanol and incubated in a dry-ice ethanol bath for 10min, pelleted by centrifugation for 10min and dissolved in 8µl water.

The appropriate primer in (5pmol in 1µl volume) was added to the DNA with 1µl 10X seq buffer and annealing carried out for 20min at 37°C. *E.coli* DNA polymerase I Klenow fragment (2units in 1X seq buffer) was added to the annealed DNA and 2µl aliquots of this mix were dispensed into 4 wells of a round bottomed 96-well microtitre plate (Nunclon) for the four chain termination reactions. Four chain termination mixes were made up, each containing a sequencing mix with one ddNTP + dNTPs (see table below for details) and 0.57µM dATP and 5µCi α^{32}PdATP in a final volume of 14µl. Aliquots of (2µl) these mixes were added to the relevant wells, the microtitre plate was spun briefly to mix the contents of the wells, then the plate was incubated at 37°C for 15min. Following this, any newly synthesised strands which had not been terminated by incorporation of a dideoxy nucleotide were extended into high molecular weight products by adding 2µl chase mix (4X cold dNTPs each at 0.25mM) and incubating at 37°C for 30min. The reactions were stopped by adding 2µl formamide dyes and the tray placed in a boiling water bath for 2min prior to electrophoresis of the samples in adjacent lanes on a denaturing polyacrylamide gel (Section 2B1.4(iii)). Gels were vacuum dried and exposed to Kodak X-OMAT S film.

Sequencing solutions:

	dA-0	dT-0	dC-0	dG-0
5mM dTTP	20 (µl)	1 (µl)	20 (µl)	20 (µl)
5mM dCTP	20	20	1	20
5mM 7-deaza dGTP	20	20	20	1
10X seq buffer	50	50	50	50
water	540	370	370	370

Sequencing mixes:

	dN-0 mixes	ddNTP	water
T seq	500 (µl)	500 (600µM ddTTP)	0
C seq	500	105 (140µM ddCTP)	395
G seq	500	155 (200µM ddGTP)	345
A seq	500	250 (140µM ddATP)	370

DNA prepared by the miniprep plasmid method (Section 2B1.2) was also sequenced using this method: 7.5-15µl miniprep DNA were denatured in a 20µl reaction volume as described above and the reactions continued as normal.

2B1.15 Southern blotting (transfer of DNA to nitrocellulose) and probing of PCR products by DNA-DNA hybridisation

Products of PCR reactions made with redundant primers were occasionally probed with redundant primers not included in the reactions or with plasmid DNAs from other sources, as detailed in the relevant sections.

(i) Transfer of DNA to nitrocellulose

Products from PCR reactions were separated on agarose gels in 1X TBE. The gels were incubated in Gel Soak I for 45min followed by Gel Soak II for 45min, both with shaking at RT. The DNA was transferred overnight onto Hybond-N+ filters (presoaked in 2X SSC before use) by placing gels on a wick of Whatman 3mm paper (soaked in 10X SSC and descending into a 10X SSC reservoir), then adding sequentially Hybond-N+ filters, two pre-soaked sheets of Whatman 3mm paper and a stack of absorbent paper towelling to the top of gels. Care was taken to eliminate air bubbles between layers and a light weight was added to the top of the stack to aid buffer transfer. After transfer, the filter was briefly rinsed in 2X SSC and the DNA was fixed on the filter and denatured by incubating the filter, DNA side up, on Whatman 3mm paper soaked in 0.4M NaOH for 20min. The filter was again rinsed in 2X SSC and kept moist.

(ii) DNA-DNA hybridisation

Hybond-N+ filters were pre-hybridised in a mix containing 5X SSC, 5X Denhardt's solution, 0.5% SDS and 20µg/ml salmon sperm DNA for 3-4hr at 45-65°C. Probe DNA (10^7-10^8 cpm, see below) was added to the mix and incubation continued overnight at the relevant temperature. The filter was washed twice in 2X SSC, 0.1% SDS for 10min at RT and then three times for 30min at the prehybridisation temperature. The filter was air dried and exposed to Kodak X-OMAT S film.

(iii) Probes

Oligonucleotide probes (50pmol) were radiolabelled in a 10-20µl reaction volume with 20µCi γ^{32}PATP using polynucleotide kinase (Section 2B1.5(iv)). The probe was

purified by a phenol and chloroform extraction and ethanol precipitation. The pellet was dissolved in 20µl water and added to the prehybridisation mix.

Plasmid DNA or restriction fragment probes were prepared using the Megaprime system (Amersham). About 25ng of DNA was mixed with 5µl Megaprime primer in a final volume of 20µl and placed in a boiling water bath for 5min. The mixture was cooled and 10µl Megaprime buffer, 50µCi α-^{32}P dCTP and 4-5units Klenow fragment were added, bringing the reaction volume to 50µl, and the mix was incubated at 37°C for 20min. The reaction was stopped by the addition of 5µl 0.2M EDTA. Bromophenol blue was added to colour the mix which was then loaded on a Sephadex G-50 'nick' column (Pharmacia) that had been equilibrated with 1X TE containing 0.1% SDS. Fractions (about 0.25ml) were collected from the column and those containing the highest counts in the initial peak were pooled and stored as probe. The probe was denatured by adding 1/20th the volume of 5M NaOH, incubating the mixture at RT for 10min and neutralising by adding 1/20th the volume of 5M HCl.

2B2. Tissue culture

2B2.1 Growth of cells

Mammalian derived cells were passaged in sterile, disposable 175cm^2 plastic flasks in the appropriate media (Section 2A.7) and were grown at 37°C in a humidified incubator under 5% CO_2. Confluent monolayers were harvested by washing the monolayer twice in 25ml trypsin:versene (1:4) (supplied by the Institute of Virology Media Services) and resuspending the cells in 10ml medium. Trypsinisation was unnecessary for ND7 cells which could be removed from flasks by gentle tapping. For continual passage, BHK and WS HeLa cells were split in a 1:10 ratio every 3-4 days, HFL cells in a 1:10 ratio every 7-10 days, U-2 OS cells in a 1:5 ratio every 4-5 days and ND7 cells in a 1:20 ratio every 3-4 days.

2B2.2 ^{35}S-methionine radiolabelling and extract preparation

Confluent monolayers of cells grown on 140mm diameter tissue culture plates were labelled with ^{35}S-methionine and soluble protein cell extracts prepared for use in standard GST 'pull-down' assays. Plates were seeded at 1.5X10^7 cells per plate in 30ml medium and incubated at 37°C overnight. The medium was removed and the cell sheet was washed twice in 15ml prewarmed (37°C) PBS-complete and then incubated with 15ml PBS complete containing 1500µCi ^{35}S-methionine for 2hr at 37°C. Following this, the cells were washed twice in cold PBS-complete and harvested in 10ml PBS-complete by scraping them from the plate. The cells were pelleted by centrifugation at 1krpm for 5mins at 4°C and resuspended in 1ml PBS-complete prior to repelleting followed by resuspension in 1ml of a buffer containing 50mM HEPES (pH 7.5), 50mM

NaCl and 0.1% NP40. The cells were lysed by sonication for 30-60sec in a soni-bath and debris pelleted by centrifugation at 3krpm for 10min at 4°C. The soluble protein extract was stored at -70°C.

Radiolabelled WS HeLa cell nuclear and cytoplasmic extracts were made by the method of Dignam et al. (1983). Labelled cells from two 140mm plates were harvested as above and resuspended in a final volume of 2ml. An aliquot of this sample (0.5ml) was treated as above on a relative scale to make a whole cell protein extract. Cells in the remaining 1.5ml were pelleted and resuspended in 0.75ml of the HEPES containing buffer and NP40 was added to 0.5% prior to incubation of the mix on ice for 5min. Cell nuclei were spun down at 2krpm for 5min at 4°C and the supernatant retained as the radiolabelled cytoplasmic extract. The pellet was resuspened in 0.75ml HEPES buffer and sonicated prior to centrifugation at 3krpm for 10min at 4°C. The supernatant was retained as the nuclear fraction.

Radiolabelling of differentiated ND7 cells was performed overnight as these cells had a lower rate of incorporation of the ^{35}S-methionine compared with the other cell types used. Undifferentiated cells were seeded on 140mm plates in differentiation medium (Section 2A7) at 1.5X10^6 cells per plate and allowed to differentitae over 3-5 days. When 80% of the cells had assumed a neuron-like morphology, the medium was removed and the cells washed twice with Low Methionine Medium (Eagle's medium containing 20% of the normal concentration of methionine, supplied by the Institute of Virology Media Services). The cells were labelled for 16hr with 1500µCi ^{35}S-methionine in Low Methionine Medium, then washed and harvested as above. Undifferentiated cells seeded at the same density were also labelled overnight to provide a suitable control.

2B2.3 Extract preparation from roller bottles of WS HeLa cells

It was necessary to prepare large amounts of soluble protein extracts from WS HeLa cells in order to purify enough 135kD protein for microsequence analysis in large scale GST 'pull-down' experiments. The cells were grown in batches of about 10 roller bottles containing 100ml medium until the cells were about 95% confluent. The medium was removed and the monolayer was washed twice in cold 25ml PBS-complete and harvested by scraping in 50ml PBS-complete. The cells were pelleted by centrifugation at 1krpm for 10min at 4°C and resuspended in PBS-complete (5ml per roller bottle) and pooled with cells from other roller bottles. The cells were pelleted again, under the same conditions, and resuspended in a buffer containing 50mM Hepes, 50mM NaCl and 0.1% NP40 (2.5ml per roller bottle). The mixture was sonicated on ice using 10sec bursts with a soniprobe until the mixture cleared indicating that the cells had lysed. Debris was pelleted by centrifugation at 3krpm for 20min at 4°C and the soluble protein extract was adjusted to 0.5M NaCl and stored at -70°C.

2B2.4 Calcium phosphate mediated transfection for immunofluorescence experiments

Cells were seeded on 13mm glass coverslips in 24-well Nunc Linbro plates at $0.5X10^5$ cells per well in 1ml of medium and incubated overnight at 37°C prior to transfection. Plasmid DNA (5µg) was made up to a volume of 70 µl in 0.1X TE and 10µl 2M $CaCl_2$ was added while whirlimixing. This mixture was added to 80µl 2X HBS, again while whirlimixing, and the mix incubated at RT for 30min before addition to cells (64µl per well). After mixing by gently swirling the plate, the cells were incubated overnight at 37°C. The medium was removed and the cells were washed in 1ml fresh medium and incubated at 37°C for 16-24hr in a fresh 1ml aliquot of medium.

2B2.5 Immunofluorescence

Transfected cell monolayers (see above) were washed once with PBS(A) and fixed for 10min at RT with 2% sucrose, 5% formaldehyde in PBS(A). This solution was removed and the cells were washed 3 times with PBS(A) and permeabilised for 10 min at RT with 0.5% NP40, 10% sucrose in PBS(A). The cells were again washed 3 times with PBS(A) supplemented with 1% calf serum (CS). Aliquots (20µl) of the relevant primary antibody diluted in PBS(A)+1% CS were then added to each coverslip for 45-60min. Again the cells were washed 3 times in PBS(A)+1% CS and incubation with a secondary antibody carried out if necessary. The mouse monoclonal antibody 11060 was used at a dilution of 1/2000 and rabbit antisera raised against 135kD peptides at dilutions of between 1/50-1/5000. The monolayer of cells was then incubated with 20µl FITC labelled goat anti-mouse IgG at a 1/100 dilution and/or TRITC labelled goat anti-rabbit IgG (1/30 dilution) (Sigma Immunochemicals) for 30min at RT in the dark. After 3 washes with PBS(A)+1% CS, the cells were air dried and mounted on glass slides with 10µl Citifluor, a glycerol/PBS solution (UKC Chemical Laboratories). Cells were examined using a Nikon MICROPHOT-SA fluorescence microscope with appropriate filters and photographed using Kodak ASA400 black and white film.

2B3 GST 'pull-down' assays and purification of the 135kD protein

2B3.1 GST fusion protein expression and preparation of bacterial extracts

A single fresh *E.coli* colony (strain BL21) transformed with the relevant pGEX plasmid (Section 2B1.7) was inoculated into 3ml L-broth and grown up overnight at 37°C with shaking. A sample (1ml) of this culture was added to 100ml 2YT broth with ampicillin at 100µg/ml and the culture was grown at 37°C with shaking for 3-4hr until the OD_{450} reached 0.6. Protein expression was induced by adding IPTG to 0.1mM and incubating under the same conditions for a further 2hr. Bacteria were harvested by centrifugation at 5krpm (Sorvall GS3 rotor) for 5min at 4°C and the cell pellet was resuspended in 2ml

PBS(A) prior to sonication in 20sec bursts with a soni-probe. Once the cells had lysed, Triton X-100 was added to 1% concentration and the lysate was incubated on ice for 5min prior to centrifugation at 9.5krpm (Sorvall SS34 rotor) for 5min at 4°C. The soluble protein extract supernatant was stored at -20°C.

2B3.2 SDS polyacrylamide gel electrophoresis (SDS PAGE) of proteins

Proteins were resolved by electrophoresis through SDS polyacrylamide minigels (Laemmli, 1970) using the Bio-Rad miniprotean II apparatus. Tricine and glycine gel systems were used interchangeably. Gel mixes were made up as follows:

Tricine gels:

Solution	10% resolving gel	12.5% resolving gel	stacking gel
49.5% acrylamide (3% cross-linker)	1.5ml	1.87ml	250µl
gel buffer	2.5ml	2.5ml	775µl
water	7.5ml	7.125ml	2.15ml

100µl 10% APS and 10µl TEMED were added to each resolving gel mix and 25µl 10% APS and 5µl TEMED were added to stacking gel mixes.

Glycine gels:

Solution	7.5% resolving gel	10% resolving gel	stacking gel
30% acrylamide (2.5% cross-linker)	2.5ml	3.33ml	0.4ml
RGB	2.5ml	2.5ml	
SGB			0.6ml
water	5ml	4.17ml	1.4ml

80µl 10% APS and 8µl TEMED were added to each resolving gel mix and 20µl 10% APS and 3µl TEMED to stacking gel mixes.

Resolving gel mixes were made, poured into glass plate sandwiches in the gel former apparatus, overlayed with butanol and allowed to set. The butanol was washed away with water and the stacking gel mixture was overlaid and a comb inserted. Protein samples were mixed with the relevant SDS gel loading buffer and placed in a boiling water bath for 2min prior to loading. Tricine gels were run at 200V and glycine gels at 100V until the tracking dye reached the bottom. The gels were stained with Coomassie blue for 5min and destained in Protein Gel Destain. Gels with radiolabelled protein samples were fixed for 10min in Protein Gel Fix, incubated in En^{3}hance for 10min, briefly washed with water and vacuum dried prior to exposure to Kodak X-OMAT S film.

2B3.3 Western blot analysis of proteins

2B3.3(i) Electroblotting to nitrocellulose

Proteins resolved on SDS PAGE minigels were transfered to nitrocellulose by the method of Towbin *et al.* (1979) in a Bio-Rad mini transblot cell. A blotting sandwich was set up such that the gel was in contact with a sheet of nitrocellulose (Schleicher and Schuell Inc.) and both were sandwiched between Whatman 3mm paper of the appropriate size. This was in turn sandwiched between sponges provided by Bio-Rad and blotting carried out at 0.25mA for 3hr.

2B3.3(ii) Immunodetection

Nitrocellulose membranes were blocked by incubation at 37°C with gentle shaking for 60min in 100ml 3% gelatin in TBS with one change of buffer after 30min. They were then washed 2X 5min at RT in TTBS before incubation overnight at RT on a shaker with the first antibody diluted appropriately in 20ml 1% gelatin in TTBS. Following this, blots were washed five times for 5min in TTBS at RT and protein A horse radish peroxidase or anti-mouse IgG whole molecule peroxidase conjugate added in a 1/1000 dilution in 20ml 1% gelatin in TTBS. After a 90min incubation at RT, again with shaking, blots were washed three times for 5min in TTBS and proteins were detected using the Amersham enhanced chemiluminescence (ECL) system. The two reagents were mixed in equal volumes and a total of 3ml poured on to the filter which was then agitated for 1min. The filters were wrapped in cling film and exposed to Kodak X-OMAT S film for 5-30sec.

2B3.4 Standard GST 'pull-down' assay

The conditions for this assay were determined experimentally (see Section 3A4). The initial step of purification of fusion proteins by interaction with glutathione agarose beads was based on the method of Smith and Johnson (1988). Glutathione agarose beads (Sigma) were prepared for use in experiments by pre-swelling in 10X volume PBS(A) for 1hr at RT and washing 3 times in excess PBS(A), spinning 20-30sec (microfuge) between washes to pellet the beads. After the final wash, the beads were resuspended in an equal volume of PBS(A) and stored as a 50% slurry for up to one month at 4°C.

The protocol determined for investigating cellular proteins which interact with segments of Vmw110 fused to GST was as follows. A 50% bead slurry (50µl) was added to 300µl bacterial extract and mixed end over end for 1hr at 4°C. The beads were recovered by a 20-30sec spin and washed 3 times in 1ml cold PBS(A) and stored on ice as a 50% slurry until use. Aliquots (10µl) were analysed by SDS gel electrophoresis (Section 2B3.2) with size standard markers to determine the quality of the bound GST fusion protein. An appropriate volume of the bead slurry was mixed end over end at 4°C for 1hr with 300µl ^{35}S-methionine radiolabelled cell extract (Section 2B2.2) in

buffer containing 0.5M NaCl. The beads were recovered by brief centrifugation, washed three times for 5min in 1ml cold 0.5M NaCl wash buffer and retained as a 50% slurry.

For experiments utilising Vmw110-GST fusion proteins, the cell extracts were routinely pre-treated by this procedure with glutathione agarose beads carrying the GST protein expressed by pGEX2TN3 vector plasmid. This step depleted the radiolabelled extract of proteins which bound non-specifically to the beads or GST.

Bound proteins were eluted from 20µl aliquots of the beads by the addition of 20µl 50mM reduced glutathione and incubating on the bench for 15min with gentle agitation every 5min. The elution step was repeated with a second volume of reduced glutathione. The eluates were mixed with SDS gel loading buffer, boiled for 2min and loaded on 10% glycine gels with 1µl 1/10 dilution radiolabelled cell extract which acted as a marker track. Electrophoresis was carried out as described in Section 2B3.2.

2B3.5 Purification of the 135kD protein

2B3.5(i) Large scale 'pull-down' experiment

This procedure was determined experimentally (see Section 3C4) but is based on the conditions of the standard 'pull-down' assay described above.

Glutathione agarose beads (4ml 50% slurry) were mixed with 5ml 2TN3 and GEXE52 extracts and incubated with end over end mixing at 4°C for 1hr. The beads were recovered by centrifugation at 3krpm for 5min at 4°C and were washed 3 times in 10ml PBS(A) and stored on ice as a 50% slurry. Samples (10µl) of each were taken for analysis by SDS PAGE as in Section 2B3.4. The 2TN3 beads were mixed with a WS Hela cell extract made from 25 roller bottles of cells (Section 2B2.3) which gave a final volume of approximately 60ml (this was split into 30ml volumes for convenience). Following end over end mixing at 4°C for 3hr, the 2TN3 beads were removed from the extract and an aliquot was washed 3 times in 0.5M NaCl wash buffer. Proteins were eluted from the beads in an equal volume of 50mM reduced glutathione and the eluate stored at -70°C. The 'pre-cleared' WS Hela cell extract was then mixed in the same manner with 4ml GEXE52 beads for approximately 42hr to ensure maximal binding of the 135kD protein to Vmw110 sequences. (A control experiment was carried out in parallel on a smaller scale in which 1ml 50% slurry GEXE52 beads were mixed with the cell resuspension buffer for the same period of time). The beads were recovered by centrifugation at 3krpm for 10min at 4°C and washed once in 10ml and twice in 5ml 0.5M NaCl Wash Buffer. After the final wash, the GEXE52 beads were collected by centrifugation and all liquid removed. Proteins were eluted in 2 identical steps by adding 2ml 50mM reduced glutathione and incubating at RT for 30min with gentle agitation of the mixture every 10min. The eluates were pooled to give a total volume of 4ml and this was reduced to 200µl by centrifuging 1ml volumes at 5krpm in Centricon

50 centrifugation units (Amicon). This reduced volume eluate was then mixed with 200µl 3X SDS gel loading buffer and boiled for 10min. Samples (300µl) were loaded on two 2.5cm wells of a 10% glycine gel with 20µl of a GEXE52 eluate from a standard 'pull-down' assay using radiolabelled extract and electrophoresed (Section 2B3.2). Relevant control samples were also loaded onto the gel.

(ii) Transfer of proteins to Problott™ membrane

Electrophoretically separated proteins were transferred to a Problott™ membrane (Applied Biosystems) in a manner similar to Western blot transfer of proteins to nitrocellulose (Section 2B3.3). The gel was first soaked for 5min in Electroblotting Buffer (Section 2A10) and a membrane of corresponding size briefly rinsed in methanol and in Electroblotting Buffer. The transblotting 'sandwich' set up as described above (Section 2B3.3(i)). Transfer was carried out for 6hr at 0.25mA in Electroblotting buffer, ensuring that the buffer was kept cool by inserting an ice block in the transblot cell. Following transfer, the membrane was rinsed in water, then briefly in methanol, before staining for 1min with 0.2% Ponceau S in 1% acetic acid. The membrane was destained for 10-15min in water until the bands could be seen. The membrane was then thoroughly air dried and photocopied before an overnight exposure to Kodak X-OMAT S film. A stained band which co-migrated with the radiolabelled 135kD protein was identified and submitted for microsequence analysis.

2B4 Expression of fragments of Vmw110 in bacteria using the T7 system, and their purification and analysis

2B4.1 Expression and crude purification of T7E52 and T7E58

A single fresh *E.coli* colony (strain BL21(DE3)pLysS) transformed with either pT7E52 or pT7E58 (Section 2B1.7) was inoculated into 10ml L-broth and grown shaking overnight at 37°C. The culture was added to 1l 2YT broth with ampicillin at 100µg/ml and the culture grown at 37°C with shaking for 3-4hr until the OD_{450} reached 0.6. Protein expression was induced by adding IPTG to 0.1mM and incubating for a further 2hr. The bacteria were harvested by centrifugation at 5krpm (Sorvall GS3 rotor) for 5min at 4°C, the cell pellet was resuspended in 10ml cold Extract Buffer and stored frozen at -20°C overnight. After thawing, 10µl 20mg/ml DNaseI, 25µl 2M $MgCl_2$ and 30µl 10mg/ml lysozyme were added to the mixture and the cells were lysed using 20sec bursts with a soniprobe. Subsequently 250µl 5M NaCl and 50µl 10% polymin P were added to the lysate which was incubated on ice for 30min. Cell debris and precipitated nucleic acids were pelleted by centrifugation at 9.5krpm for 5min at 4°C. Saturated ammonium sulphate was added to the supernatant to 40% volume and the mixture stirred on ice for 30min. Precipitated proteins were pelleted by centrifugation at 9.5krpm for 10min at 4°C and the pellet was resuspended in 2ml 50mM HEPES (pH

7.5), 50mM NaCl, 0.01% CHAPS, 1mM PMSF, 0.1mM DTT. The extract was filtered through a 0.45µm millipore filter and stored at -20°C.

2B4.2 FPLC purification of T7E52 and T7E58

T7E52 and T7E58 were purified by two ion exchange chromatography steps and a gel filtration step. Columns were pre-equilibrated with the relevant buffer which had been filtered through a 22µm filter and de-gassed, until the UV absorbance of the eluted buffer reached a steady state.

(i) ion exchange chromatography

Crude samples of extracts containing the T7E52 protein were loaded onto a 20ml Mono-Q anion exchange column which had been pre-equilibrated in Column Buffer: 50mM HEPES (pH 7.5), 50mM NaCl, 0.01% CHAPS, 1mM PMSF, 0.01mM DTT. After loading, the column was washed with Column Buffer (10ml) and proteins were eluted on a linear 0.05-1.0M NaCl gradient in column buffer (20ml). Flow-through fractions containing T7E52 (as indicated by SDS PAGE analysis-Section 2B3.2) were pooled and loaded on a 20ml Mono-S cation exchange column which had also been pre-equilibrated in Column Buffer. The column was washed with column buffer (10ml) and a 0.05-1.0M NaCl gradient in Column Buffer (20ml) applied. Fractions (1ml) were collected and analysed by SDS gel electrophoresis. The procedure was similar for purification of the T7E58 protein except the initial crude extract was loaded on the Mono-S column and pooled flow-through fractions on the Mono-Q column.

(ii) gel filtration

Purified protein samples from the ion exchange chromatography columns were loaded in 300-500µl volumes on a Superdex 75 column which had been pre-equilibrated in a buffer containing 50mM HEPES (pH 7.5), 1M NaCl, 0.01% CHAPS, 1mM PMSF and 0.01mM DTT. Fractions (1ml) of the eluate were collected and samples were analysed by SDS PAGE (Section 2B3.2) prior to storage at -20°C.

2B4.3 Glutaraldehyde cross-linking analysis

Glutaraldehyde covalently cross-links amino groups of lysine residues of proteins in close proximity and can therefore be used to determine the multimeric nature of proteins. The concentrations of purified T7E52 and T7E58 proteins were estimated from SDS PAGE analysis in comparison with known amounts of marker proteins (Section 2B3.2) and 20µl volumes containing 1µg or 5µg protein were incubated for 30min at 20°C with glutaraldehyde concentrations ranging from 0.001% to 0.01% . Glycine was added to a concentration of 50mM to stop the reaction by competitively inhibiting protein:protein cross-linking and the samples were analysed by SDS PAGE using 12.5% polyacrylamide tricine gels (Section 2B3.2). The products were detected

either by Western blotting using MAb 10503 at 1/5000 dilution (Section 2B3.3(ii)) or they were visualised directly by Coomassie blue staining of the gel.

2B4.4 Glycerol gradient centrifugation and dot blot analysis

About 20-30µg purified T7E52 and T7E58 proteins were loaded on 10-30% glycerol gradients poured in 5ml centrifuge tubes in a buffer containing 1M NaCl, 50mM HEPES (pH 7.5) with 20µg each of the size standards bovine serum albumin (BSA) and carbonic anhydrase (CA). Gradients were centrifuged at 40krpm for 46-48hr at 4°C (Sorvall AH650 rotor), then 200µl fractions were collected from the top of the tube. Aliquots (20µl) of fractions were analysed by SDS PAGE (Section 2B3.2) to determine the comparative sedimentation rates of the proteins. The peak positions of proteins T7E52 and T7E58 were further verified by dot blot analysis: 50µl samples of gradient fractions were adsorbed onto nitrocellulose using a BRL Hybri-Dot manifold apparatus, then the filters were blocked twice for 30min in 100ml 3% gelatin in TBS at 37°C with gentle shaking. Antibody incubations were carried out as described for Western blot analysis (Section 2B3.3). The primary antibody was MAb 10503 used at 1/5000 dilution and the secondary antibody was anti-mouse IgG whole molecule peroxidase conjugate used at 1/1000 dilution. Proteins were detected by ECL.

2B5 Library screening

A bacteriophage λ ZAPII HeLa cDNA expression library (Section 2A4) was screened with 135kD rabbit antipeptide antisera (Section 3D3) and with a 53mer oligonucleotide (Section 3E3) designed from peptide sequence information in an attempt to isolate a cDNA clone encoding the 135kD protein sequence. The *E.coli* bacterial strain used for these experiments was XL-1 Blue (Section 2A5).

2B5.1 Titration of library

The library titre was determined as follows: 2-3 fresh XL-1 blue colonies grown on LB agar plates with tetracycline at 50µg/ml were inoculated into 50ml 2YT broth supplemented with 0.2% maltose and grown overnight with shaking at 37°C. The maltose induces expression of the maltose operon which contains the *lamb* gene encoding the bacteriophage λ receptor which is presented on the bacterial cell wall. The cells were then pelleted at 4krpm for 10min at RT and resuspended in 20ml sterile 0.01M $MgSO_4$ at RT before storage at 4°C for up to 3 days. The OD_{600} of the cell suspension was read and the bacteria were diluted to OD_{600}=2. Dilutions of library bacteriophage were prepared in 2YT broth and 100µl dilutions mixed with 100µl bacterial suspension in a shaking incubator at 37°C for 20min to adsorb the bacteriophage to the cells. Top Agar (3ml, prewarmed to 37°C) was added and the

mixture which was poured onto dry 90mm LB agar plates and allowed to set. Plates were incubated overnight at 37°C and the plaques on the bacterial lawn were counted.

2B5.2 Antibody screening

The bacteriophage λ library was plated out on 140mm plates at $7X10^4$ pfu per plate by scaling up the procedure above: bacteriophage in 470µl 2YT broth were mixed with 230µl bacteria and, following adsorption, 7ml 47°C Top Agar were added and the mix was poured onto the plates. Plates were incubated at 42°C for 3-4hr until plaques became visible. Circular nitrocellulose discs (137mm diameter) were marked with a ball point pen, soaked in 10mM IPTG, air dried and placed on the agar, taking care not to trap air between the disc and the agar. The plates were then marked with lines corresponding to those on the disc (to enable the plate and disc to be lined up later for picking plaques) and incubated at 37°C to allow protein expression and transfer to the membrane. The plates were then cooled at 4°C for 30-45min prior to lifting the discs which were washed briefly in TBS. Blocking was carried out at 37°C in a shaking incubator in one of three ways (in an attempt to improve the results): (i) 2X 30min in 100ml 3% gelatin in TBS, or (ii) 2X 30min in 5% dried milk, 0.1% NP40 in PBS(A) or, (iii) in the same buffer as in (ii) with 0.01% sodium azide for 30min and overnight. Following this, discs were washed twice for 5min at RT in TTBS and incubated overnight at RT with the r664/2 rabbit antiserum (see Section 3D3) diluted to 1/1000 in 30ml 1%gelatin in TTBS. After five 5min washes in TTBS, the discs were incubated for 90min with protein A horse radish peroxidase at a 1/1000 dilution in 30ml 1% gelatin in TTBS. They were then washed in TTBS three times for 5min and proteins detected by the ECL method (Section 2B3.3(ii)) using 5ml total volume of the mixed reagents.

Plaques thought to contain bacteriophage expressing peptides recognised by the antisera were picked into 1ml 2YT which was vortexed thoroughly and stored at 4°C. Serial dilutions were made and the samples were titrated for a second round of screening on 90mm plates. The protocol outlined above was followed on an appropriately smaller scale to isolate single plaques thought to express 135kD derived polypeptides.

2B5.3 DNA-DNA hybridisation screening

(i) radiolabeling of probe

The 53mer oligonucleotide was 5' end labelled in a reaction containing 20nmol DNA, 100µCi $\gamma^{32}P$ ATP with T4 polynucleotide kinase in the appropriate buffer. Following heat inactivation of the enzyme by incubation at 70°C for 10min, the reaction mix was loaded on a Sephadex G-50 'nick' column (Section2B1.15(iii)) to separate labelled

oligonucleotide from unincorporated γ^{32}P ATP. The probe fractions were pooled and approximately 10nmol was used for each 140mm diameter disc.

(ii) Transfer of library DNA to nitrocellulose filters

As for the antibody screening procedure, the bacteriophage λ library was plated out on 140mm plates at $7X10^4$ pfu per plate. Plates were incubated at 37°C for about 4hr until plaques appeared and then at 4°C overnight. The 137mm diameter nitrocellulose discs were marked with ballpoint pen, placed on the agar for 10min, and removed and air dried for 10min. A duplicate disc was also lifted from each plate. The discs were placed, DNA side up, on Whatman 3mm paper soaked in 0.2M NaOH, 1.5M NaCl for 2min, then they were transferred to 3mm paper soaked in 0.4M Tris.HCl (pH 7.6), 2X SSC for 2min and finally to 3mm paper soaked in 2X SSC. Following baking in a vacuum oven at 80°C for 90min, prehybridisation was carried out at the appropriate temperature for 4hr in 5X SSC, 5X Denhardt's solution, 0.5% SDS and 20μg/ml salmon sperm DNA before addition of the probe. The incubation was continued at the same temperature overnight, then the discs were washed for 10min at RT and at the hybridisation temperature for 2hr in 2X SSC, containing 0.1% SDS. The filters were sealed in plastic bags and exposed overnight to Kodak X-OMAT S film with an intensifying screen. Duplicate filter autoradiographs were compared and plaques to which the probe had hybridised were picked as in Section 2B5.2 and plated out on 90mm plates for re-screening.

CHAPTER 3 RESULTS

3A Expression of C-terminal residues of Vmw110 as GST fusion proteins and investigations of their abilities to interact with cellular proteins in GST 'pull-down' assays

3A1 Introduction

The aim of this project was to identify and characterise cellular proteins which interact with the HSV-1 immediate-early protein Vmw110. Two observations prior to the start of this work indicated that Vmw110 may interact with cellular proteins via residues in its C-terminal end. Firstly, immunofluorescence experiments with a deletion mutant virus, D14, which is missing Vmw110 residues 680-720, showed that the Vmw110 protein expressed by this virus no longer localised to ND10 domains at early times of infection but was diffusely spread throughout the nucleus (Everett and Maul, 1994). Secondly, in transfection assay systems, insertion and deletion mutations in the C-terminal 120 residues significantly decreased the ability of Vmw110 to transactivate gene expression, particularly in synergy with Vmw175 (Everett, 1987a, 1988a; see Section 1D5.1). A run of serine residues immediately prior to the C-terminal 180 residues of Vmw110 indicates that this is possibly a domain boundary. My initial experiments involved bacterial expression and purification of the putative C-terminal domain of Vmw110 prior to investigating interactions between it and host cell proteins.

There are a variety of methods which aim to detect specific protein:protein interactions, and one of the most widely used involves the construction of fusion proteins between the protein of interest and *Schistosoma japonicum* glutathione S-transferase. The expressed GST fusion proteins can be purified from bacterial lysates by adsorption to glutathione agarose beads (Smith and Johnson, 1988). The beads serve as an affinity matrix, allowing proteins to bind to the immobilised target fusion protein. Bound proteins can be partially purified by a simple centrifugation step, hence the name 'pull-down' assay.

Purified GST fusion proteins have been used in a variety of assays either to investigate known protein:protein interactions or, as described in this Thesis, to identify novel interactions. An example of the former case includes the expression of GST fusions with SV40 large T antigen residues; these were used to define more clearly the regions of SV40 large T antigen with which the DNA binding proteins TEF-1 and TBP interact (Gruda *et al.*, 1993). In another example, 'pull-down' assays using a GST-SW15 fusion protein and partially purified yeast cell extracts supplemented with a radiolabelled DNA probe were carried out; this led to the identification of a previously cloned protein, GRF10, which interacts with SW15 to form a DNA binding complex (Brazas and Stillman, 1993).

In the investigations carried out in this project, GST fusion proteins immobilised on glutathione agarose beads were used in a 'pull-down' assay of the type outlined in Figure 3A1 in an attempt to identify novel interactions between Vmw110 residues and cellular proteins: beads-bound fusion proteins were mixed with radiolabelled cellular protein extracts, then the complexes were isolated by centrifugation and washed in a suitable buffer. Cellular proteins which had bound to the fusion proteins were analysed by polyacrylamide gel electrophoresis. The use of whole cell extracts in the experiments ensured, as far as possible, that any interactions detected between Vmw110 residues and cellular proteins were not artifacts which arose as a result of over-representation of particular cellular proteins. This method of identifying specific protein:protein interactions has been employed previously: use of a GST-pRb fusion protein showed that the RB1 gene product interacts with at least seven cellular proteins (Kaelin *et al.*, 1991). A 60kD protein, RBP60, was later identified as an essential component of the pRb-E2F DNA binding complex in gel retardation assays (Ray *et al.*, 1992). Although a specific link between the proteins identified by these groups has not yet been established, the different methods utilised provide an example of a way in which an interaction seen in one experimental system can (perhaps) be verified in another. This is important because it lessens the possibility of an observed interaction being an artifact of the experimental system employed.

The experiments in this Section describe the optimisation of the 'pull-down' assay conditions and subsequent use of the assay in defining more precisely Vmw110 residues involved in interacting with a 135kD cellular protein. The assay was also used to investigate the half-life and cellular location of the 135kD protein. Finally, a collaborative experiment with other members of the group showed that the 135kD protein pulled down in GST assays is likely to be the same as that co-immune precipitated with intact, purified Vmw110.

Figure 3A1

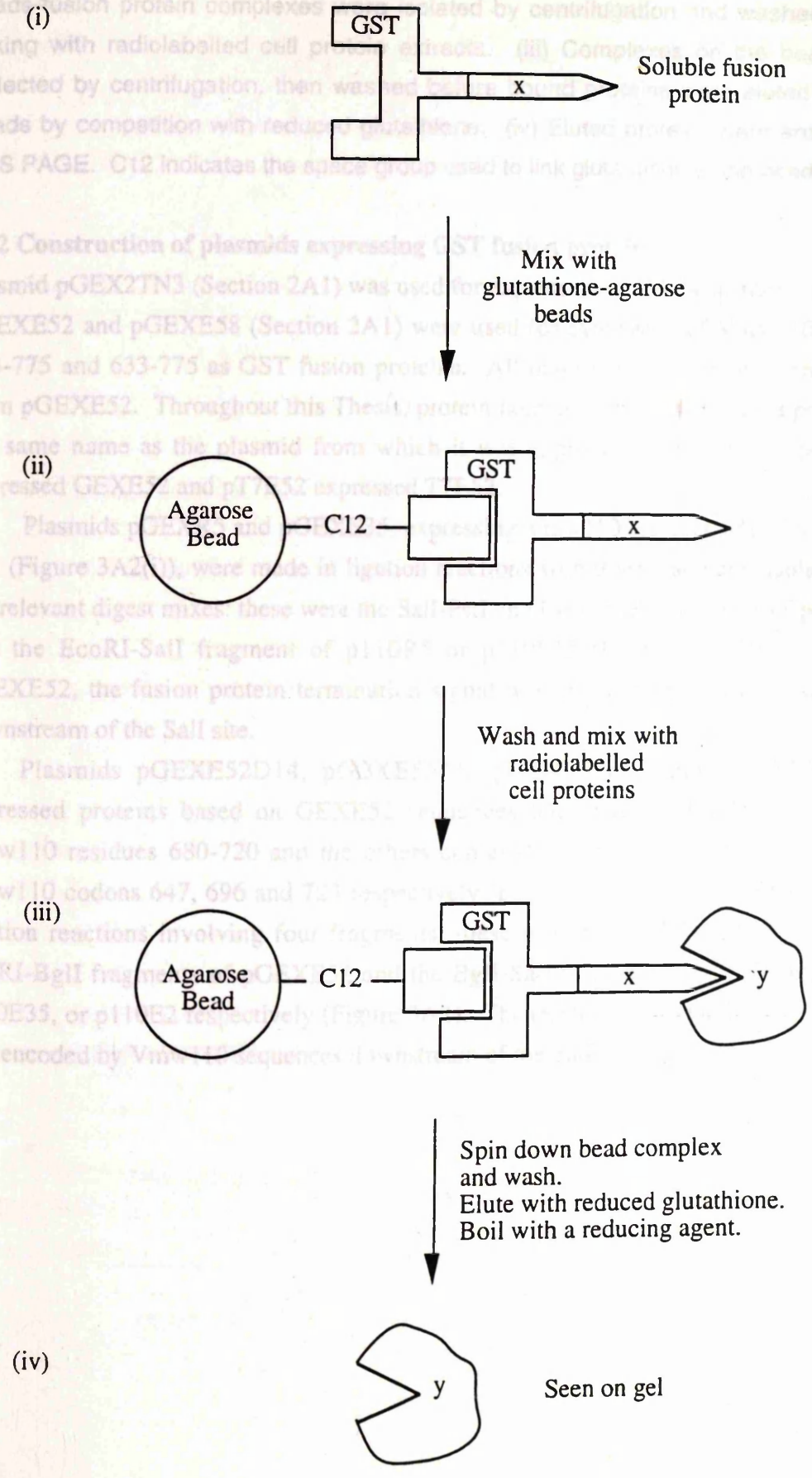

(i)
GST
x
Soluble fusion protein
Mix with glutathione-agarose beads
(ii)
Agarose Bead
C12
GST
x
Wash and mix with radiolabelled cell proteins
(iii)
Agarose Bead
C12
GST
x
y
Spin down bead complex and wash.
Elute with reduced glutathione.
Boil with a reducing agent.
(iv)
y
Seen on gel

Figure 3A1 Outline of the GST 'pull-down' experimental strategy. (i) A bacterial extract containing a GST fusion protein was mixed with glutathione agarose beads and (ii) beads-fusion protein complexes were isolated by centrifugation and washed prior to mixing with radiolabelled cell protein extracts. (iii) Complexes on the beads were collected by centrifugation, then washed before bound proteins were eluted from the beads by competition with reduced glutathione. (iv) Eluted proteins were analysed by SDS PAGE. C12 indicates the space group used to link glutathione to the beads.

3A2 Construction of plasmids expressing GST fusion proteins

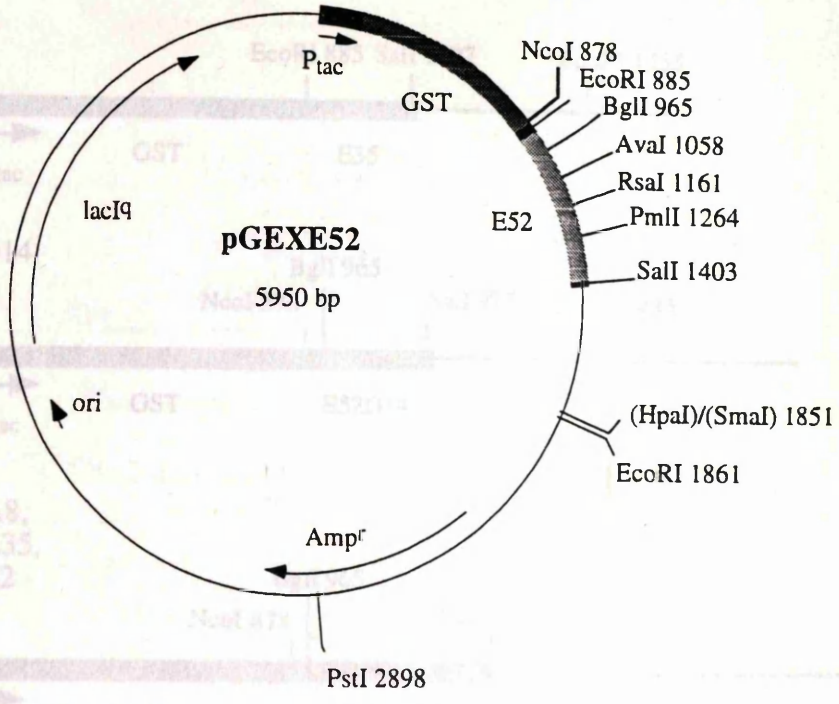
Plasmid pGEX2TN3 (Section 2A1) was used for expression of GST sequences. Plasmids pGEXE52 and pGEXE58 (Section 2A1) were used for expression of Vmw110 residues 594-775 and 633-775 as GST fusion proteins. All other pGEX plasmids were derived from pGEXE52. Throughout this Thesis, protein nomenclature is such that a protein has the same name as the plasmid from which it was expressed, for example, pGEXE52 expressed GEXE52 and pT7E52 expressed T7E52.

Plasmids pGEXR5 and pGEXE35, expressing Vmw110 residues 681-775 and 696-775 (Figure 3A2(i)), were made in ligation reactions with three fragments isolated from the relevant digest mixes: these were the SalI-PstI and PstI-EcoRI fragments of pGEXE52 and the EcoRI-SalI fragment of p110R5 or p110E35 (Figure 3A2(i)B). As with pGEXE52, the fusion protein termination signal was encoded by Vmw110 sequences downstream of the SalI site.

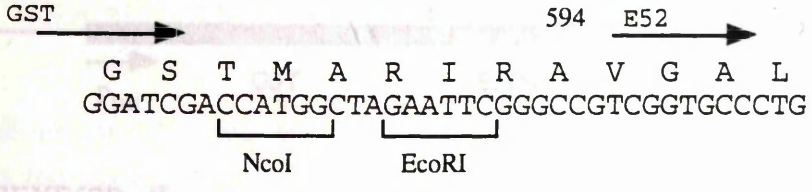
Plasmids pGEXE52D14, pGEXE52A8, pGEXE52E35 and pGEXE52E2 all expressed proteins based on GEXE52 sequences but protein E52D14 was missing Vmw110 residues 680-720 and the others contained 12bp EcoRI linker insertions at Vmw110 codons 647, 696 and 723 respectively (Figure 3A2). These were all made in ligation reactions involving four fragments: these were the SalI-PstI, PstI-EcoRI and EcoRI-BglI fragments of pGEXE52 and the BglI-SalI fragment of p110D14, p110A8, p110E35, or p110E2 respectively (Figure 3A2). The termination signal in each case was that encoded by Vmw110 sequences downstream of the SalI site.

Figure 3A2

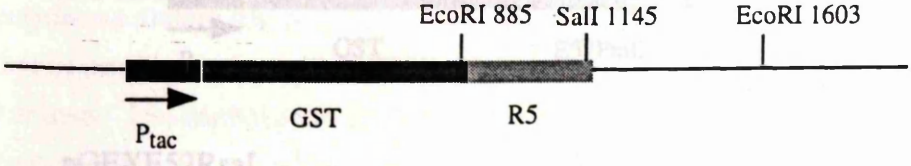
(i)



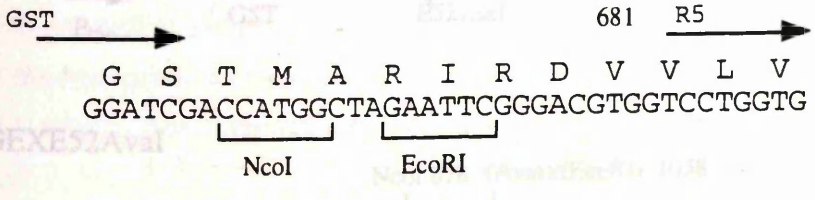
GST-E52 junction

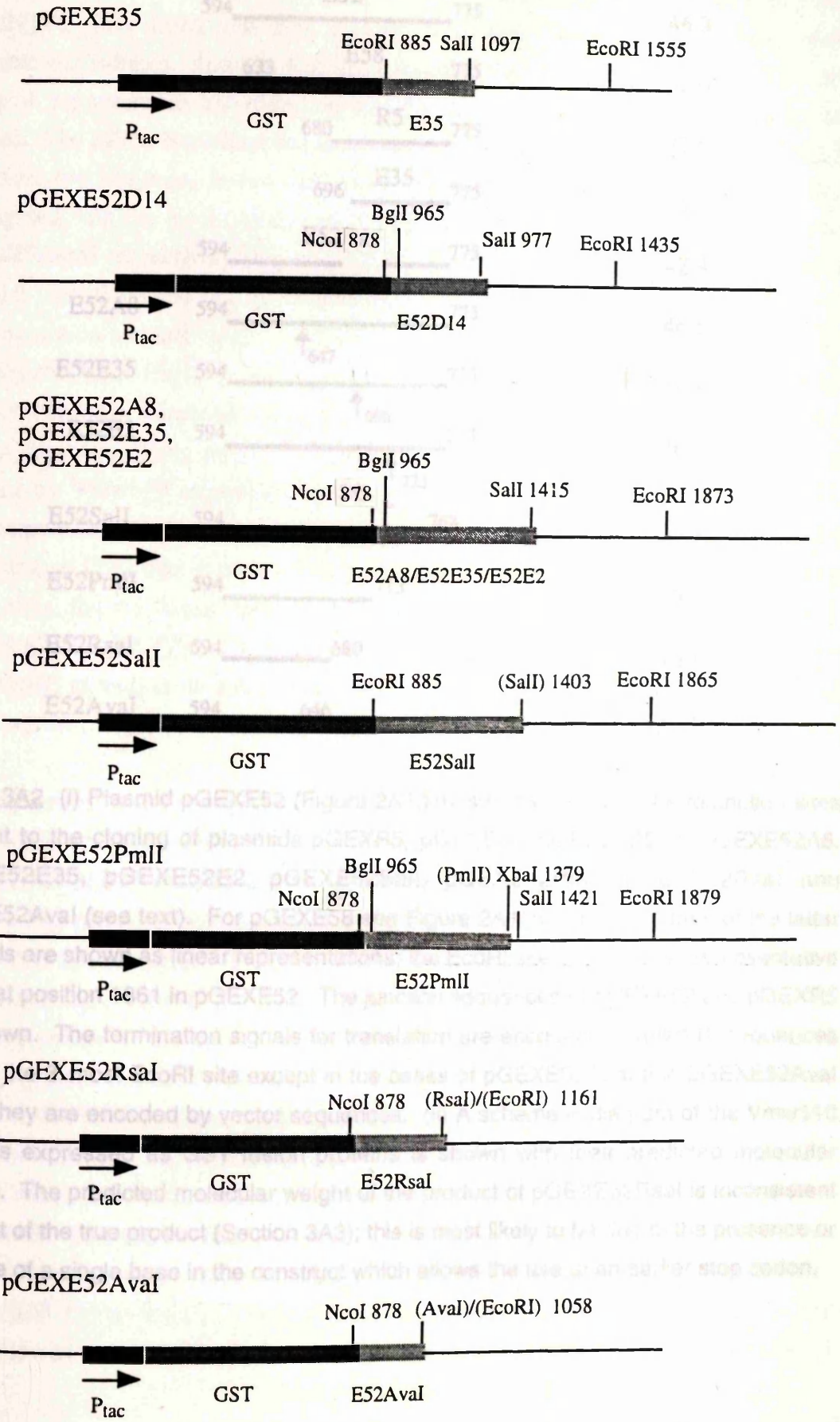


pGEXR5

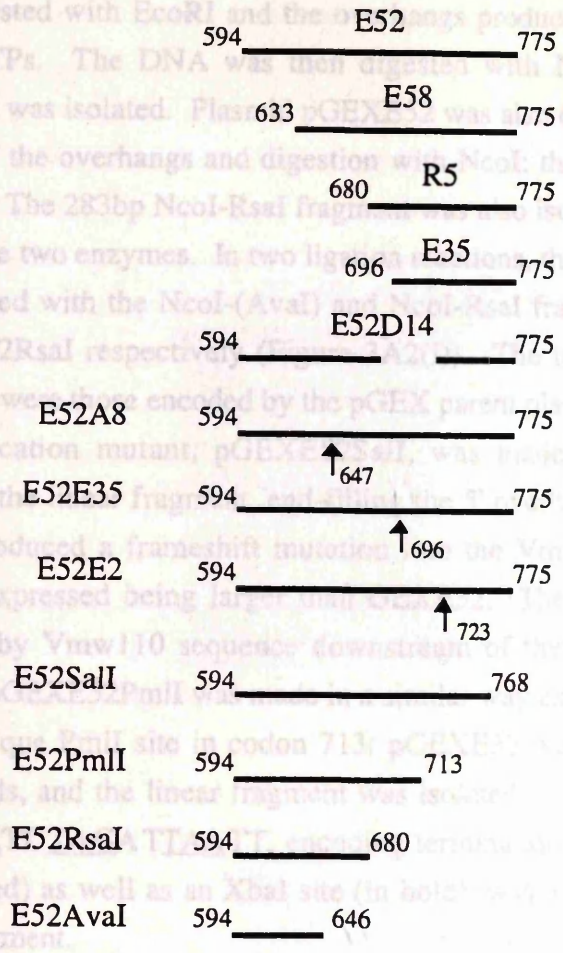


GST-R5 junction




pGEXE35
EcoRI 885
SalI 1097
EcoRI 1555
Ptac
GST
E35
pGEXE52D14
BglI 965
NcoI 878
SalI 977
EcoRI 1435
Ptac
GST
E52D14
pGEXE52A8,
pGEXE52E35,
pGEXE52E2
BglI 965
NcoI 878
SalI 1415
EcoRI 1873
Ptac
GST
E52A8/E52E35/E52E2
pGEXE52SalI
EcoRI 885
(SalI) 1403
EcoRI 1865
Ptac
GST
E52SalI
pGEXE52PmlI
BglI 965
(PmlI) XbaI 1379
NcoI 878
SalI 1421
EcoRI 1879
Ptac
GST
E52PmlI
pGEXE52RsaI
NcoI 878
(RsaI)/(EcoRI) 1161
Ptac
GST
E52RsaI
pGEXE52AvaI
NcoI 878
(AvaI)/(EcoRI) 1058
Ptac
GST
E52AvaI

(ii)



	Predicted fusion protein size /kD
E52	46.3
E58	42.0
R5	37.6
E35	35.8
E52D14	42.4
E52A8	46.8
E52E35	46.8
E52E2	46.8
E52SalI	53.2
E52PmlI	39.7
E52RsaI	40.1
E52AvaI	33.1

Figure 3A2 (i) Plasmid pGEXE52 (Figure 2A1(iii)) is shown, with all the restriction sites relevent to the cloning of plasmids pGEXR5, pGEXE35, pGEXE52D14, pGEXE52A8, pGEXE52E35, pGEXE52E2, pGEXE52SalI, pGEXE52PmlI, pGEXE52RsaI and pGEXE52AvaI (see text). For pGEXE58 see Figure 2A1(ii). The structures of the latter plasmids are shown as linear representations; the EcoRI site in each case representative of that at position 1861 in pGEXE52. The junction sequences of pGEXE52 and pGEXR5 are shown. The termination signals for translation are encoded by Vmw110 sequences prior to the 3' most EcoRI site except in the cases of pGEXE52RsaI and pGEXE52AvaI where they are encoded by vector sequences. (ii) A schematic diagram of the Vmw110 residues expressed as GST fusion proteins is shown with their predicted molecular weights. The predicted molecular weight of the product of pGEXE52RsaI is inconsistent with that of the true product (Section 3A3); this is most likely to be due to the presence or absence of a single base in the construct which allows the use of an earlier stop codon.

The GEXE52 truncation mutants pGEXE52RsaI and pGEXE52AvaI, expressing Vmw110 residues 594-680 and 584-646 respectively, were made as follows: pGEXE52 was digested with EcoRI and the overhangs produced filled in using Klenow fragment and dNTPs. The DNA was then digested with NcoI and the NcoI-(EcoRI) vector fragment was isolated. Plasmid pGEXE52 was also digested with AvaI followed by end-filling of the overhangs and digestion with NcoI: the 180bp NcoI-(AvaI) fragment was isolated. The 283bp NcoI-RsaI fragment was also isolated from a digestion of pGEXE52 with these two enzymes. In two ligation reactions, the pGEXE52 NcoI-(EcoRI) fragment was ligated with the NcoI-(AvaI) and NcoI-RsaI fragments to give pGEXE52AvaI and pGEXE52RsaI respectively (Figure 3A2(i)). The termination codons utilised in these plasmids were those encoded by the pGEX parent plasmid, pGEX2TN3.

The truncation mutant, pGEXE52SalI, was made by cutting pGEXE52 with SalI, isolating the linear fragment, end-filling the 5' overhangs and religating the 'flush' ends. This introduced a frameshift mutation into the Vmw110 sequences with the resultant protein expressed being larger than GEXE52. The termination signal, however, was encoded by Vmw110 sequence downstream of the position of the mutated SalI site. Plasmid pGEXE52PmlI was made in a similar way except that XbaI linkers were inserted at the unique PmlI site in codon 713: pGEXE52 was digested with PmlI, which gives 'flush' ends, and the linear fragment was isolated. An 18bp self complementary linker, AATTAA**TCTAGA**TTAATT, encoding termination codons in all three reading frames (underlined) as well as an XbaI site (in bold), was added to a ligation reaction with the linear fragment.

3A3 Expression of GST fusion proteins

The plasmid constructs described in Section 3A2 were transformed into the *E.coli* strain BL21 (Section 2B1.7). Cultures were grown, then fusion protein expression was induced from the P_{tac} promoter by the addition of IPTG and protein extracts were prepared as described in Section 2B3.1. Extracts containing GST protein were prepared from *E.coli* BL21 transformed with pGEX2TN3 for use in control experiments.

Expression of the fusion proteins was ascertained by SDS-PAGE analysis of bacterial protein extracts. The gels were stained with Coomassie blue and GST fusion proteins of the predicted sizes were observed amongst a background of bacterial proteins (results not shown). To confirm expression of the expected proteins, samples of bacterial extracts were Western blotted onto nitrocellulose following resolution of proteins by SDS-PAGE, and GST-Vmw110 fusion proteins were detected using the mouse monoclonal antibodies 10503 or 10810: the former recognises an epitope between residues 696-775 of Vmw110 and the latter an epitope between residues 594-633. The results showed that all of the constructs expressed proteins of the sizes expected relative

to the GEXE52 fusion protein and that the GST protein ('c' tracks) was not detected by either antibody (Figure 3A3(i)).

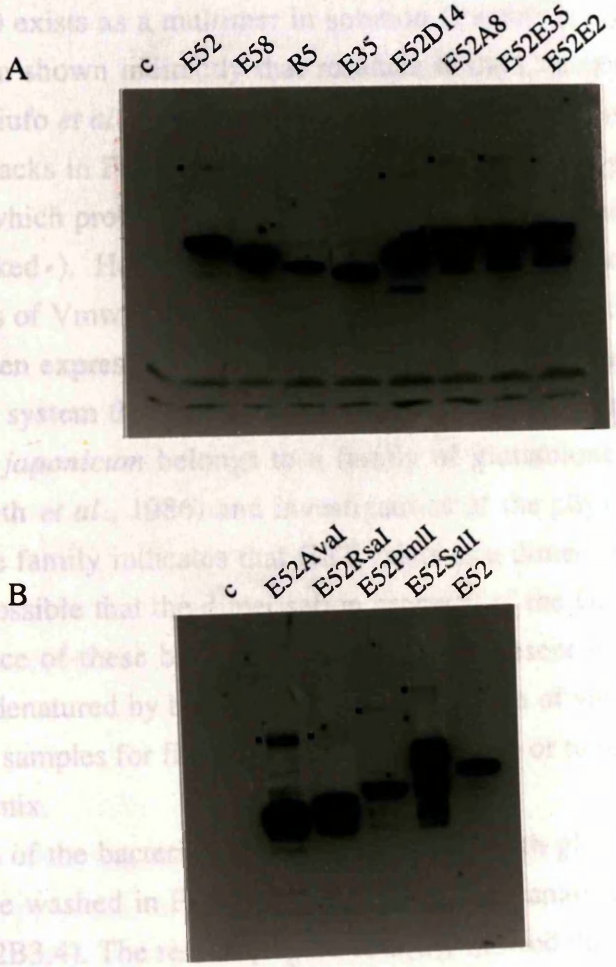


Figure 3A3(i) Western blot analysis of bacterial extracts containing GST fusion proteins. From 100ml cultures induced with IPTG, 2ml of bacterial protein extracts were prepared and 5µl samples were subjected to SDS PAGE and the proteins Western blotted onto nitrocellulose. In panels A and B the major band in each track is the GST fusion protein indicated by the label above each track. The 'c' tracks show the bacterial extract containing GST sequences alone. The monoclonal antibody 10503 was used to detect the proteins in A and 10810 those in B. Both antibodies were used at a dilution of 1/5000. Bands representing dimerised fusion proteins are marked '•'.

It was evident from the blots that the level of fusion protein expression from any one plasmid construct could vary: for example, compare the GEXE52AvaI with GEXE52. This was reflected in the amounts of protein which bound to the glutathione agarose beads (see below). Some of the fusion proteins showed evidence of low levels of degradation: for example, the GEXE52 insertion mutants. The higher level of degradation seen with the GEXE52SalI fusion protein was lessened with use of a freshly prepared extract.

Vmw110 exists as a multimer in solution (Everett *et al.*, 1991; Chen *et al.*, 1992) and it has been shown indirectly that residues in the C-terminal end of the protein are responsible (Ciufo *et al*, 1994) (Sections 1D11 and 3B1). It is of interest to note that in many of the tracks in Figure 3A3(i), above the major fusion protein band there is a less intense band which probably represents fusion proteins present in a dimeric form (these bands are marked ▪). However, it is difficult to draw conclusions from these results as to which residues of Vmw110 are necessary for multimerisation: E58 sequences appear not to dimerse when expressed as fusion proteins yet when expressed in isolation using the T7 expression system this domain forms multimers in solution (Section 3B). The GST enzyme of *S. japonicum* belongs to a family of glutathione transferase enzymes (EC 2.5.1.18) (Smith *et al.*, 1986) and investigations of the physical properties of a human member of the family indicates that GST exists as a dimer in solution (Warholm *et al.*, 1983). It is possible that the dimerisation property of the GST sequences is responsible for the presence of these bands and that they are present only in samples which were incompletely denatured by boiling. It would have been of value to repeat the experiment but to boil the samples for five minutes rather than two or to use higher amounts of 2-ME in the boiling mix.

Aliquots of the bacterial extracts were mixed with glutathione agarose beads, then the beads were washed in PBS(A) and samples were analysed on SDS polyacrylamide gels (Section 2B3.4). The results (Figure 3A3(ii)) showed that the various fusion proteins bound to the beads to different extents: for example, compare GEXE58 with GEXR5. The degradation seen with some fusion proteins, particularly with GEXR5, GEXE35 and GEXE52D14, was minimised by the use of fresh extracts. It was necessary to be careful that in the 'pull-down' assays, equivalent amounts of the various beads-bound fusion proteins were used. Consequently, the beads-fusion protein complexes were always analysed by SDS PAGE prior to use in 'pull-down' assays.

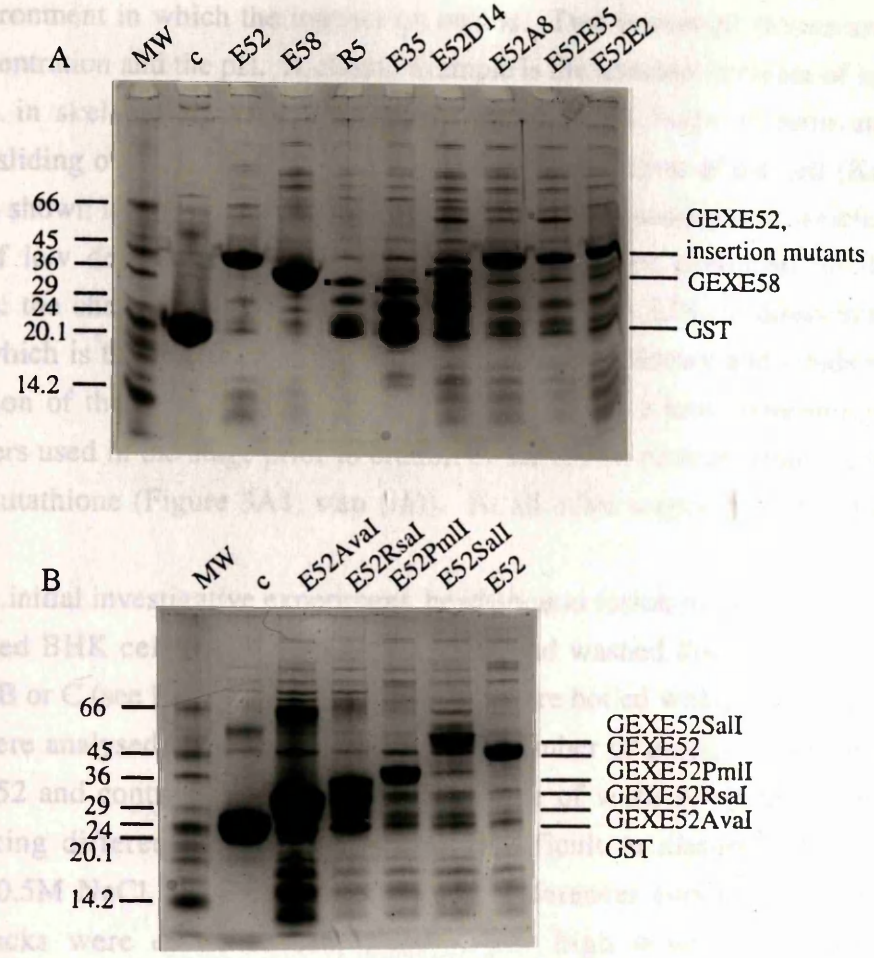


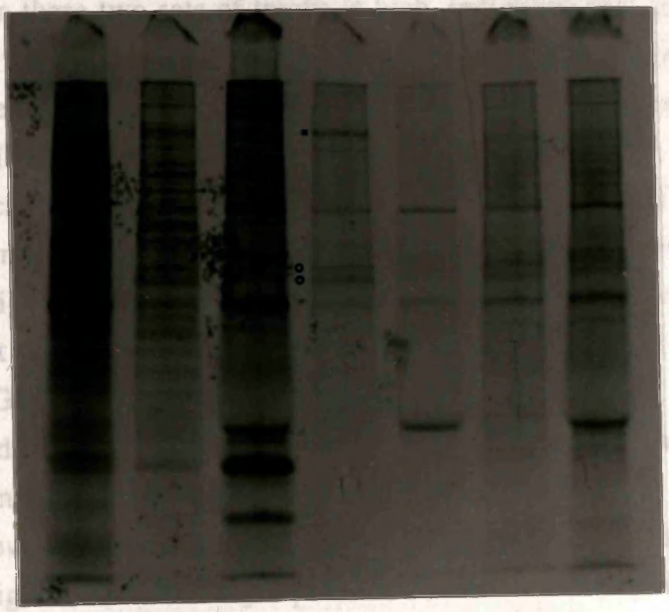
Figure 3A3(ii) Coomassie stained polyacrylamide gels showing samples of fusion proteins bound to beads. Aliquots of bacterial extracts (300µl) were incubated with 10µl of a 50% slurry of glutathione agarose beads for 1hr at 4°C. The beads were washed three times in 1ml cold PBS(A), boiled with boiling mix and samples were loaded on 12.5% polyacrylamide gels. The bands in each track showing beads-bound fusion protein are indicated by the labels at the sides of each photograph, except in the cases of GEXR5, GEXE35 and GEXE52D14 where they are marked • . Degradation products are most evident in these latter tracks. The 'c' tracks show beads-bound GST protein in a control experiment. 'MW' indicates molecular weight marker proteins whose sizes are shown.

3A4 Optimisation of the GST 'pull-down' assay

The extent to which two proteins interact with each other within a cell depends upon the microenvironment in which the interaction occurs. Two important factors are the local ionic concentration and the pH. A classic example is the transient increase of intracellular Ca^{2+} seen in skeletal muscle cells of vertebrates which leads to actin and myosin filaments sliding over one another with subsequent contraction of the cell (Katz, 1966). Another is shown in the change in pH inside a macrophage endocytotic vesicle following binding of low density lipoprotein (LDL) to the receptor presented on the plasma membrane: the change in pH inside the vesicle causes the LDL to dissociate from the receptor which is then recycled to the plasma membrane (Brown and Goldstein, 1986). Optimisation of the 'pull-down' assay involved varying the ionic concentration of the wash buffers used in the stage prior to elution of the fusion proteins from the beads with reduced glutathione (Figure 3A1, step (iii)). At all other stages the ionic strength was low.

In an initial investigative experiment, beads-bound fusion proteins were mixed with radiolabelled BHK cell extracts (Section 2B2.2) and washed three times in one of the buffers A, B or C (see Figure 3A4(i)). The beads were boiled with gel loading buffer and samples were analysed by SDS PAGE. A large number of proteins remained bound to the GEXE52 and control (GST) beads with the use of wash A which contained 0.1M NaCl, making differences between the tracks difficult to discern. Wash B, which contained 0.5M NaCl, was more stringent and differences between the GEXE52 and control tracks were easier to see; there is one high molecular weight band of approximately 135kD (denoted ▪) in the GEXE52 track which is not present in the control track and also two lower molecular weight bands (denoted ∘). In this experiment, the latter two bands were also present in the control track of GST beads washed in wash C, containing LiCl. The 135kD band was also visible in the wash A E52 track but it was more difficult to see due to the high background.

As the high molecular weight protein consistently remained bound to the E52 beads in a 0.5M NaCl wash, the assay was further modified such that beads were mixed with radiolabelled cellular proteins in a 0.5M NaCl buffer. The presence of bands in the control tracks indicated that cellular proteins bound to either the glutathione agarose beads, the GST protein or both and so extracts were 'pre-cleared' of these proteins prior to incubation with GEXE52 beads by incubation with the GST beads. To be sure that the proteins seen in the experiments were interacting with the GST fusion proteins, complexes were eluted from the glutathione agarose beads by competition with reduced glutathione. Under these conditions (the standard GST 'pull-down' assay, Section 2B3.4) two high molecular weight proteins bound more strongly to the GEXE52 beads and were still not present in the control tracks; the additional protein seen compared to that seen in the experiment of Figure 3A4(i) was estimated to be approximately 150kD.



Wash A	Wash B	Wash C
50mM Tris pH8	50mM Tris pH8	0.6M LiCl
100mM NaCl	0.5M NaCl	0.1M Tris pH8
1mM EDTA	1mM EDTA	0.1% BME
0.5% NP40	0.5% NP40	

Each buffer also contained protease inhibitors;
1mM PMSF, 0.5µg/ml leupeptin, 40µg/ml bestatin

Figure 3A4(i) Investigative GST 'pull-down' assay. Aliquots (20µl) of a 50% slurry of control GST beads and GEXE52 beads were added to 300µl quantities of ^{35}S-radiolabelled BHK cell extracts (Section 2B2.2) and incubated for 1hr at 4°C. Beads were then spun down and washed three times for 5min in 1ml of one of the three wash buffers. Boiling mix was then added and the samples loaded on a 12.5% polyacrylamide gel which was dried down and exposed to film following electrophoresis. A 1µl sample of the ^{35}S-radiolabelled BHK cell extract was run in the far left-hand track. The tracks marked 'E52' show proteins bound to the GST beads and those marked 'c' proteins bound to the GST beads. • denotes the high molecular weight band discussed in the text and ∘ the two lower molecular weight bands.

Figure 3A4(ii) shows the results of a typical 'pull-down' experiment carried out with the GEXE52 and GEXE58 fusion proteins using the standard assay conditions. The background in these two sets of tracks is high, indicating that these fusion proteins are very 'sticky'. It was found that the background in these experiments varied widely, although experiments carried out within the same 2-4 week period were generally found to show similar background proteins. The increased number of differences seen in experiments carried out a few months apart may be a reflection of the different batches of cells used to make the radiolabelled extracts. A close examination of the experiment in Figure 3A4(ii) shows that many of the background bands are present in the control (c) tracks, albeit more faintly. The major band in the control tracks is likely to be mammalian GST which is the same size as the helminth protein. This band was present only in 'pull-down' experiments where proteins were analysed on 12.5% acrylamide gels and was absent in those analysed on gels of a lower percentage.

'Pull-down' experiments with the GEXE52 fusion protein showed that the binding of the two high molecular weight proteins seen in initial experiments was completely reproducible. Other bands were also seen (examples marked •) and the interactions of these proteins with Vmw110 may be significant. However, they were not pursued further, either because they were present inconsistently or because they were very faint and an alteration in the assay conditions would probably have been necessary in assisting further investigation.

Of the two high molecular weight bands pulled down by GEXE52, the lower of the two, the 135kD band, is missing in the GEXE58 track. This shows that residues 594-633 of Vmw110 are essential for the interaction with this protein in this system. The details of 135kD interaction with Vmw110 residues expressed as GST fusion proteins were invesigated further and are fully discussed in Section 3A6.

The sizes of radiolabelled cellular proteins pulled down by the GST fusion proteins in these experiments were determined by comparing them with characteristic proteins in the extract tracks (marked 'ex'). The sizes of these proteins were determined by comparing their migration positions on a gel with those of known standards: an example of this analysis is shown in Figure 3A4(iii).

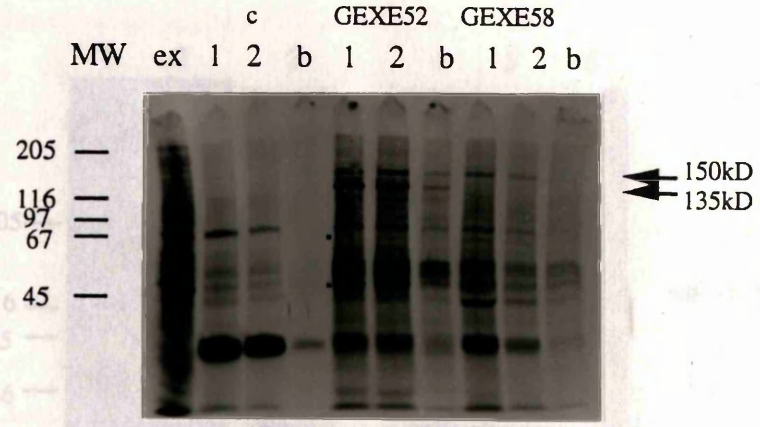


Figure 3A4(ii) GST 'pull-down' assay performed under standard assay conditions. Positions of size standards indicated under 'MW' were ascertained as described in Figure 3A4(iii), following the sizing of predominant bands in the 'ex' track in which a sample of the ^{35}S-radiolabelled BHK cell extract was run. In each set of tracks, 1,2 show proteins eluted from the relevant beads (c (GST), GEXE52, GEXE58) in two successive steps with reduced glutathione. The 'B' tracks indicate proteins remaining on the beads following elution. Two proteins consistently pulled down by the GEXE52 beads are marked as 150kD and 135kD protein bands. Bands marked · in the E52 track show those which may also be significant but because of their relative low abundance, or the fact that they were present inconsistently, they were not analysed further. The strong low molecular weight band present in the 'c' tracks and to an extent in the E52 and E58 tracks is likely to be BHK cell GST enzyme which is approximately the same size as *Schistosoma japonicum* GST.

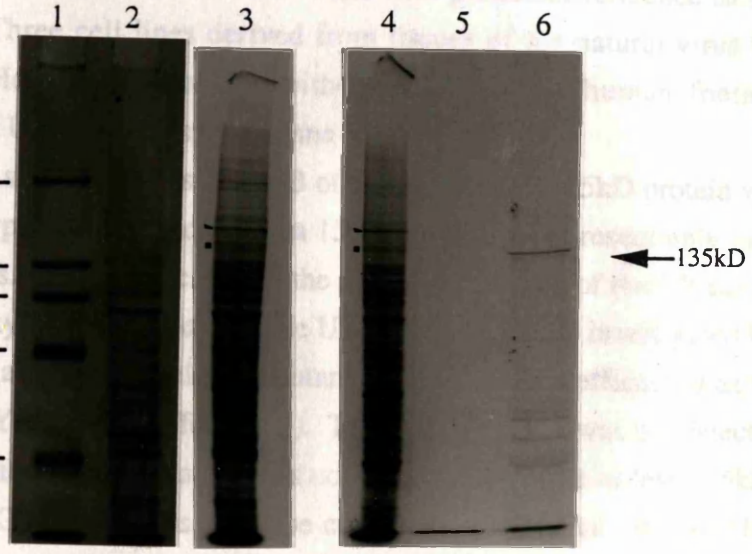


Figure 3A4(iii) Size determination of the 135kD protein. The three panels shown are all of 7.5% polyacrylamide gels. The left-hand panel shows a Coomassie stained gel which was dried down and exposed to Kodak X-OMAT S film to give the centre panel; track 1 shows molecular weight standards where sizes are marked and track 2 a sample of ^{35}S-methionine radiolabelled WS HeLa cell proteins. Track 3 is an autoradiograph of track 2 - the most abundant proteins were visible in both tracks which enabled a direct comparison of track 3 with track 1. The right-hand panel shows a standard GST 'pull-down' assay; radiolabelled cell extract was run as a standard in track 4, proteins eluted from GST beads in track 5 and proteins eluted from GEXE52 beads in track 6. The position of the 135kD protein is marked. This protein was found to consistently co-migrate with a faint group of bands marked • which was below a characteristic band marked - in the WS HeLa cell extracts. A plot of logMW of the standard proteins against distance migrated shows the 135kD protein to migrate at approximately 130kD. The protein was more accurately sized by comparison with the migration position of ICP6 (HSV-1 large subunit of ribonucleotide reductase); ICP6 has a molecular weight of 136kD and the 135kD cellular protein migrates just below it on polyacrylamide gels.

3A5 An investigation of the GEXE52-135kD protein:protein interaction in a number of different cell types

In the previous Section, strong and specific protein:protein interactions were observed between GEXE52 and two BHK cell proteins, of molecular weights 150kD and 135kD. As interactions between Vmw110 and cellular proteins are potentially important for the biology of the virus (Section 1D12), the standard 'pull-down' assay was carried out using a number of different radiolabelled cell extracts with particular reference to looking at human cell lines. Three cell lines derived from tissues of the natural virus host were investigated: WS HeLa (a squamous epithelial line), HFL (human foetal lung, a fibroblastic line) and U-2 OS (an osteosarcoma line).

The results are shown in Parts A and B of Figure 3A5. A 135kD protein was pulled down from all cell types investgated whilst a 150kD protein was present only in the BHK and HFL cell extracts. The significance of the apparent presence of the 150kD protein in only a subset of cell types is not known. The U-2 OS cell line was investigated because it has been shown that a Vmw110 deletion mutant virus plaques as efficiently as wild-type virus on these cells (Yao and Schaffer, 1995). The 150kD protein was not detected in this cell type, and the results showed that there is not significantly more or less 135kD protein pulled down by GEXE52 from this cell type compared with others. It could be argued that the GEXE52 beads were saturated with the 135kD protein and were therefore unable to bind more. However, an investigative experiment during the purification of this protein from WS HeLa cell extracts showed that the amounts of 135kD protein pulled down were limited by the amount of the 135kD protein present in cell extracts rather than by the amount of GEXE52 protein mixed with cell extracts (Section 3C3).

The ND7 cell line was also investigated (Figure 3A5.C). This is a neuronal line originally obtained by the fusion of neuroblastoma cells with primary rat sensory neurons (Wood *et al*, 1990). Experiments were carried out with this line in order to ascertain whether or not the 150kD and 135kD proteins were present in both undifferentiated and differentiated cell extracts. It was thought that the absence or presence of either of these two proteins in differentiated cell extracts compared with undifferentiated cell extracts may shed some light on the mechanism by which Vmw110 acts during the lytic-latent switch in the life cycle of HSV-1. However, the results showed that, as with the WS HeLa and U-2 OS cell extracts, the 150kD protein was not present in either cell extract and also that the 135kD protein was present in similar amounts in both types of ND7 cell extract.

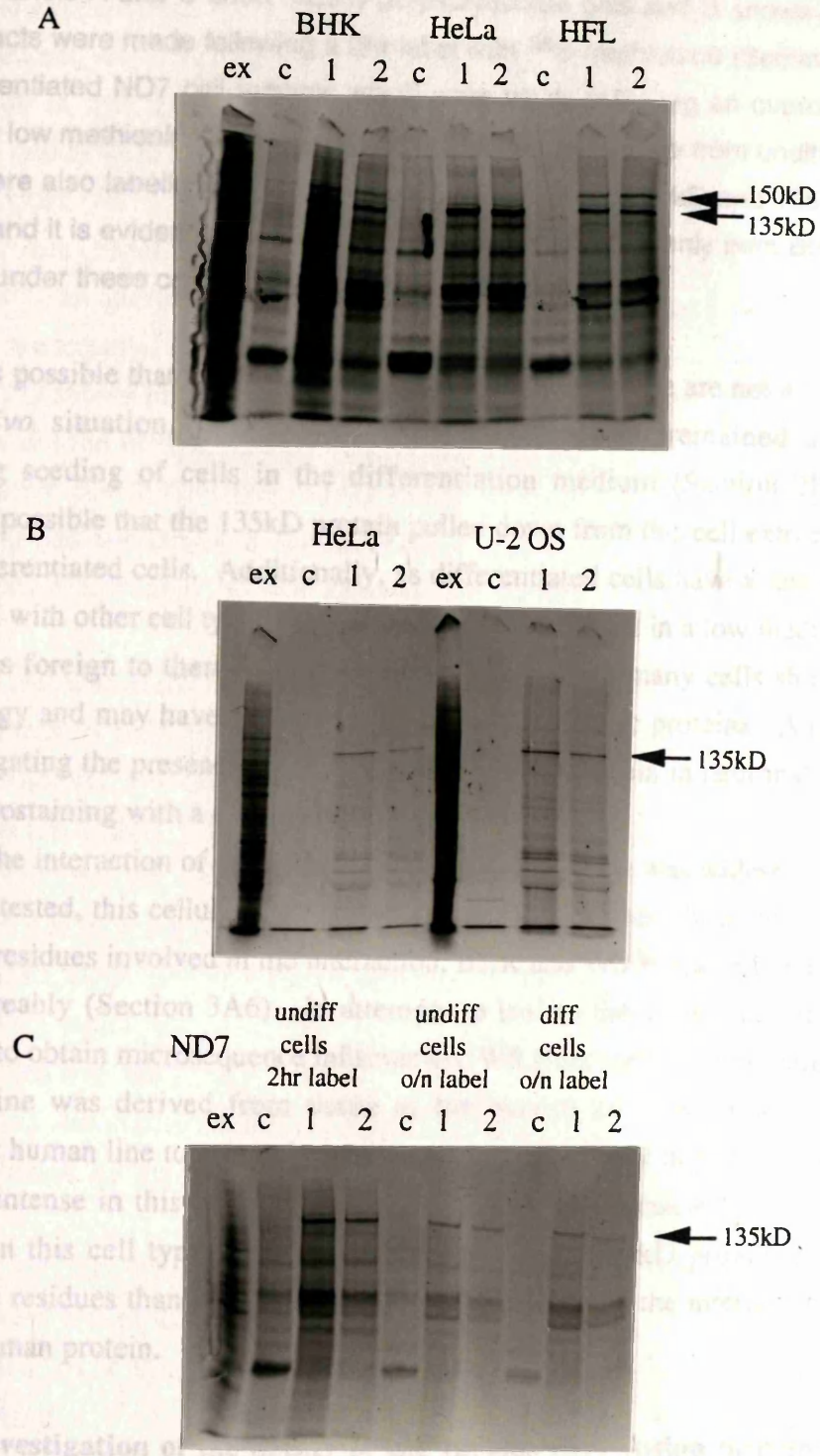


Figure 3A5 GST 'pull-down' experiments with GEXE52 and ^{35}S-methionine radiolabelled extracts derived from a number of different cell types. These experiments show the results of standard GST 'pull-down' assays (Section 2B3.4) using the cell extract indicated above each experiment. The 'ex' tracks show radiolabelled whole cell protein samples, in A from BHK cells, B from WS HeLa and U-2OS cells and in C from an undifferentiated ND7 cell extract. The 'c' tracks show proteins eluted from GST beads with reduced glutathione and 1,2 proteins eluted from GEXE52 beads in two successive

steps. Panels A and C show 12.5% polyacrylamide gels and B shows a 7.5% gel. All cell extracts were made following a 2hr label with ^{35}S-methionine (Section 2B2.2) except for differentiated ND7 cell extracts which were made following an overnight labelling of cells in a low methionine medium. A control extract was made from undifferentiated cells which were also labelled overnight. The positions of the 150kD and 135kD proteins are marked and it is evident that a 150kD protein is pulled down only from BHK and HFL cell extracts under these conditions.

It is possible that the experiments with the ND7 cell line are not a true reflection of the *in vivo* situation. Approximately 20% of the cells remained undifferentiated following seeding of cells in the differentiation medium (Section 2B2.1) and it is therefore possible that the 135kD protein pulled down from this cell extract is contributed by undifferentiated cells. Additionally, as differentiated cells have a low metabolic rate, compared with other cell types, they were labelled overnight in a low methionine medium which was foreign to them (Section 2B2.2). As a result, many cells showed an altered morphology and may have been expressing uncharacteristic proteins. A conclusive way of investigating the presence or absence of the 135kD protein in neuronal cells would be by immunostaining with a suitable anti-135kD antibody.

As the interaction of GEXE52 with the 135kD protein was widespread amongst the cell types tested, this cellular protein was investigated further. In an investigation of the Vmw110 residues involved in the interaction, BHK and WS HeLa cell extracts were used interchangeably (Section 3A6). In attempts to isolate the 135kD protein in sufficient quantities to obtain microsequence information, WS HeLa cell extracts were used because this cell line was derived from tissue of the natural host and it was also the most convenient human line to grow. In addition, it was found that in general the 135kD band was most intense in this cell type. This may have been because the protein was more abundant in this cell type, because the WS HeLa cell 135kD protein contained more methionine residues than that of other cell types, or because the interaction was stronger with the human protein.

3A6 An investigation of the ability of the various GST fusion proteins to bind the 135kD protein

Having established the existence of a strong and specific interaction between GEXE52 and a 135kD cellular protein, standard GST 'pull-down' assays were carried out with all of the GST fusion proteins (Section 2A2) to investigate which residues of the C-terminal domain of Vmw110 were necessary for the interaction. The results are shown in Figure 3A6. Radiolabelled BHK cell extracts were used for the experiments in A, B and C and radiolabelled WS HeLa cell extracts for the experiments in D, E and F. G summarises the results shown in A-F.

It is evident from the results shown in A that GEXE58 did not interact with the 135kD protein, which indicated that residues 594-633 of Vmw110 were necessary for the interaction in this system. As expected, the smaller fusion proteins, GEXE35 and GEXR5, which were also missing Vmw110 residues 594-633, did not interact with the 135kD protein (Panel C). GEXE52D14 was found to bind weakly but reproducibly to the 135kD protein (Panel B). The most likely explanation for this is that the deleted residues contribute to an altered conformation of the domain rather than that the deleted residues, 670-720, are required for binding.

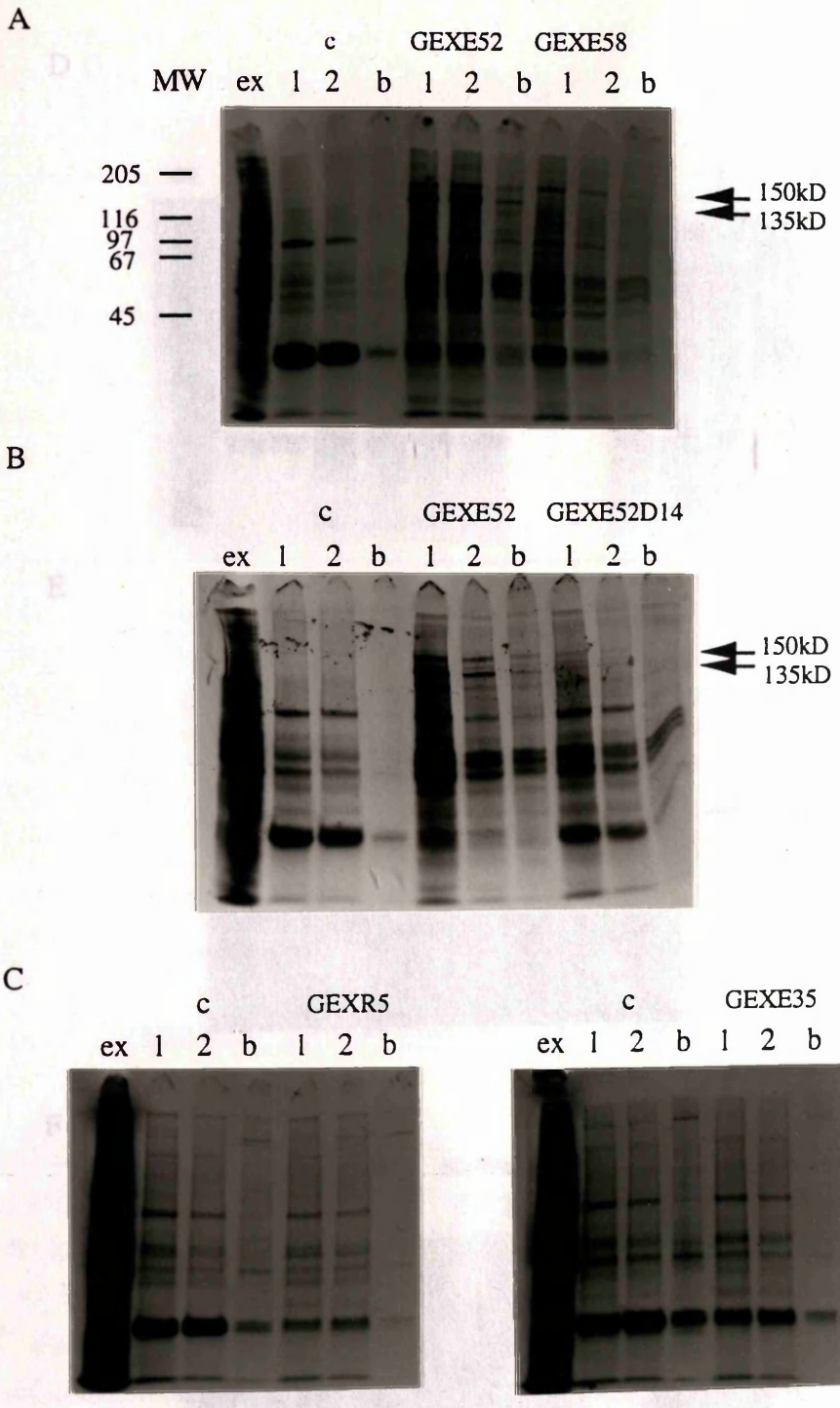
Part D shows that the GEXE52 truncation mutant GEXE52SalI bound the 135kD protein as did the insertion mutant GEXE52E2 whilst GEXE52E35 did not. The strong bands visible just below the 135kD bands in these gels were peculiar only to this experiment and are another example of the variabilty in background which was seen in the 'pull-down' experiments. It is possible that the band represents a 135kD break-down product.

The result with the GEXE52E35 mutant was surprising in view of the fact that the truncation mutant GEXE52AvaI bound the 135kD protein (Panel E). This indicated that Vmw110 residues 594-646 were sufficient for the interaction, yet the four amino acid insertion in GEXE52E35 was at residue 696. It is possible that an insertion mutation can alter the conformation of a domain in such a way as to render it non-functional whilst deletions in a similar region do not adversely affect its folding. Similarly, the insertion at 647 in GEXE52A8 (Panel E) also reduced 135kD binding. As expected from the GEXE52AvaI result, the truncation mutants GEXE52PmlI and GEXE52RsaI (Panel F) also interacted with the 135kD protein.

Panel G summarises these results. It can be concluded that Vmw110 residues 594-633 are essential for the interaction with the135kD protein and that residues 594-646 are sufficient.

Although the interaction with the 150kD protein was not investigated in detail, it was concluded from the experiments shown in Panel A that Vmw110 residues 633-680 were necessary for this interaction because GEXE58 bound the 150kD protein but GEXR5 did not.

Figure 3A6


A
c
GEXE52
GEXE58
MW ex 1 2 b 1 2 b 1 2 b
205
116
97
67
45
150kD
135kD
B
c
GEXE52
GEXE52D14
ex 1 2 b 1 2 b 1 2 b
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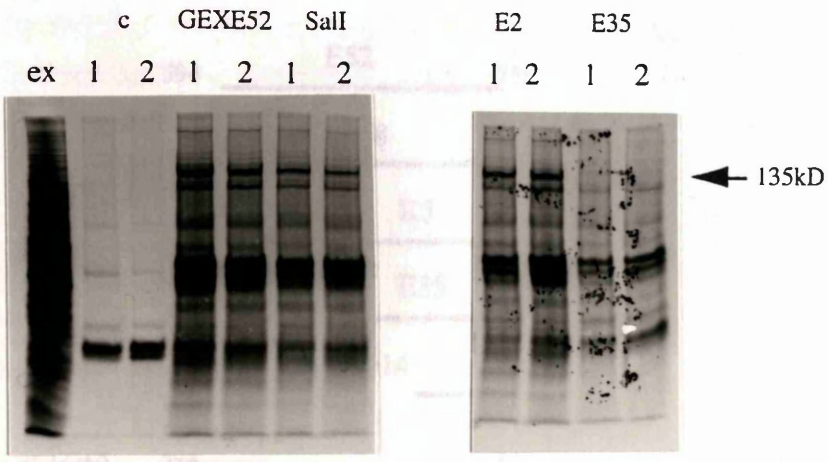
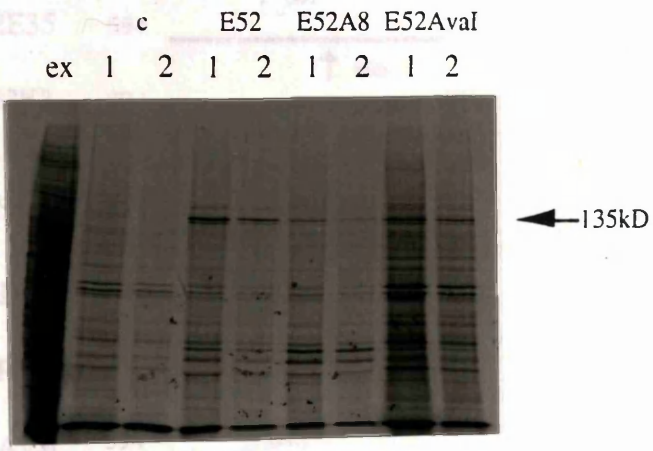
135kD
C
c
GEXR5
ex 1 2 b 1 2 b
c
GEXE35
ex 1 2 b 1 2 b

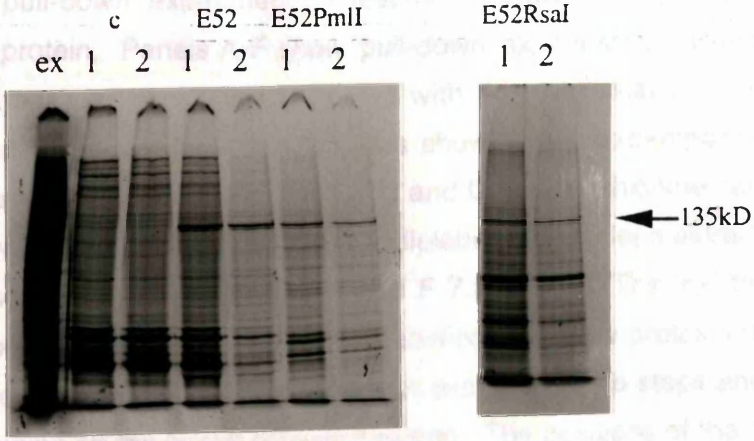
D


c
GEXE52
SalI
E2
E35
ex 1 2 1 2 1 2
1 2 1 2
135kD

E


c
E52
E52A8
E52AvaI
ex 1 2 1 2 1 2 1 2
135kD

F


c
E52
E52PmlI
E52RsaI
ex 1 2 1 2 1 2
1 2
135kD

G

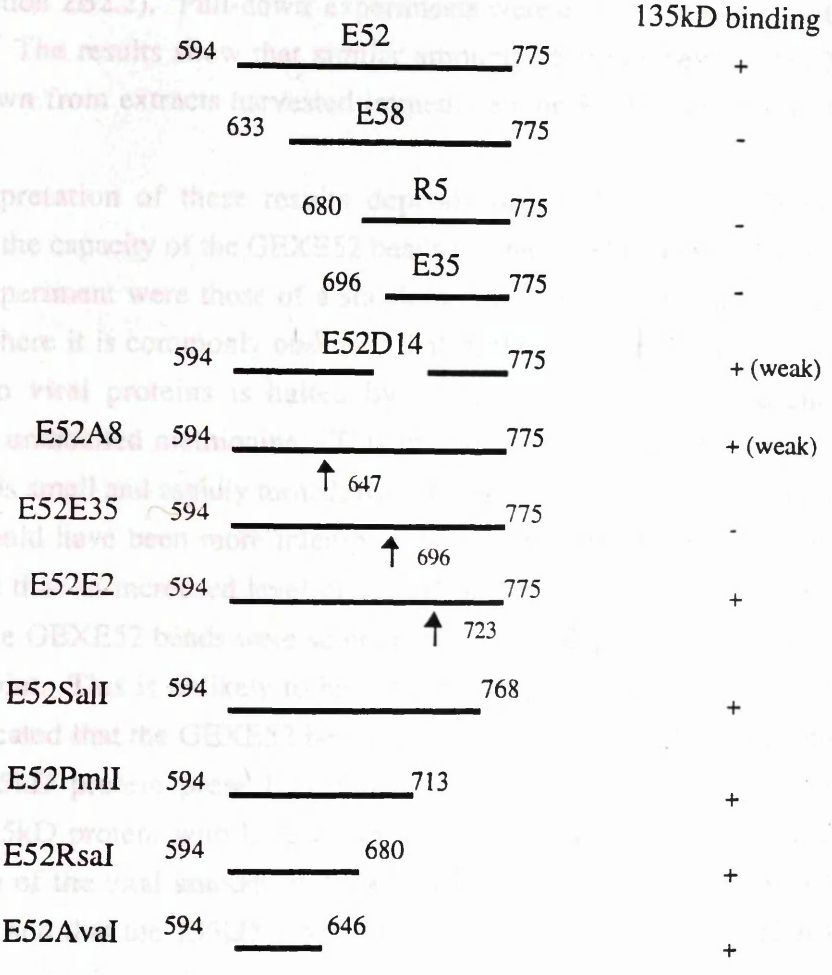


Figure 3A6 GST 'pull-down' experiments investigating Vmw110 residues involved in binding the 135kD protein. Panels A-F show 'pull-down' experiments carried out using the standard assay conditions (Section 2B3.4) with the GST fusion proteins used indicated above each set of tracks. The 'c' tracks show control experiments with GST beads. Panel G summarises the results. In A, B and C, ^{35}S-methionine radiolabelled BHK cell extracts were used and in D, E and F, radiolabelled WS HeLa extracts. Panels A-D show 12.5% polyacrylamide gels and E and F 7.5% gels. The 'ex' tracks show samples of radiolabelled cellular proteins, tracks labelled 1,2, show proteins eluted from the relevant GEX beads with reduced glutathione in two successive steps and 'b' tracks show proteins remaining on the beads following elution. The positions of the 150kD and 135kD proteins are shown where relevant.

3A7 An investigation of the half-life of the 135kD protein in cells

An adaptation of the standard GST 'pull-down' assay enabled an investigation of the stability of the 135kD protein in tissue culture cells. Following the standard 2 hour labelling procedure with ^{35}S-methionine, WS HeLa cells were either harvested as usual or the label mix was replaced with normal growth medium and the cell extracts made 5 or 12 hours later (Section 2B2.2). 'Pull-down' experiments were carried out using the GEXE52 fusion protein. The results show that similar amounts of radiolabelled 135kD protein were pulled down from extracts harvested immediately or 5 or 12 hours after labelling (Figure 3A7).

The interpretation of these results depends on the intracellular pool size of methionine and the capacity of the GEXE52 beads for the 135kD protein. The conditions used for this experiment were those of a standard pulse-chase experiment to label viral polypeptides, where it is commonly observed that further incorporation of radiolabelled methionine into viral proteins is halted by its removal from the medium and its replacement by unlabelled methionine. This implies that the intracellular pool size of ^{35}S-methionine is small and rapidly turned over; if this was not the case, the radiolabelled 135kD band would have been more intense at the 5hr time point than it actually is. It could be argued that an increased level of 135kD binding was not seen at the 5hr time point because the GEXE52 beads were saturated with 135kD protein at the level seen at the zero time point. This is unlikely to have been the case as the experiment shown in Figure 3C3 indicated that the GEXE52 beads used in the 'pull-down' experiments bound most of the 135kD protein present in cell extracts. Therefore, the persistence of radiolabelled 135kD protein with little or no indication of its depletion showed that a small proportion of the total amount of 135kD protein present in the cell was labelled during the pulse and that the 135kD protein was stable, having a half-life of at least 12 hours.

The significance of this observation can only be speculated at. Proteins with a short half-life are often those with key regulatory roles in the cell and whose intracellular concentrations are regulated by changes in their rates of synthesis, for example, some of the proteins involved in cell cycle control (Amon *et al.*, 1993). It is possible therefore that the interaction between Vmw110 and the 135kD protein is long lasting relative to many other protein:protein interactions in the cell (hours rather than minutes) and that Vmw110 exerts its effects during virus infection in a complex with this cellular protein.

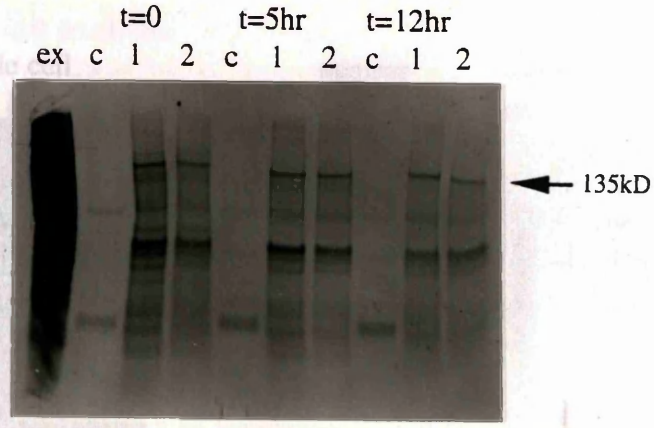


Figure 3A7 Investigation of the half life of the 135kD protein. WS HeLa cells were labelled for 2hr with ^{35}S-methionine (Section 2B2.2) and harvested immediately for extract preparation (t=0) or the label medium was replaced with normal growth medium and the cells harvested after 5 or 12hr. Standard 'pull-down' assays were carried out using GEXE52 beads. The 'ex' track shows radiolabelled whole cell proteins from the t=0 extract. The 'c' tracks show proteins eluted from GST beads with reduced glutathione in control experiments and tracks labelled 1,2, show proteins eluted from GST beads in successive elution steps.

3A8 An investigation of the cellular location of the 135kD protein

In a crude investigation of the location of the 135kD protein in the cell, 'pull-down' experiments using the GEXE52 fusion protein were carried out with labelled nuclear and cytoplasmic WS HeLa cell extracts. These extracts were prepared using the method of Dignam *et al.* (1983) (Section 2B2.2).

The results show that the 135kD protein was present in both cell fractions (Figure 3A8). As the experiment was performed using using nuclear and cytoplasmic extracts from the same number of cells, it can be concluded that there is more 135kD protein in the cytoplasm than in the nucleus. The reason for this and its significance is unknown, but the quantities of the 135kD protein present in this extract appear to be far too large to be explained by leakage from nuclei during extract preparation. It would, however, have been of value to test the extracts for characteristic nuclear and cytoplasmic proteins.

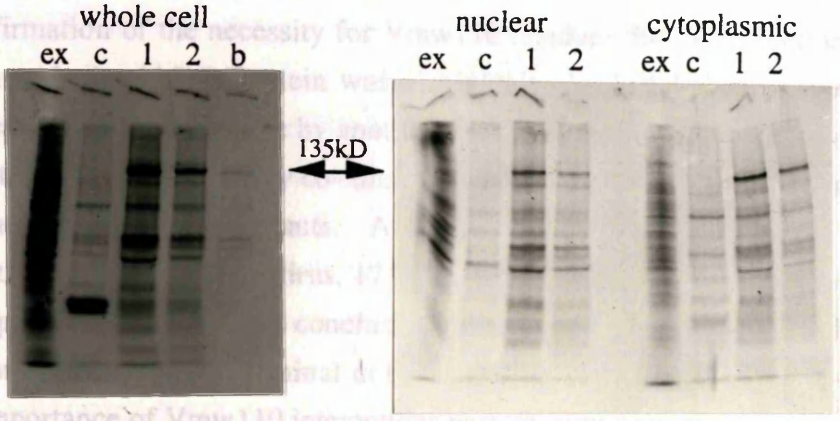


Figure 3A8 A GST 'pull-down' experiment using radiolabelled nuclear and cytoplasmic cell extracts. The extracts used in this experiment were prepared by the method of Dignam *et al.* (1983) (Section 2B2.2). Standard GST 'pull-down' assays were carried out using the GEXE52 fusion protein. The 'ex' tracks show samples of the relevant radiolabelled cellular proteins, tracks labelled 1,2, show proteins eluted from GEXE52 beads with reduced glutathione in two successive steps and 'b' tracks show proteins remaining on the beads after elution. The position of the 135kD protein is indicated.

3A9 The interaction of a 135kD protein with intact Vmw110

As with any experiment involving the use of isolated protein domains, it was important to provide evidence that the behaviour of the C-terminal domain of Vmw110 in the GST 'pull-down' assays was representative of its behavior in the intact protein. It was possible that the interactions seen between some of the Vmw110-GST fusion proteins and the 135kD cellular protein were a property peculiar to the fusion proteins and that Vmw110 residues did not behave as they do in the intact protein. Experiments carried out by other members of the group showed that a 135kD protein was co-immune precipitated from HSV-1 infected cell extracts using the anti-Vmw110 monoclonal antibody 11060 (Meredith *et al.*, 1994). The result was similar when purified Vmw110 (Everett *et al.*, 1991b) was added exogenously to cell extracts.

A collaborative depletion binding experiment was carried out to investigate whether or not the 135kD protein pulled down by GEXE52 was the same as that co-immune precipitated with exogenously added Vmw110. Following a 'pull-down' experiment with GEXE52 beads, purified Vmw110 protein was added to the 135kD protein-depleted cell extract and immune-precipitated with MAb 11060. The amounts of 135kD protein co-immune precipitated were much less than in a control experiment (compare lanes 4 and 5

of Figure 3A9). The experiment was repeated in the reverse order with the same result (compare lanes 9 and 10 of Figure 3A9). These experiments indicated that the 135kD proteins observed in the two experimental systems were likely to be the same. This could be conclusively shown in a Western blot with an anti-135kD antibody but unfortunately, such an antibody was not available.

Confirmation of the necessity for Vmw110 residues 594-633 (Section 3A6) in the interaction with the 135kD protein was also obtained. A deletion mutant virus, D12, lacking these residues, was made by another member of the group and it was found that a 135kD protein was not detectably co-immune precipitated with Vmw110 from virus D12-infected radiolabelled cell extracts. A 135kD protein was precipitated in a control experiment using the wild-type virus, 17^+ (Meredith *et al.*, 1995). This experiment used a genetic approach to support the conclusion that the 135kD proteins which bind to intact Vmw110 and its isolated C-terminal domain are likely to be the same. In view of the potential importance of Vmw110 interactions with cellular proteins in the life cycle of the virus, particularly with reference to the lytic-latent switch, these results provided grounds for further investigation of the 135kD protein.

Interestingly, the 150kD protein from BHK cells was not detected in immune precipitates with Vmw110 either from infected cell extracts or when Vmw110 was added exogenously. This suggests either that the interaction with the 150kD protein seen in the GST 'pull-down' assays was artifactual or that binding conditions in the immune precipitation experiments were unsuitable or insufficiently sensitive for the interaction to be detected.

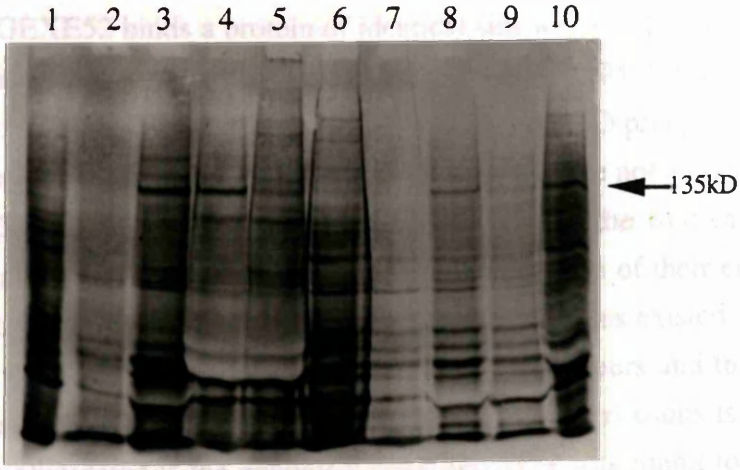


Figure 3A9 Reciprocal depletion experiment. Tracks 1-5: a radiolabelled BHK cell extract was made (Section 2B2.2) and precleared with GST beads. To one half of this extract, purified Vmw110 was added and immune precipitated with monoclonal antibody 11060. A 'pull-down' experiment with GEXE52 beads was carried out with the other half, followed by an immune precipitation with added Vmw110 using monoclonal antibody 11060. Track 1 shows a sample of radiolabelled BHK cell proteins. Track 2 shows

proteins eluted from GST beads with reduced glutathione. Track 3 shows proteins eluted from GEXE52 beads. Track 4 shows proteins co-immune precipitated with exogenously added Vmw110 following extract 'preclearing' with GST beads. Track 5 shows proteins co-immune precipitated with exogenously added Vmw110 from an extract precleared with GST and GEXE52 beads. Tracks 6-10: reciprocal experiment to that shown in tracks 1-5. A radiolabelled BHK cell dounce soluble extract was made (Meredith *et al.*, 1994) and split into two. A standard GST 'pull-down' assay was carried out with one sample, using GEXE52 beads. Vmw110 was added to the other sample and immune precipitated with monoclonal antibody 11060 before addition of GEXE52 beads for a 'pull-down' assay. Track 6 shows a sample of radiolabelled dounce extracted proteins. Track 7 shows proteins eluted from GST beads with reduced glutathione. Track 8 shows proteins eluted from GEXE52 beads. Track 9 shows proteins eluted from GEXE52 beads following an immune-precipitation with added Vmw110 and 11060. Track 10 shows proteins co-immune precipitated from a dounce soluble extract with Vmw110 using 11060. The radiolabelled BHK cell and BHK dounce soluble extracts were made in different buffers so it was necessary to alter the NaCl concentration of extracts as the experiment proceeded. The position of the 135kD protein is marked.

3A10 Discussion

The results in this Section show that Vmw110 strongly and specifically interacts with a 135kD cellular protein via residues in its C-terminal portion: intact Vmw110 co-immune precipitates with a 135kD protein from both BHK and WS HeLa cell extracts (Meredith *et al.*, 1994) and GEXE52 binds a protein of identical size which is likely to be the same. The Vmw110 residues involved in this interaction are between 594-775 and residues 594-646 are sufficient. The GEXE52 protein interacted with a 135kD protein in all cell types tested which is perhaps surprising considering the cell lines were not all derived from the same species. Characterisation of these 135kD proteins, in the first instance with a suitable panel of anti-135kD antibodies and then by comparison of their encoding DNA sequences, would clarify whether or not any structural similarities existed. In WS HeLa cells, the protein was shown to have a half-life of at least 12 hours and to be present in both the nucleus and cytoplasm. The significance of these observations is unknown but could possibly be elucidated if the encoding DNA sequence was found to be similar to that of a well characterised protein.

The GEXE52 protein also interacted with a 150kD protein from BHK and HFL cell extracts. From the data shown, it was concluded that Vmw110 residues 633-680 were necessary for this interaction. Time constraints did not allow as extensive an investigation of this protein as the 135kD protein, but it is possible that its interaction with C-terminal residues of Vmw110 is significant. It could be speculated that it is a

precursor of the 135kD protein and that the metabolic peculiarities of some cell types allow its accumulation.

It is likely that other cellular proteins interact with Vmw110. For example, the two bands marked · in Figure 3A4(ii) were present in a number of experiments although their intensities varied. These proteins may have been present in lower amounts than either the 135kD or 150kD proteins, or their interactions with Vmw110 may have been transient. Specific interactions with very low amounts of protein would have been obscured by the high background. A study of some of these proteins may be amenable by altering the conditions of the GST 'pull-down' assay. An alteration of immune-precipitation conditions might also uncover further interactions and, at least in principle, the use of an antibody other than 11060 might reveal a protein which interacts with residues close to the 11060 epitope. Use of the yeast Two-Hybrid System would be an efficient way of detecting proteins which interact transiently with Vmw110 (Fields and Song, 1989).

3B Expression of C-terminal residues of Vmw110 from a T7 vector and a physical analysis of their nature

3B1 Introduction

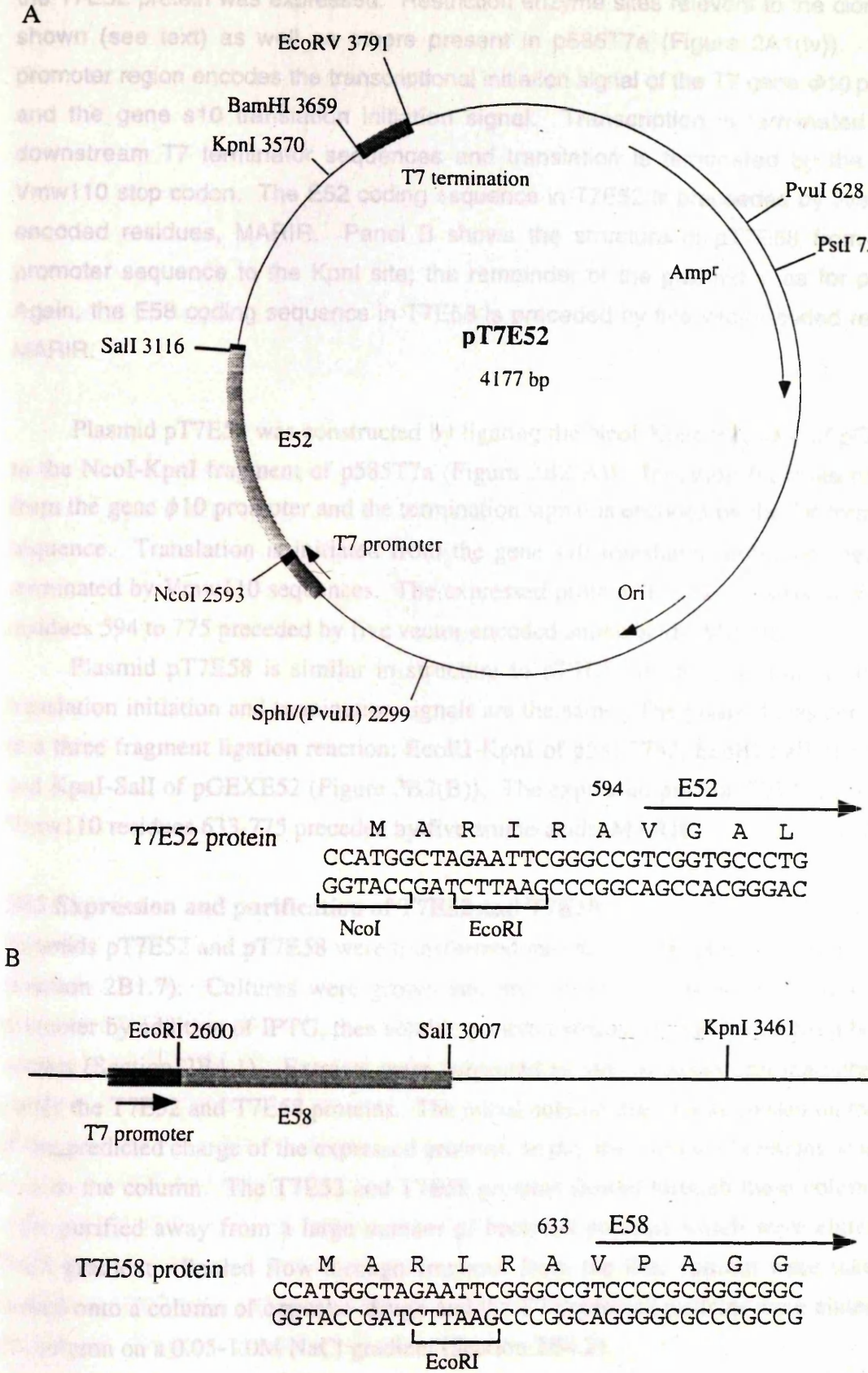
The aim of the experiments in this section was to analyse the physical nature of the C-terminal domain of Vmw110. Intact Vmw110 has been shown to exist as a multimer in solution (Everett *et al.*, 1991b; Chen *et al.*, 1992) and a multimerisation domain has been mapped to the C-terminal end of the protein (Ciufo *et al.*, 1994) (Section 1D11). The domain was mapped in a series of experiments to residues 617-711: the experiments involved immuneprecipitation and cross-linking of *in vitro* translated domains of Vmw110 as well as far-Western blotting. As this region overlapped residues 594-633 which were found to be involved in binding the 135kD protein (Section 3A6), it was of interest to determine whether or not the residues responsible for these two properties of Vmw110 were the same. The ability of the C-terminal domain of Vmw110 to multimerise was investigated by the more direct and traditional biochemical methods of gel filtration chromatography and glycerol gradient centrifugation using purified proteins expressed from a T7 vector.

The Vmw110 sequences from pGEXE52 (residues 594-775) and pGEXE58 (residues 633-775) were cloned into T7 expression vectors and the expressed proteins were purified by ion exchange chromatography. Gel filtration chromatography and glycerol gradient centrifugation showed that the T7E52 and T7E58 proteins existed in multimeric form. In an attempt to resolve the nature of the multimers, glutaraldehyde cross-linking experiments were also carried out.

3B2 Construction of pT7E52 and pT7E58

The T7 expression vectors were originally made by cloning fragments of T7 DNA into the unique BamHI site of pBR322 (Studier *et al.*, 1990). The T7 fragments were the gene ϕ10 promoter plus the s10 translation initiation region for the gene 10 protein and the Tϕ transcription termination signal. Linker sequences, giving rise to a number of different vectors, were inserted between the s10 translation initiation region and the Tϕ terminaton signal. Plasmid p585T7a was derived from pET8c via an intermediate, p585.4 (Section 2A1), such that the pUC19 MCR was inserted between the T7 s10 initiation and Tϕ termination signals. Plasmid p585T7b is similar to p585T7a except a frameshift has been introduced prior to the MCR.

Figure 3B2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BamHI 3659
KpnI 3570
T7 termination
PvuI 628
PstI 754
Ampr
SalI 3116
pT7E52
4177 bp
E52
T7 promoter
NcoI 2593
Ori
SphI/(PvuII) 2299
594
E52
T7E52 protein
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CCATGGCTAGAATTCGGGCCGTCGGTGCCCTG
GGTACCGATCTTAAGCCCGGCAGCCACGGGAC
NcoI
EcoRI
B
EcoRI 2600
SalI 3007
KpnI 3461
E58
T7 promoter
633
E58
T7E58 protein
M A R I R A V P A G G
CCATGGCTAGAATTCGGGCCGTCCCCGCGGGCGGC
GGTACCGATCTTAAGCCCGGCAGGGGCGCCCGCCG
EcoRI

Figure 3B2 Construction of pT7E52 and pT7E58. Panel A shows pT7E52 from which the T7E52 protein was expressed. Restriction enzyme sites relevent to the cloning are shown (see text) as well as others present in p585T7a (Figure 2A1(iv)). The T7 promoter region encodes the transcriptional initiation signal of the T7 gene Φ10 promoter and the gene s10 translation initiation signal. Transcription is terminated by the downstream T7 terminator sequences and translation is terminated by the normal Vmw110 stop codon. The E52 coding sequence in T7E52 is preceeded by five vector-encoded residues, MARIR. Panel B shows the structure of pT7E58 from the T7 promoter sequence to the KpnI site; the remainder of the plasmid is as for pT7E52. Again, the E58 coding sequence in T7E58 is preceded by five vector-coded residues, MARIR.

Plasmid pT7E52 was constructed by ligating the NcoI-KpnI fragment of pGEXE52 to the NcoI-KpnI fragment of p585T7a (Figure 3B2(A)). Initiation for transcription is from the gene ϕ10 promoter and the termination signal is encoded by the Tϕ termination sequence. Translation is initiated from the gene s10 translation initiation region and terminated by Vmw110 sequences. The expressed protein, T7E52, consists of Vmw110 residues 594 to 775 preceded by five vector-encoded amino acids, MARIR.

Plasmid pT7E58 is similar in structure to pT7E52 in that the transcription and translation initiation and termination signals are the same. The plasmid was constructed in a three fragment ligation reaction: EcoRI-KpnI of p585T7b2, EcoRI-SalI of p110E58 and KpnI-SalI of pGEXE52 (Figure 3B2(B)). The expressed protein, T7E58, consists of Vmw110 residues 633-775 preceded by five amino acids, MARIR.

3B3 Expression and purification of T7E52 and T7E58

Plasmids pT7E52 and pT7E58 were transformed into the *E.coli* strain BL21(DE3)pLysS (Section 2B1.7). Cultures were grown and protein expression induced from the T7 promoter by addition of IPTG, then soluble protein extracts were prepared from bacterial lysates (Section 2B4.1). Extracts were subjected to ion exchange chromatography to purify the T7E52 and T7E58 proteins. The initial column matrix was chosen on the basis of the predicted charge of the expressed proteins, so that the expressed proteins would not bind to the column. The T7E52 and T7E58 proteins flowed through these columns and were purified away from a large number of bacterial proteins which were eluted on a NaCl gradient. Pooled flow-through fractions from the first column were taken and loaded onto a column of opposite charge and the T7 expressed proteins were eluted from the column on a 0.05-1.0M NaCl gradient (Section 2B4.2).

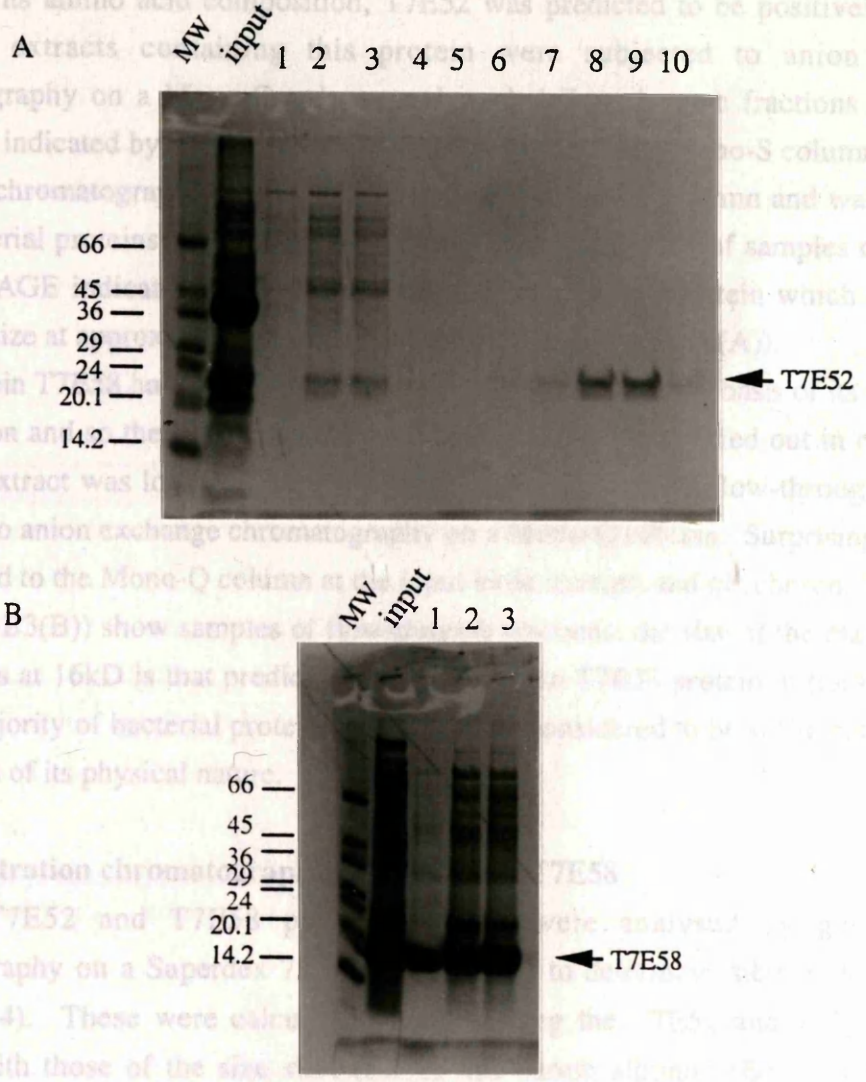


Figure 3B3 Purification of T7E52 and T7E58 proteins. (A) T7E52: a 2ml bacterial extract containing T7E52 was subjected to anion exchange chromatography on a 20ml Mono-Q column and pooled flow-through fractions (6ml total volume) loaded on a 20ml Mono-S column (Section 2B4.2). Fractions (2ml) were collected and 10µl samples were analysed by SDS PAGE on a 12.5% tricine gel - tracks 1-10. Standard molecular weight proteins, MW, whose sizes are marked were run. An 'input' sample, 5µl of pooled flow through fractions from the Mono-Q column, was also run. The position of T7E52 in tracks 8 and 9 is marked. (B) T7E58: bacterial extract (0.5ml) containing protein T7E58 was subjected to cation exchange chromatography on a 1ml Mono-S column and pooled flow-through fractions (6ml) were loaded on a 1ml Mono-Q column (Section 2B4.2). Fractions (2ml) were collected and 10µl samples analysed by SDS PAGE on a 12.5% tricine gel. Samples in tracks 1-3 were the first three collected and show proteins which did not bind to theMono-Q column. The position of T7E58 in these fractions is shown. 'MW' indicates a track with molecular weight markers and the 'input' track contains a sample of the pooled flow-through fractions from the Mono-S column.

Based on its amino acid composition, T7E52 was predicted to be positively charged. Bacterial extracts containing this protein were subjected to anion exchange chromatography on a Mono-Q column and pooled flow-through fractions containing T7E52 (as indicated by SDS PAGE analysis) were loaded on a Mono-S column for cation exchange chromatography. The T7E52 protein bound to this column and was separated from bacterial proteins on a 0.05-1.0M NaCl gradient. Analysis of samples of fractions by SDS PAGE indicated those which contained the purified protein which was of the predicted size at approximately 20kD (tracks 8 and 9 of Figure 3B3(A)).

Protein T7E58 had a predicted overall negative charge on the basis of its amino acid composition and so the purification procedure for T7E52 was carried out in reverse: the bacterial extract was loaded onto a Mono-S column and pooled flow-through fractions subjected to anion exchange chromatography on a Mono-Q column. Surprisingly, T7E58 did not bind to the Mono-Q column at the input ionic strength and pH chosen. Tracks 1-3 of Figure 3B3(B)) show samples of flow-through fractions: the size of the major band in these tracks at 16kD is that predicted for T7E58. The T7E58 protein in track 1, having had the majority of bacterial proteins removed, was considered to be sufficiently purified for analysis of its physical nature.

3B4 Gel filtration chromatography of T7E52 and T7E58

Purified T7E52 and T7E58 protein samples were analysed by gel filtration chromatography on a Superdex 75 column in order to determine their molecular sizes (Figure 3B4). These were calculated by comparing the T7E52 and T7E58 elution volumes with those of the size standards bovine serum albumin (BSA) and carbonic anhydrase (CA) which were analysed as a mixture (Figure 3B4(A)). The presence of particular proteins in peak fractions was determined by SDS PAGE (results not shown).

Peak 2 in Figure 3B4(A) corresponds with the exclusion volume of the column and contains the dimer of BSA (MW 132kD). Peak 3 at 9.66ml and peak 4 at 11.90ml correspond to the elution volumes of BSA (MW 66kD) and CA (MW 29kD) respectively. T7E52 was eluted at 9.47ml (peak 2 of Figure 3B4(B)) and T7E58 at 9.84ml (peak 3 of Figure 3B4(C)). Peak 2 in the T7E58 analysis corresponds with the exclusion volume of the column and contained bacterial proteins too large to enter the beads of the column matrix. The sizes of the peaks in this run showed that the column was overloaded (Figure 2 of Meredith *et al.*, 1995 shows a better trace). Based on the elution volumes of the standard proteins, T7E52 and T7E58 were calculated to have apparent molecular sizes of 70kD and 62kD respectively.

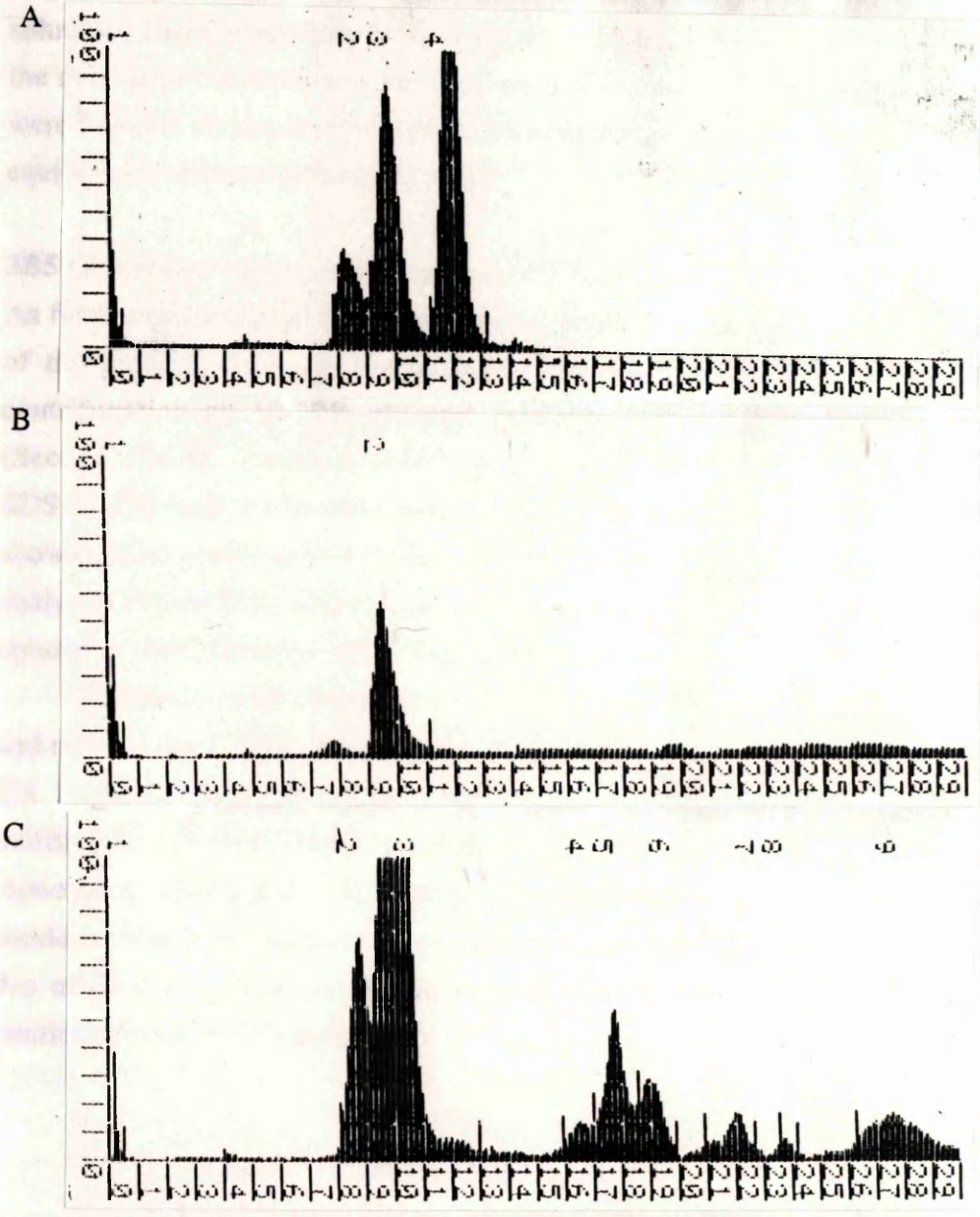


Figure 3B4 Gel filtration chromatography of T7E52 and T7E58 on a superdex 75 column. Panel A shows a run with 50µg each of the size standards bovine serum albumin (66kD) and carbonic anhydrase (29kD). Peak 2 at 8.10ml corresponds to the void volume of the column and contains the BSA dimer. Peak 3 at 9.68ml corresponds to monomeric BSA and peak 4 at 11.92ml corresponds to CA. B and C show runs with purified T7E52 and T7E58 proteins; 300µl aliquots of purified proteins from the ion exchange chromatography were run. T7E52 was eluted at 9.47ml, giving a molecular weight of 70kD and T7E58 at 9.84ml giving a molecular weight of 62kD. These were much larger than the predicted monomeric molecular weights of 19.9kD and 16.0kD respectively.

As the predicted molecular weights of T7E52 and T7E58 are 19.9kD and 16.0kD respectively (Section 3B4) it was concluded that these proteins existed as multimers in solution. These were likely to be of higher order than dimers but it was not possible from the data to to conclude whether they were tri- or tetrameric. The fact that the proteins were found in single, dominant peaks showed that the multimers were stable and not in an equilibrium between different species.

3B5 Glycerol gradient centrifugation of T7E52 and T7E58

As further confirmation of the multimeric nature of T7E52 and T7E58, purified samples of the proteins from the ion exchange chromatography columns were subjected to centrifugation on 10-30% glycerol gradients with the size standards BSA and CA (Section 2B4.4). Fractions were collected from the gradients and samples subjected to SDS PAGE analysis to determine the migration positions of the proteins (results not shown). The positions of T7E52 and T7E58 on the gradient were confirmed by dot blot analysis (Figure 3B5) with the monoclonal antibody 10503 which recognises a Vmw110 epitope in the C-terminal end of the protein.

The results showed that most of the T7E52 protein was contained in fractions 9-13 and most of the T7E58 protein was in fractions 8-14, whilst the control proteins BSA and CA migrated between fractions 10-14 and 7-9 respectively. This confirmed the multimeric nature of these proteins as concluded from the gel filtration analyses: monomeric T7E52 and T7E58, at predicted molecular weights of 19.9kD and 16.0kD, would have sedimented more slowly than CA of molecular weight 29.0kD. However, as this method was more crude than the gel filtration analyses, the precise nature of the multimers could not be determined accurately.

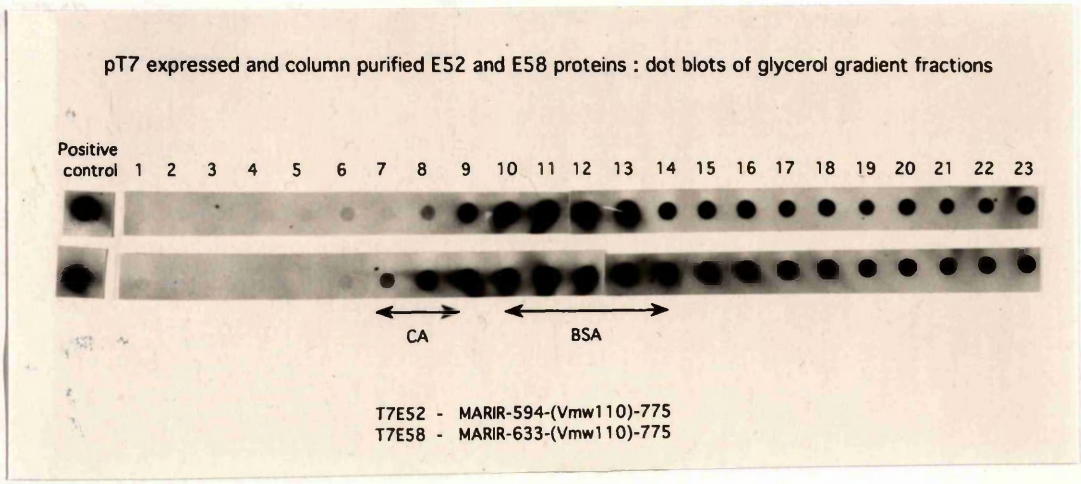


Figure 3B5 Glycerol gradient centrifugation of T7E52 and T7E58. Approximately 25µg column purified T7E52 or T7E58 were centrifuged down a 5ml 10-30% glycerol gradient

with 20µg each of the size standards carbonic anhydrase (CA) and bovine serum albumin (BSA) at 40krpm for 48hr (Section 2B4.4). Fractions (200µl) (1-23) were taken from the top of the gradient and samples analysed by SDS PAGE to determine the migration positions of CA and BSA (results not shown). Aliquots (50µl) were dot blotted onto nitrocellulose and T7 expressed Vmw110 sequences detected by ECL as for Western blotting (Section 2B3.3); the primary antibody was the monoclonal antibody 10503. The positive controls show samples of a dilution of the gradient input aliquot.

3B6 Glutaraldehyde cross-linking of T7E52 and T7E58

In an attempt to resolve the question of the multimeric nature of T7E52 and T7E58, glutaraldehyde cross-linking experiments were carried out with samples of the purified proteins eluted from the Superdex 75 column (Section 2B4.3). Cross-linked proteins were analysed by SDS PAGE and detected by Coomassie staining when high concentrations of protein were used (approximately 250µg/ml) or by Western blotting following electrophoresis, using the MAb 10503 when low concentrations of protein were used (approximately 50µg/ml).

In Figure 2B5(A) it can be seen that T7E58 was readily cross-linked when present at 250µg/ml, particularly with increasing concentrations of glutaraldehyde. A control experiment with carbonic anhydrase (CA) present at a similar concentration was carried out to show that the cross-linking seen was not an artifact of high protein concentrations. These results were confirmed in an experiment in which smaller amounts of T7E52 and T7E58 (50µg/ml) were cross-linked in the presence of increasing concentrations of glutaraldehyde and the products analysed by Western blotting (Panel B). The blot shows that both proteins readily formed multimers but that there was an absence of a dominant species. Consequently, this method did not resolve the question of the precise physical nature of T7E52 and T7E58. This will require a more sophisticated biophysical analysis, such as NMR spectroscopy, X-ray crystallography or light scattering analysis.

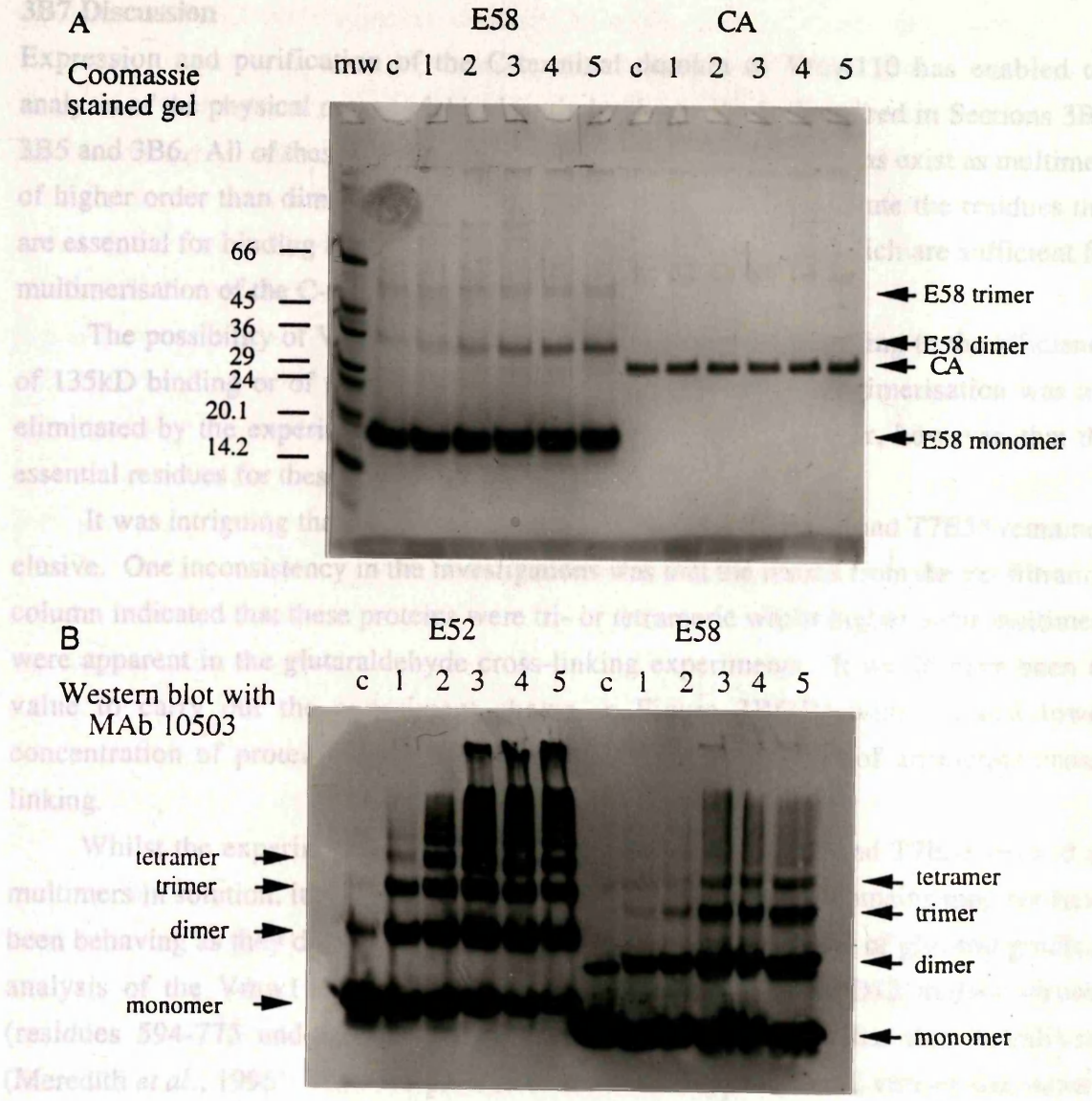


Figure 3B6 Glutaraldehyde cross-linking of T7E52 and T7E58. Samples of T7E52 and T7E58 purified from the Superdex 75 column were incubated in 20µl volumes at 250µg/ml (Panel A) or 50µg/ml (Panel B) with glutaraldehyde at 0.001%-0.01% (tracks 1-5) for 30min at 20°C. The reactions were stopped by addition of glycine to a final concentration of 50mM. Panel A shows a Coomassie stained gel of an experiment carried out with T7E58 at the higher protein concentration. The control protein used was carbonic anhydrase which is a monomeric at concentrations between 50µg/ml and 250µg/ml. The 'c' tracks in both experiments show proteins which were not cross-linked with glutaraldehyde. Panel B shows a Western blot of cross-linked products in a similar experiment to that in Panel A but with lower protein concentrations.

3B7 Discussion

Expression and purification of the C-terminal domain of Vmw110 has enabled the analysis of the physical nature of this domain by the methods described in Sections 3B4, 3B5 and 3B6. All of these indicate that the T7E52 and T7E58 proteins exist as multimers of higher order than dimers in solution. These results clearly separate the residues that are essential for binding the 135kD protein (594-633) from those which are sufficient for multimerisation of the C-terminal domain (633-775).

The possibility of Vmw110 residues outside 594-633 contributing to the efficiency of 135kD binding or of those outside 633-775 contributing to multimerisation was not eliminated by the experiments described in this Thesis. It was clear, however, that the essential residues for these two properties of Vmw110 were different.

It was intriguing that the precise multimeric natures of T7E52 and T7E58 remained elusive. One inconsistency in the investigations was that the results from the gel filtration column indicated that these proteins were tri- or tetrameric whilst higher order multimers were apparent in the glutaraldehyde cross-linking experiments. It would have been of value to carry out the experiment shown in Figure 3B6(B) with a much lower concentration of protein to completely eliminate the possibility of artifactual cross-linking.

Whilst the experiments in this Section showed that T7E52 and T7E58 existed as multimers in solution, it remained a possibility that these isolated domains may not have been behaving as they do in the intact protein. However, the results of glycerol gradient analysis of the Vmw110 proteins expressed by the E52X and D12 mutant viruses (residues 594-775 and 594-633 deleted respectively) indicate that this is unlikely (Meredith *et al.*, 1995): Vmw110 protein from the wild-type and D12 viruses sedimented at similar rates whilst the Vmw110 protein from the E52X virus sedimented much more slowly. This showed that the fast sedimentation rate of Vmw110 was not due primarily to the formation of a complex with the 135kD protein and was consistent with the idea that Vmw110 residues 594-633 are not required for multimerisation. It is possible that the sedimentation rates of Vmw110 expressed from the wild-type and from the D12 viruses are affected by the formation of a complex with a cellular protein which interacts with Vmw110 residues between 633-775. However, in conjunction with the data presented in this Thesis, it is reasonable to conclude that multimerisation is a property of the C-terminal domain of Vmw110. Additionally, the consistency between the observation that the Vmw110 protein of the D12 virus does not co-immune precipitate the 135kD protein and the data from 'pull-down' assays with GEXE52 and GEXE58 proteins, lends itself to the assurance that the C-terminal domain of Vmw110 folds correctly when expressed in isolation.

The results of the experiments presented in this Section are also consistent with those of Ciufo *et al.* (1994) who mapped a Vmw110 multimerisation domain to residues

between 617-711 with adjacent residues on either side of these contributing to the efficiency of multimer formation in some of their assays. It is likely that in mapping the domain to between residues 633-775 in the direct biochemical analyses presented here, the N-terminal boundary has been more clearly defined.

3C Purification of the WS HeLa cell 135kD protein

3C1 Introduction

In view of the potential biological significance of the interaction of Vmw110 with the 135kD protein, it became necessary to identify the cellular protein so that the interaction could be investigated further. The experiments in this section describe the purification of the 135kD protein from WS HeLa cells and subsequent microsequence analysis. The sequence information was used to raise antipeptide sera as well as in the design of redundant PCR primers for use in cloning a cDNA (Sections 3C4 and 3C5). It was quickly found that although the amounts of 135kD protein pulled down in a standard GST 'pull-down' assay were visible on a Coomassie stained gel, the quantities were too low for microsequence analysis. The yield was increased by scaling up the 'pull-down' assay. Proteins were subjected to SDS PAGE and blotted to a PVDF membrane (Problott™) which was stained to show the position of the 135kD protein. The protein was submitted for sequence analysis by Dr. Matsudaira (MIT).

3C2 The 135kD protein in BHK cells is visible on a Coomassie stained protein gel

In order to obtain microsequence information from bands of protein immobilised on Problott™ membrane, it was necessary to blot microgram quantities from a polyacrylamide gel. The quantity of 135kD protein pulled down by GEXE52 beads from BHK cell extracts in a standard GST 'pull-down' assay was investigated by Coomassie staining the analytical polyacrylamide gel. The gel was vacuum dried and exposed to Kodak X-OMAT S film (Figure 3C2).

Careful positioning of the autoradiograph on the dried, stained gel showed that the 135kD protein was visible as a faintly stained band which precisely migrated with the radiolabelled 135kD band (compare the A tracks of Panels A and B). The protein was absent from all of the control tracks, 'control', 'B' and 'C'. The proteins in the tracks marked 'control' show those eluted from GST beads in the usual control experiment. Proteins in the 'B' tracks were eluted from GEXE52 beads in an experiment in which the beads were mixed with the BHK cell resuspension buffer instead of radiolabelled BHK cell proteins. These tracks show bacterial proteins which bind to the fusion protein and it is evident that there are no major bacterial bands in the region of the 135kD protein. This meant that the 135kD protein could be purified by isolation from a gel or blot as it would be unlikely to contain large quantities of contaminating proteins. In another control, proteins were eluted from glutathione agarose beads which had been mixed with radiolabelled proteins ('C' tracks). This ensured that the 135kD cellular protein was eluted from GEXE52 beads because it bound to the fusion protein and not to the beads themselves. The only band present in these tracks was mammalian GST.

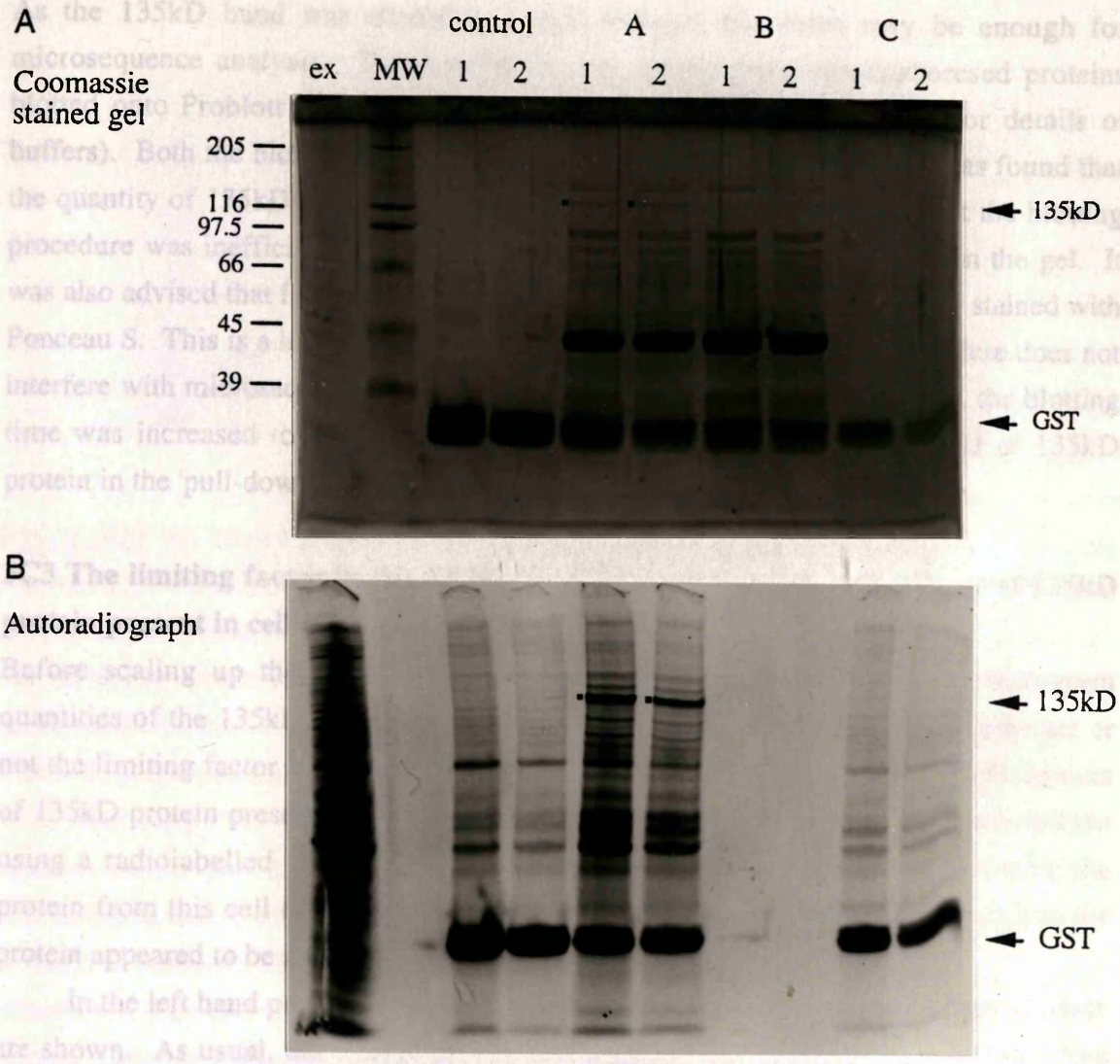


Figure 3C2 Visualisation of the 135kD protein from BHK cells on a Coomassie stained polyacrylamide gel. A standard GST 'pull-down' assay was carried out and proteins eluted from the beads analysed by SDS PAGE on a 12.5% polyacrylamide gel (Section 2B3.4). The gel was stained with Coomassie blue and destained (Panel A) and vacuum dried and exposed to Kodak X-OMAT S film to give the autoradiograph shown in Panel B. The 'ex' track shows a sample of the radiolabelled BHK cell proteins whose sizes are indicated. The remaining tracks which are in pairs marked 1,2 show proteins eluted from beads in two successive steps with reduced glutathione. Proteins eluted from GST beads were run in the control tracks and those eluted from GEXE52 beads in the A tracks. A comparison of the autoradiograph with the stained gel shows the presence of a faintly stained 135kD band, marked ▪ , in the A tracks. The B tracks show proteins eluted from GEXE52 beads in a control experiment in which GEXE52 beads were mixed with BHK cell resuspension buffer; consequently these tracks are blank in Panel B. In the C tracks, the proteins shown are those eluted in a control experiment from glutathione agarose beads alone which had been mixed with a radiolabelled cellular extract. The major band present in these tracks is mammalian GST.

As the 135kD band was stainable, it was thought that there may be enough for microsequence analysis. The experiment was repeated and electrophoresed proteins blotted onto Problott™ membrane for 3 hours (See Section 2B3.5(ii) for details of buffers). Both the blot and the gel were stained with Coomassie blue. It was found that the quantity of 135kD protein on the gel was too small for analysis and that the blotting procedure was inefficient, as approximately 50% of the protein remained on the gel. It was also advised that for eventual microsequence analysis, the blot should be stained with Ponceau S. This is a less sensitive stain than Coomassie blue, but the procedure does not interfere with microsequence analysis. As a consequence of this experiment, the blotting time was increased to 6 hours and investigations into increasing the yield of 135kD protein in the 'pull-down' experiments were made.

3C3 The limiting factor in the GEXE52-135kD interaction is the amount of 135kD protein present in cell extracts

Before scaling up the standard GST 'pull-down' assay for isolation of microgram quantities of the 135kD protein, an experiment was carried out to determine whether or not the limiting factor in the GEXE52-135kD protein:protein interaction was the amount of 135kD protein present in cell extracts (Figure 3C3). The experiment was carried out using a radiolabelled WS HeLa cell extract. It was previously decided to purify the protein from this cell type because these cells were derived from human tissue and the protein appeared to be most abundant in this extract (Section 3A5).

In the left hand panel of Figure 3C3, the results of a standard GST 'pull-down' assay are shown. As usual, the 135kD protein was eluted from GEXE52 beads but not from control GST beads. A second quantity of GEXE52 beads were mixed with the 135kD protein 'pre-cleared' extract and proteins eluted from these beads are shown in the 'A' tracks of the right hand panel. This result showed that incubating radiolabelled extracts with GEXE52 beads considerably depleted the levels of 135kD protein present. In a converse experiment, proteins were eluted from GEXE52 beads which had been incubated successively in two aliquots of radiolabelled WS HeLa cell proteins that had been 'pre-cleared' with GST beads ('B' tracks). In comparison with the standard assay, similar amounts of the 135kD protein were eluted from these beads. The conclusion drawn from this experiment was that the limiting factor in the GEXE52-135kD protein:protein interaction was the small quantity of 135kD protein present in cell extracts.

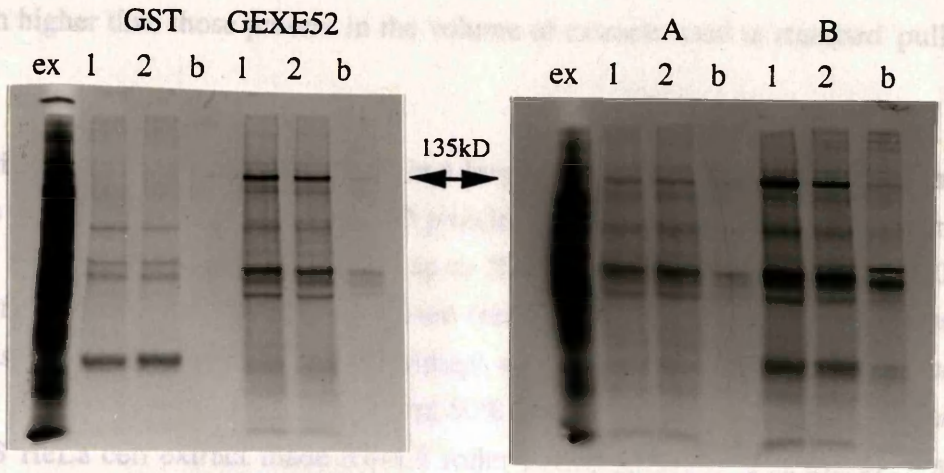


Figure 3C3 An investigation of the limiting factor in the GEXE52-135kD protein:protein interaction. A standard 'pull-down' assay was carried out and proteins eluted from GST and GEXE52 beads loaded on a 10% polyacrylamide gel (left-hand panel). In both gels, the 'ex' tracks show samples of the ^{35}S-methionine radiolabelled cell extracts used in these experiments. In each group of three tracks, 1,2 show proteins eluted from beads in two successive steps with reduced glutathione and in the 'b' tracks, the beads were run. The position of the 135kD protein is indicated. The experiment under A shows proteins eluted from GEXE52 beads which were mixed with an extract which had been preincubated with GEXE52 beads as well as GST beads. The 135kD protein is present in smaller amounts compared to the standard assay. Proteins in the B tracks were eluted from GEXE52 beads which were passed through two aliquots of the WS HeLa cell extract. Greater amounts of the 135kD protein were present in this eluate compared with the standard eluate.

Based on this result, attempts were made to isolate sufficient quantities of the 135kD protein for microsequencing by increasing the amounts of cell extract used in the experiments. In one experiment, an unlabelled extract was prepared from a roller bottle of WS HeLa cells in a final volume of 5ml. The extract was 'pre-cleared' for three hours with 50µl GST beads and incubated overnight, with end over end shaking, with 20µl GEXE52 beads. Proteins eluted from the beads were subjected to SDS PAGE and blotted onto Problott™ membrane and stained with Ponceau S. A 135kD band was not visible (results not shown) so a number of subsequent experiments were carried out in which small quantities of GEXE52 beads (up to 50µl) were passed through cell extracts from up to 10 roller bottles of WS HeLa cells. These attempts were also unsuccessful and led to experiments in which the amounts of GEXE52 beads were also increased (Section 3C4). The reasons for the problems encountered in these latter experiments were unknown: it may have been that the GEXE52 beads were saturated with the 135kD protein at levels

not much higher than those present in the volume of extracts used in standard 'pull-down' assays.

3C4 Purification of the 135kD protein in a large scale GST 'pull down' experiment

Attempts were made to purify the 135kD protein in a number of 'pull-down' experiments of larger scale than the standard assay: up to 500µl GEXE52 beads and up to 10 roller bottles of WS HeLa cell extract were used (results not shown). These attempts were unsuccessful and an investigative experiment was carried out in which the volume of GEXE52 beads was increased further; 1ml 50% slurry GEXE52 beads were mixed with 20ml WS HeLa cell extract made from 5 roller bottles of cells. The extract was 'pre-cleared' with 1ml 50% slurry GST beads by end over end mixing for three hours and then incubated with the GEXE52 beads overnight. Following washing of the beads three times in 5ml 0.5M NaCl wash buffer, proteins were eluted from the beads twice in 0.5ml 50mM reduced glutathione to give a final volume of 1ml. The eluate was concentrated by centrifugation at 5krpm in a Centricon 50 centrifugation unit such that the volume was reduced to 40µl. The sample was run on a 10% polyacrylamide gel with appropriate controls (Figure 3C4(i)). The results showed that the 135kD protein was efficiently concentrated by this method and did not break down (track 1) and that it was not present in any of the control tracks: track 2 shows an experiment in which the GEXE52 beads were incubated with the cell extract resuspension buffer and track 3 shows proteins eluted from GST beads. A sample of the GEXE52 bacterial protein extract was run in track 4 to show that there were no major bacterial bands of 135kD.

This experiment was repeated on a larger scale (Section 2B3.5): 4ml GEXE52 beads were used to bind the 135kD protein from 60ml unlabelled WS HeLa cell extract made from cells grown in 25 roller bottles (Section 2B2.3). The extract was 'pre-cleared' with GST beads and incubated with the GEXE52 beads for 40 hours. During this period, proteins were eluted from 20µl samples of the GEXE52 beads to ascertain that the 135kD protein had bound (results not shown). After washing the beads in the 0.5M NaCl wash, proteins were eluted from the beads in two stages with reduced glutathione and the eluate volume was reduced to 200µl as in the previous experiment. The sample was mixed with radiolabelled proteins eluted from GEXE52 beads in a standard GST 'pull-down' assay and subjected to SDS PAGE with appropriate control samples on a 10% polyacrylamide gel. The gel was run at 50V rather than the usual 100V (Section 2B3.2) to minimise band smearing. Electrophoresed proteins were blotted onto Problott™ membrane which was stained with Ponceau S which revealed a heavily stained high molecular weight band (Figure 3C4(ii)A). An overnight exposure of the blot to Kodak X-OMAT S film confirmed that this was the 135kD protein (compare panels A and B of Figure 3C4(ii)). The blot was sent to MIT for microsequence analysis of the 135kD protein by Dr. Matsudaira.

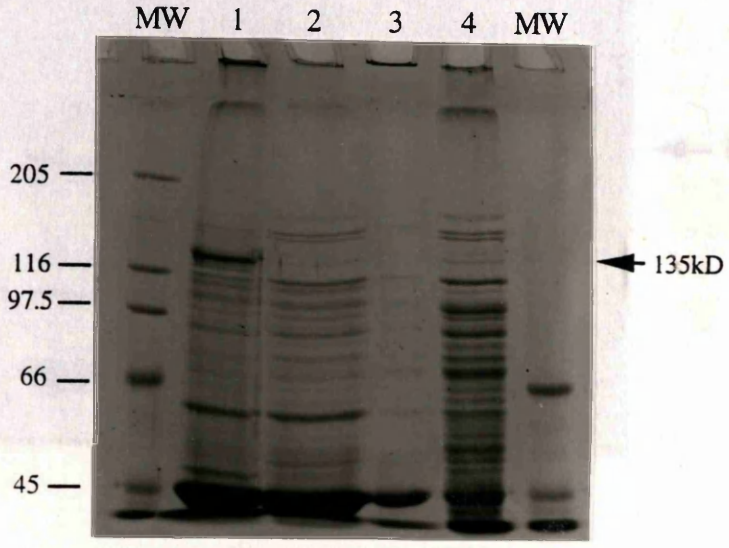


Figure 3C4(i) An investigative GST 'pull-down' experiment for purification of the 135kD protein. A 1ml slurry of GST and GEXE52 beads were made by mixing 1ml glutathione agarose beads with 3ml of the relevent bacterial extracts for 1hr at 4°C. A WS HeLa cell extract (20ml), made from 5 roller bottles of cells, was 'pre-cleared' for 3hr with the GST beads and then incubated with GEXE52 beads overnught. GST and GEXE52 beads were washed three times in 5ml 0.5M NaCl wash buffer (Section 2A10) and proteins eluted in two 15min incubations with 0.5ml 50mM reduced glutathione. The elution volumes were concentrated to 40µl and the proteins were electrophoresed on a 10% polyacrylamide gel. Track 1 shows proteins eluted from the GEXE52 beads and track 3 those from GST beads. A parallel control experiment was carried out in which GST and GEXE52 beads were incubated with WS HeLa cell resusupension buffer. Protein eluted from the GEXE52 beads were run in track 2. In track 4 a sample of the GEXE52 bacterial extract was run. The MW track shows standard proteins whose sizes are indicated. The position of the 135kD protein is marked; this protein is present only in track 1 and not in any of the control tracks.

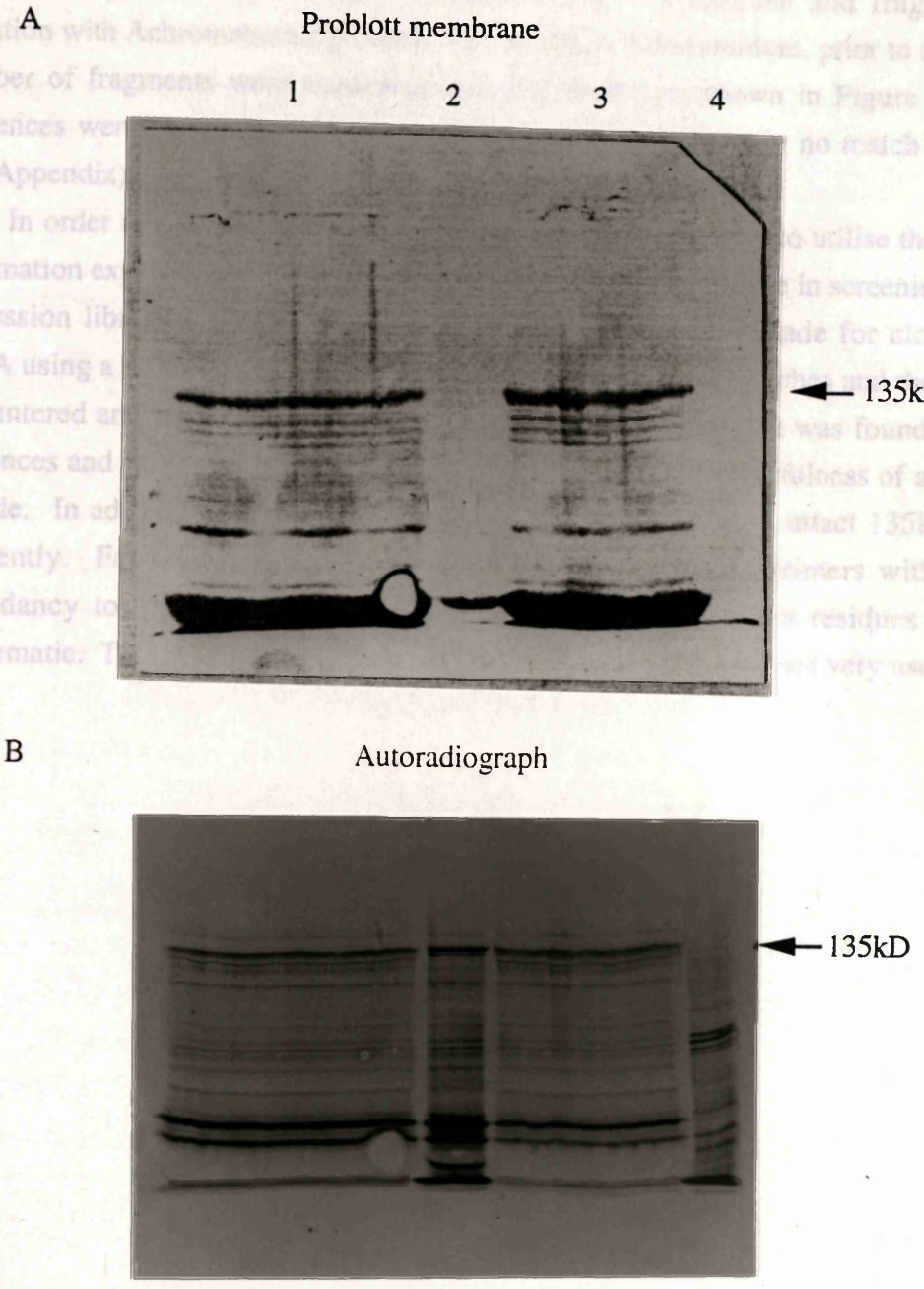


Figure 3C4(ii) Purification of the 135kD protein. Purification of the 135kD protein was carried out as described in Section 2B3.5. The concentrated GEXE52 eluate was mixed with proteins eluted from GEXE52 beads in a standard 'pull-down' assay carried out with radiolabelled cell extrac and electrophoresed on a 10% polyacrylamide gel (tracks 1 and 3). Track 2 shows proteins eluted from GEXE52 beads in a standard 'pull-down' assay. The heavily stained high molecular weight band in tracks 1 and 3 on the Problott membrane (Panel A) was ascertained as being the 135kD protein by comparison with an autoradiograph of the blot (Panel B).

3C5 Peptide sequences

The 135kD protein was eluted from the Problott™ membrane and fragmented by digestion with Achromobacter protease, which cuts at lysine residues, prior to analysis. A number of fragments were sequenced and the results are shown in Figure 3C5. The sequences were used to screen the available database but initially no match was found (see Appendix).

In order to obtain a cDNA clone, it was therefore necessary to utilise the sequence information experimentally. Antipeptide antisera were raised for use in screening a cDNA expression library (Section 3B4) and redundant primers were made for cloning from cDNA using a PCR method (Section 3B5). Details of these approaches and the problems encountered are discussed in the relevant sections. In summary, it was found that short sequences and unknown residues within a sequence limited the usefulness of a particular peptide. In addition the antipeptide sera might not recognise the intact 135kD protein efficiently. For the PCR experiments it was necessary to use primers with minimal redundancy to prevent non-specific priming events so unknown residues (X) were problematic. This meant that peptides Glasgow 39, 40 and 23 were not very useful.

Figure 3C5

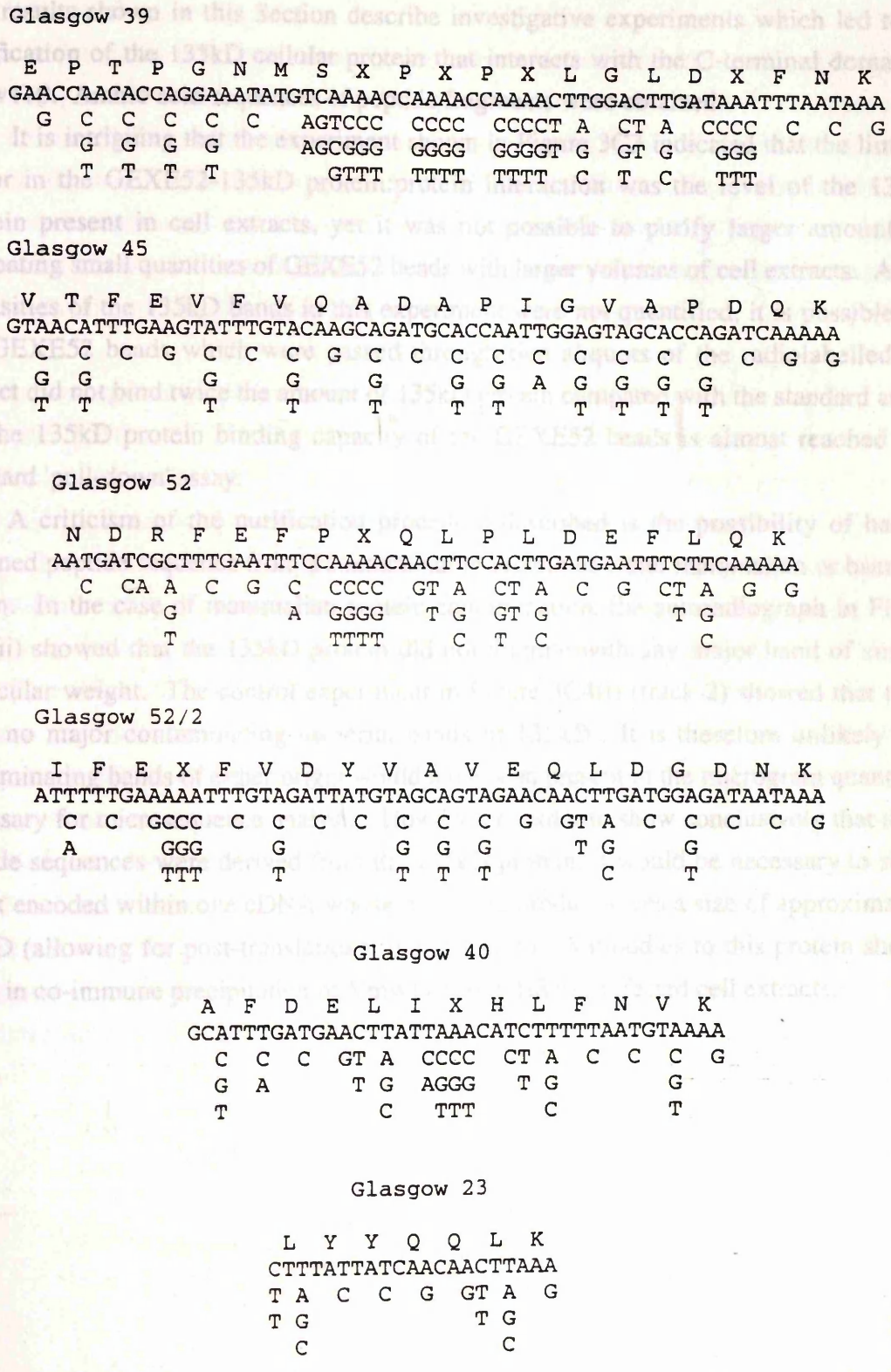


Figure 3C5 135kD peptide sequences. The sequences of the six peptides are shown using the single letter amino acid symbols (See Abbreviations). All potential codons for each amino acid are also shown, indicating the level of redundancy for design of PCR primers.

3C6 Discussion

The results shown in this Section describe investigative experiments which led to the purification of the 135kD cellular protein that interacts with the C-terminal domain of Vmw110. Amino acid sequences of peptide fragments were obtained.

It is intriguing that the experiment shown in Figure 3C3 indicated that the limiting factor in the GEXE52-135kD protein:protein interaction was the level of the 135kD protein present in cell extracts, yet it was not possible to purify larger amounts by incubating small quantities of GEXE52 beads with larger volumes of cell extracts. As the intensities of the 135kD bands in this experiment were not quantified, it is possible that the GEXE52 beads which were passed through two aliquots of the radiolabelled cell extract did not bind twice the amount of 135kD protein compared with the standard assay, i.e. the 135kD protein binding capacity of the GEXE52 beads is almost reached in a standard 'pull-down' assay.

A criticism of the purification procedure described is the possibility of having obtained peptide sequence from a contaminating protein of either mammalian or bacterial origin. In the case of mammalian protein contamination, the autoradiograph in Figure 3C4(ii) showed that the 135kD protein did not migrate with any major band of similar molecular weight. The control experiment in Figure 3C4(i) (track 2) showed that there were no major contaminating bacterial bands of 135kD. It is therefore unlikely that contaminating bands of either origin would have been present in the microgram quantities necessary for microsequence analysis. However, in order to show conclusively that these peptide sequences were derived from the 135kD protein, it would be necessary to show all six encoded within one cDNA whose translated product gives a size of approximately 135kD (allowing for post-translational modification). Antibodies to this protein should result in co-immune precipitation of Vmw110 from HSV-1 infected cell extracts.

3D Anti-135kD protein antisera

3D1 Introduction

The experiments in this section describe attempts to identify an anti-135kD antibody from an existing panel of antibodies which were known to detect various ND10 antigens and to raise an anti-135kD antipeptide serum in rabbits. A strong, specific monoclonal antibody or an antiserum which detected the 135kD protein in Western blots was required for further investigation of the Vmw110-135kD interaction: firstly, in screening a cDNA expression library to obtain a clone; secondly in immune precipitation experiments; and thirdly in immunofluorescence experiments.

3D2 Screening anti-ND10 monoclonal antibodies

It was thought that the 135kD protein may be an ND10 protein because it interacted with the C-terminal end of Vmw110 (Section 3A). Residues in the C-terminal end of Vmw110 were shown in immunofluorescence experiments to be required for targetting Vmw110 to these domains (Section 1D9.3). Antibodies which detected ND10 antigens in immunofluorescence experiments were used in Western blot analyses of standard GST 'pull-down' assays in an attempt to identify an anti-135kD protein antibody.

Two monoclonal antibodies, MAb 1150 and MAb 5E10, which detect ND10 antigens of 55kD and 126kD respectively (Ascoli and Maul, 1991; Stuurman *et al.*, 1992), were obtained from Professor G. Maul (Wistar Institute) and used to screen Western blots of standard GST 'pull-down' assays. The 126kD protein has been identified as an isoform of PML (Dyck *et al.*, 1994). It was considered unlikely that MAb 1150 would interact with the 135kD protein, unless the 55kD ND10 antigen was a 135kD protein breakdown product, but it was possible that 5E10 may have done and that the size discrepancy between the 126kD antigen and the 135kD protein had arisen from experimental error.

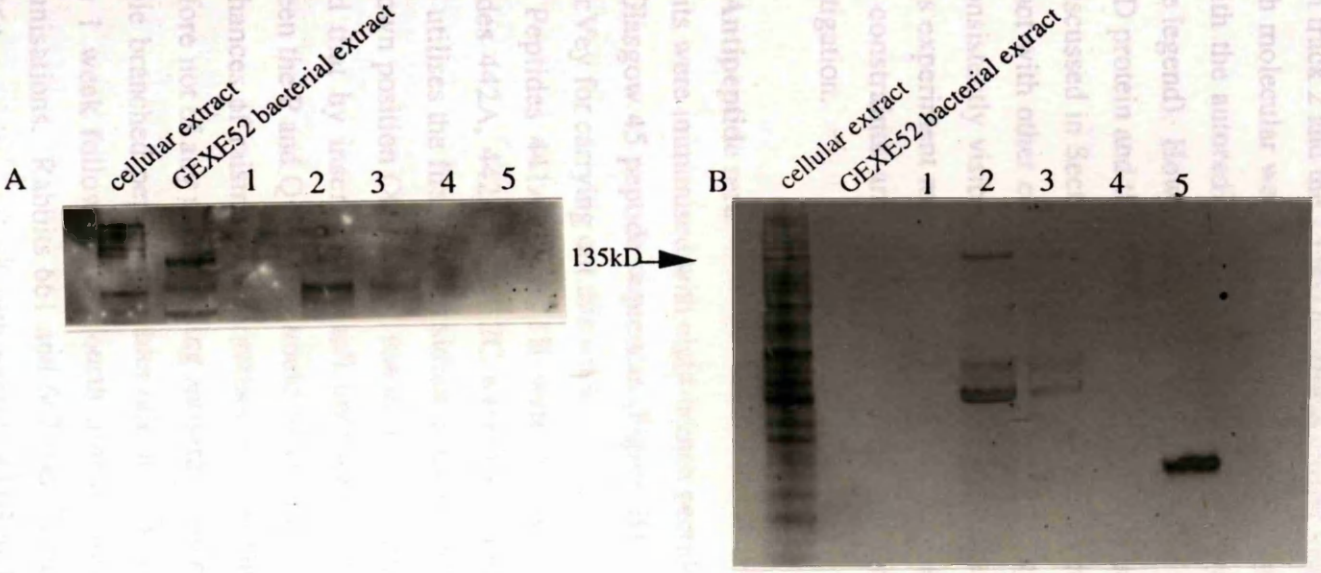


Figure 3D2 Investigation of the anti-PML antibody 5E10 in a Western blot analysis of a standard GST 'pull-down' assay. A standard GST 'pull-down' assay was carried out (Section 2B3.4) and protein samples electrophoresed on a 12.5% polyacrylamide gel prior to Western blotting (Section 2B3.3). A strip of the blot from the high molecular weight region was probed with the monclonal antibody 5E10 (Panel A) and following ECL the blot was stripped, rejoined with the lower portion of the filter and exposed to Kodak X-OMAT S film. Tracks 2 and 3 show proteins eluted from GEXE52 beads in two successive elutions with reduced glutathione. The position of the 135kD band is indicated in Panel B and it is evident by comparison with Panel A that the high molecular weight bands in the corresponding tracks of Panel A are not the same. All other tracks are controls: 2µl 1/10 dilution of radiolabelled WS HeLa cell proteins and 5µl of the GEXE52 bacterial extract were run in the indicated tracks. In track 1, a 10µl sample 50% slurry GEXE52 beads were run and in 4 a similar sample of GST beads. Track 5 shows proteins eluted from GST beads.

As expected, MAb 1150 did not detect the 135kD band in the GEXE52 eluate (results not shown). The results with MAb 5E10 are shown in Figure 3D2. Panel A shows the Western blot and B an autoradiograph of the same blot after it was stripped to remove any remaining chemiluminescence and reunited with the portion containing lower molecular weight proteins (Section 2B3.3). Due to the small amounts of antibody available, only a strip of the blot was probed. Proteins eluted from GEXE52 beads were run in track 2 and the 135kD protein is clearly visible in the autoradiograph (B). There is a high molecular weight band in the corresponding track of the Western blot (A) which, as with the autoradiograph, is not present in any of the control tracks 1, 4 and 5 (see figure legend). However, a careful comparison of the two original films indicated that the 135kD protein and the major band in track 2 of the Western blot had different mobilities. As discussed in Section 3A10 it is possible that the C-terminal 180 residues of Vmw110 interact with other cellular proteins which are not pulled down in sufficient quantities to be consistently visible on an autoradiograph. The protein detected by monoclonal 5E10 in this experiment may be an example and it is possible that this is PML. Unfortunately, time constraints and the unavailability of alternative antibodies prevented further investigation.

3D3 Antipeptide sera

Rabbits were immunised with eight-branch peptides which were based on the Glasgow 52 and Glasgow 45 peptide sequences (Figure 3D3(i)). The author wishes to acknowledge G. McVey for carrying out this work.

Peptides 441A and 441B were designed from the Glasgow 52 sequence and peptides 442A, 442B and 442C were based upon the Glasgow 45 sequence. Peptide 441B utilises the first seven residues of Glasgow 52. Insertion of a glycine residue at the unknown position (X) enabled use of more Glasgow 52 residues in peptide 441A. It was hoped that by inserting a small uncharged amino acid at this position, the spacing between the P and Q residues would reflect that in the intact protein which would increase the chances of raising a good antiserum. Peptides 442A and 442B were insoluble and therefore not of any use in raising antisera. Two rabbits were immunised with each of the soluble branched peptides at intervals of approximately 2 weeks. Blood samples were taken 1 week following the fourth immunisation as well as after the sixth and tenth immunisations. Rabbits 661 and 662 were immunised with peptide 441A, rabbits 663 and 664 were immunised with peptide 441B and rabbits 665 and 666 were immunised with peptide 442C.

A. Glasgow 52

NDRFEFPXQLPLDEFLQK

441A: $(NDRFEFPGQLPLDEFL)_8K_7$ soluble (in water)

441B: $(NDRFEFP)_8K_7$ soluble

B. Glasgow 45

VTFEVFVQADAPIGVAAPDQK

442A: $(VTFEVFVQADAPIGVA)_8K_7$ insoluble

442B: $(VTFEVFVQ)_8K_7$ insoluble

442C: $(ADAPIGVA)_8K_7$ soluble

C

Peptide	Rabbit numbers
441A	r661, r662
441B	r663, r664
442C	r665, r666

Figure 3D3(i) Peptides designed for raising antisera to the 135kD protein in rabbits. Panel A shows the amino acid composition of peptides 441A and 441B which were based on the sequence of the Glasgow 52 peptide. Peptide 441A contains a glycine residue (G) at the unknown position (X). Panel B shows the structures of the three peptides designed from the sequence information of the Glasgow 45 peptide. Peptides used for raising antisera were those which were soluble in water and the numbers of the rabbits immunised with these peptides are shown in Panel C.

Standard 'pull-down' experiments were carried out with appropriate controls and gels were Western blotted and blots probed with the rabbit antisera. Initially, the antisera were used at dilutions of 1/100 to 1/1000 and then at dilutions as low as 1/10, without successful detection of the 135kD protein. It was possible that the quantities of 135kD protein on the blots were too low to be detected by the antisera so a larger scale 'pull-down' experiment was carried out using the method described in Figure 3C4(i). The concentrated GEXE52 eluate was mixed with a small quantity of radiolabelled proteins from a standard assay, and samples were electrophoresed and Western blotted. Each serum was tested and the blot stripped between each experiment. One serum, r664 which was raised against peptide 441B, detected the 135kD protein in the GEXE52 eluate (compare tracks 4 and 5 of Figure 3D3(ii)) at dilutions as low as 1/5000. The 135kD

protein was not picked up in the control GEXE52 eluate (track 3) or the GEXE52 bacterial extract (track 2). The serum did not detect a 135kD protein in the WS HeLa cell protein extract, giving instead weak detection of multiple cell proteins (track 1). This confirmed that the interaction of this antiserum with the 135kD protein was weak at best. Further immunisation of the rabbit with the 441B peptide did not improve the quality of the serum. An attempt was made by A. Orr to improve its quality by passing 10ml of the serum, diluted 1/10 with 10mM Tris.HCl (pH 7.5) to give 100ml, through a column containing the 441B peptide coupled to CNBr-activated Sepharose 4B (Pharmacia). Proteins which interacted with the column were eluted with 100mM glycine, pH 2.5 and then with 100mM triethylamine, pH 11.5. Unfortunately, this procedure did not improve the quality of the antibody sufficiently: the eluate failed to interact with the 135kD protein on Western blots of standard 'pull-down' assays.

Although the r664 serum did not detect low levels of the 135kD protein on Western blots, it was used to screen the λZAPII WS HeLa cell cDNA expression library (Section 2B5.2). It was thought that high local peptide concentrations would make this approach to cloning a cDNA feasible. Variations on the standard protocol were carried out but specific clones were not detected.

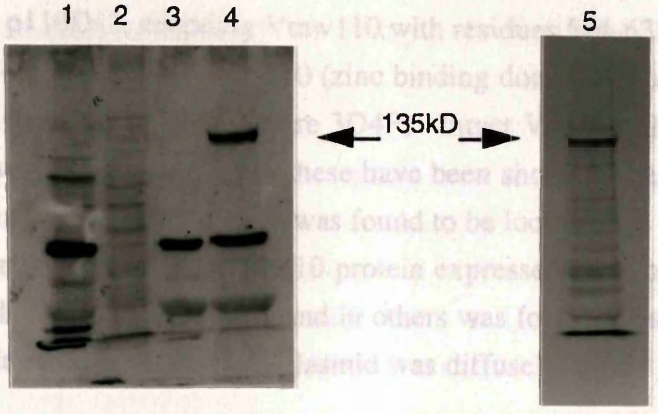


Figure 3D3(ii) Western blot of a GST 'pull-down' assay with rabbit 664 serum raised against 441B. A GST 'pull-down' experiment was carried out such that 1ml GEXE52 beads bound the 135kD protein from a cell extract prepared from five roller bottles of WS HeLa cells (Section 3C4(i)). The rabbit 664 serum was used at a dilution of 1/5000 in the Western blot shown in the left-hand panel. In track 1, 20µl of the GEXE52 bacterial extract were run and in track 2 a 20µl sample of the WS HeLa cell extract. Tracks 3 and 4 show proteins eluted from GEXE52 beads (the eluates were concentrated in a Centricon 50 unit) with track 3 being the control as the beads were mixed with WS HeLa cell resuspension buffer. The track 4 eluate was mixed with proteins eluted from GEXE52 in a standard assay and track 5 shows an autoradiograph of track 4 following stripping of the blot. A comparison of tracks 4 and 5 confirmed that the high molecular weight band detected in track 4 was the 135kD protein.

3D4 Immunofluorescence

The r664 serum was used in immunofluorescence experiments in an attempt to investigate the location of the 135kD protein in WS HeLa cells (Section 2B2.5). While the lack of a strong interaction between the r664 serum and the 135kD protein on Western blots suggested that these experiments might be difficult or inconclusive, it was thought worthwhile to make an attempt as the antibody might have been more specific or stronger in immunofluorescence experiments, particularly if the 135kD protein was present in locally high concentrations.

The location of the 135kD protein in untransfected cells was investigated by probing cells with serum obtained from rabbit 664 prior to immunisation with the 441B peptide and with the r664 antiserum (Figure 3D4A, left and right hand panels respectively). Unfortunately, there was no significant difference between the results obtained with the pre-immune and anti-peptide antisera and no conclusion as to the normal intracellular location of the 135kD protein could be drawn.

In another series of experiments, cells were transfected with plasmids encoding Vmw110 sequences (Section 2B2.4). These plasmids were p111, encoding intact Vmw110 protein, p110D12, encoding Vmw110 with residues 594-633 deleted, p110FXE, encoding Vmw110 with residues 106-150 (zinc binding domain) deleted and p110E52X, encoding Vmw110 residues 1-593 (Figure 3D4F). Intact Vmw110 localised to punctate domains in the nucleus (Figure 3D4B); these have been shown to be ND10 (Maul *et al.*, 1993). The protein product of p110D12 was found to be localised to punctate domains in the nucleus (Figure 3D4C). The Vmw110 protein expressed from p110 FXE located to punctate nuclear domains in some cells and in others was found in the cytoplasm (Figure 3D4D). The product of the p110E52X plasmid was diffusely spread through the nucleus (Figure 3D4E).

In cells transfected with plasmid p111, the r664 serum gave a signal which colocalised with Vmw110 at discrete sites within the nucleus (Figure 3D4B). The pre-immune serum did not give such a signal (results not shown). Control experiments were carried out to check that the TRITC conjugated goat anti-rabbit antibody was not non-specifically binding MAb 11060 and that FITC conjugated goat anti-mouse antibody was not binding to rabbit antibodies.

Similar colocalisation of the signal from the r664 serum was also obtained with Vmw110 expressed from p110D12, at ND10 (Figure 3D4C). This was surprising as the Vmw110 protein expressed from this plasmid lacks the residues shown to be necessary for interacting with the 135kD protein (594-633): this was shown in GST 'pull-down' assays (Section 3A6) and in immune precipitation experiments with intact Vmw110 (Section 3A9). It is possible that Vmw110 residues other than 594-633 also interact with the 135kD protein, and that conditions within the cell are more favourable for this interaction than those in immune precipitation reactions. An alternative explanation is that the r664 serum non-specifically detects Vmw110. This problem has been encountered previously with an entirely different rabbit serum (R. Everett, personal communication) (r414) which had been raised against a human PML peptide sequence. Anti-serum r414 detected ND10 in the presence of Vmw110 in all cell types tested, including a PML deficient cell line. Like r664, r414 also gave non-specific diffuse fluorescence in untransfected cells. This indicates that extreme caution should be taken regarding the interpretation of immunofluorescence experiments carried out with rabbit sera, especially when high concentrations of Vmw110 are present.

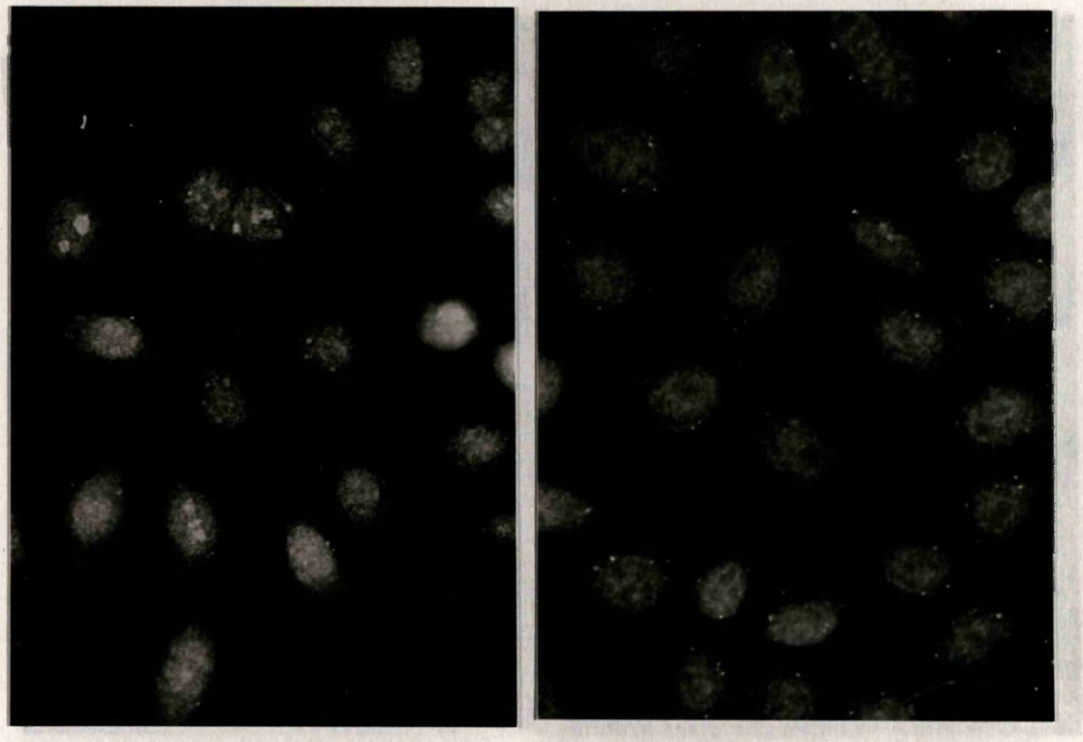
The r664 serum gave a signal which very weakly colocalised with Vmw110 expressed from p110FXE (Figure 3D4D). The distribution of the RING finger mutant Vmw110 in these experiments was unusual, in that in some cells the protein was present in the cytoplasm while it is usually located at ND10 within the nucleus (compare the two different phenotypes in Figure 3D4D, left hand panel). The reasons for the altered distribution are unknown but they correlate with the amount of protein present. While the

r664 signal was easily seen to colocalise with wild type Vmw110 in punctate nuclear dots, this was not evident with the RING finger mutant.

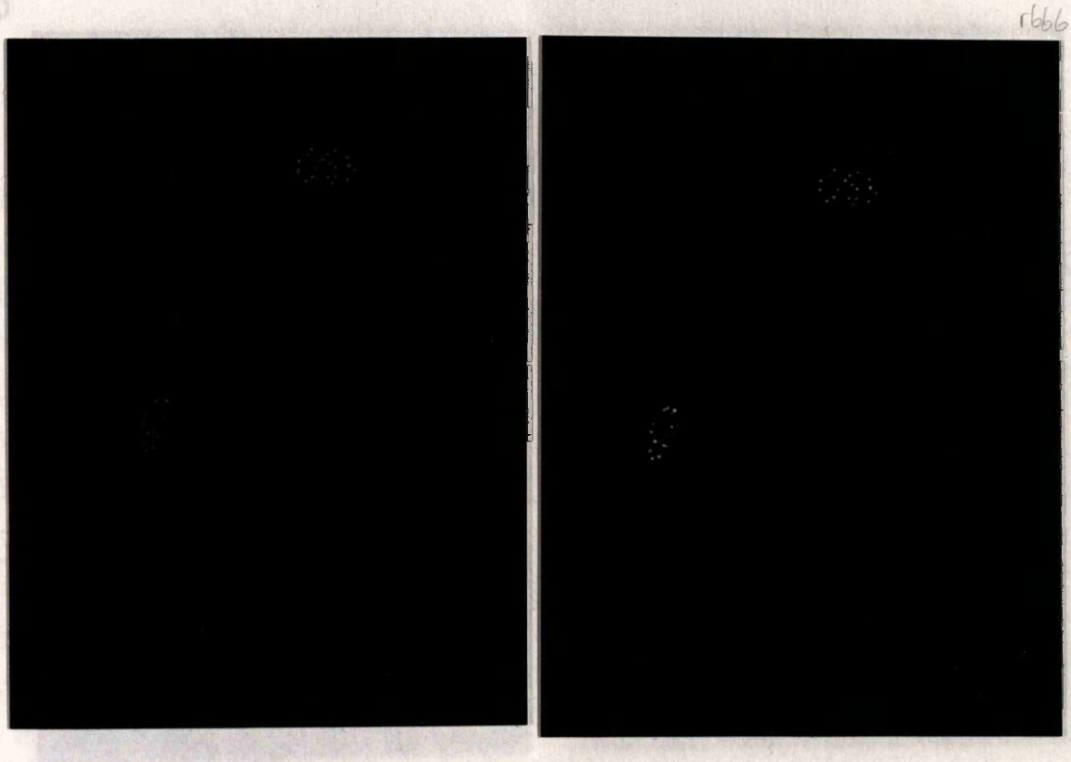
As expected, co-localisation between Vmw110 expressed from p110E52X and the 135kD protein was not seen (Figure 3D4E).

These results are difficult to interpret, particularly with the knowledge that the r664 serum may be detecting Vmw110 non-specifically, although this was not obvious from channel cross-over control experiments or Western blots (R. Everett, personal communication). If the non-specific interaction was weak, this would explain the apparent lack of colocalisation of the r664 signal with Vmw110 expressed from p110E52X as the Vmw110 protein is not concentrated in punctate domains. Before conclusions as to the normal cellular location of the 135kD protein can be drawn, it is necessary to repeat these experiments with a stronger anti-135kD serum, or even better, a monoclonal antibody.

A



B



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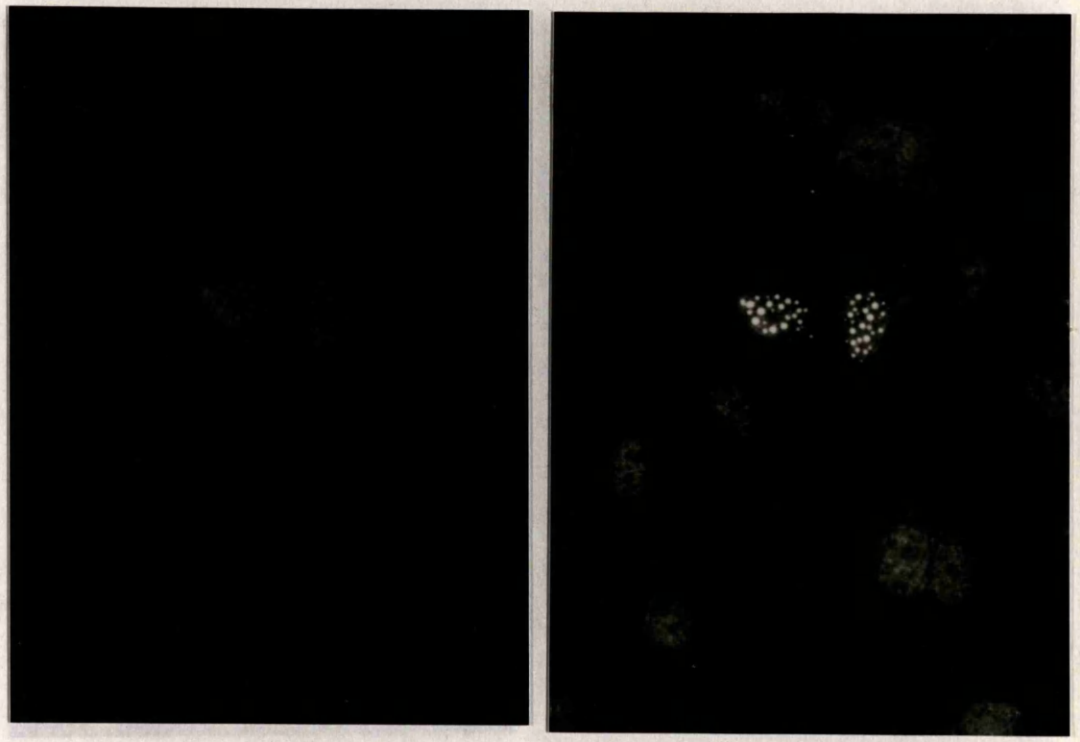
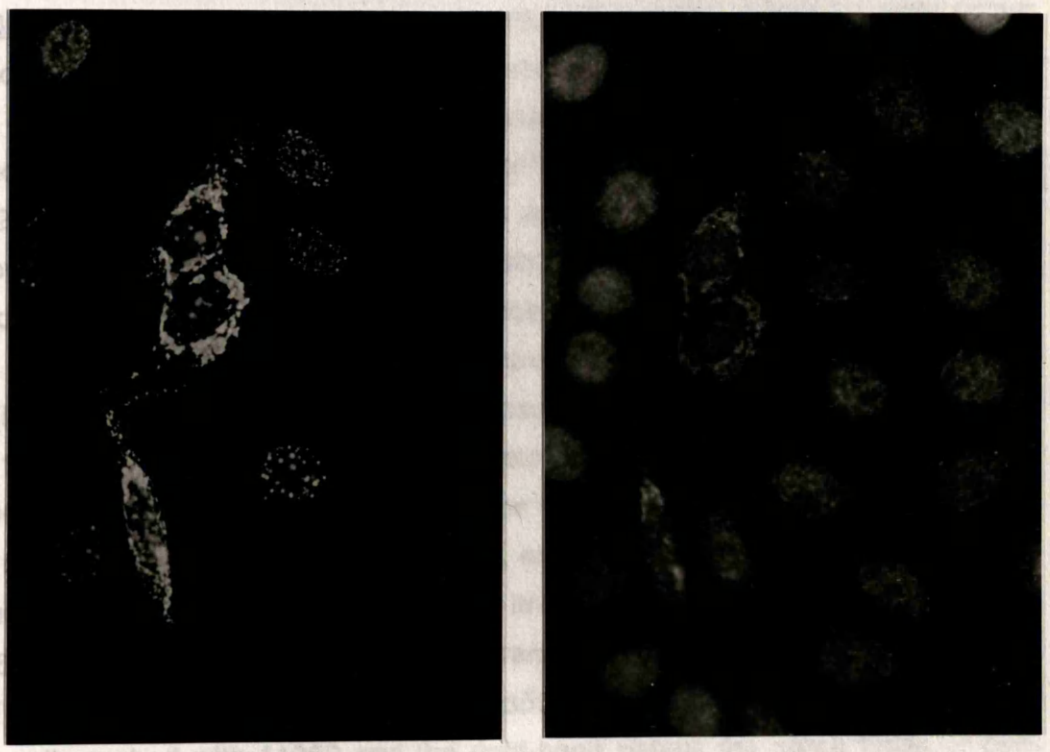


D



E

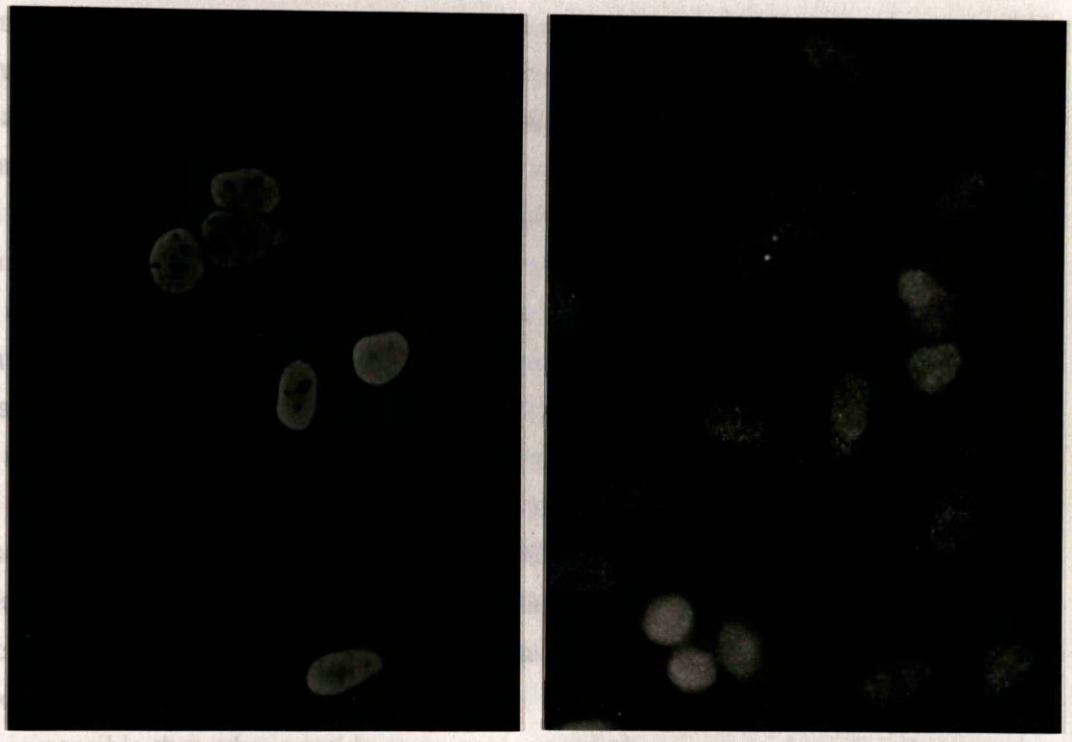


Figure 3D4 Immunofluorescence experiments carried out using the anti-135kD antipeptide serum r664. WS HeLa cells were seeded in 24 well Nunc Linbro plates at $0.5X10^5$ cells per well in 1ml medium and transfected with the relevent plasmids as described in Section 2B2.4. Immunofluorescence was carried out as described in Section 2B2.4; monoclonal antibody 11060 was used at a dilution of 1/2000 and r664 at 1/100 (lower dilutions did not improve the results). FITC labelled goat anti-mouse IgG was used to detect 11060 binding and TRITC labelled goat anti-rabbit igG to detect r664 binding. Panel A shows untransfected cells probed with the preimmune serum at 1/100 dilution (left-hand panel) and r664 (right-hand panel). Panel B shows cells transfected with p111, from which wild type Vmw110 is expressed; the left-hand panel was probed with 11060 and the right-hand panel shows the same field of cells probed with r664. Panel C shows cells transfected with p110D12, expressing Vmw110 with residues 594-633 deleted; the left-hand panel shows 11060 immunofluorescence and the right-hand panel r664 fluorescence. Panel D shows cells transfected with p110FXE (Δ106-150) and Panel E cells transfected with p110E52X (Δ594-775), again, the left-hand panels showing cells probed with 11060 and the right-hand panels the same fields of cells probed with r664.

3D5 Discussion

The results in this Section show attempts to identify a monoclonal antibody or a rabbit antiserum to the 135kD protein. Unfortunately, neither of the monoclonal antibodies raised against ND10 antigens detected the 135kD protein in Western blots of standard GST 'pull-down' assays. Of the rabbit anti-sera raised against 135kD peptides, only r664 gave a positive result and this was obtained only when large amounts of the 135kD protein were present. This meant that the serum was very weak and explains why a cDNA clone was not detected on screening the cDNA expression library. Also, it may well have been the case that any clones identified in the library would have been detected as a result of non-specific interactions between the serum and expressed peptides. This would not have been discovered until several rounds of plaque purification had been carried out and the clone sequenced. The weak reactivity of the r664 serum also explains why the normal cellular location of the 135kD protein could not be established in immunofluorescence experiments. The precedence of the potential problems of non-specific binding indicated by the r414 serum made it difficult to draw conclusions from these experiments.

It is possible that other peptide sequences would have been more immunogenic, but the possibility that some rabbit sera non-specifically detect Vmw110 in immunofluorescence experiments did not make further attempts at raising an antipeptide rabbit serum attractive. Therefore, attempts to clone a cDNA by other means were made (Section 3E).

3E Cloning a cDNA encoding the 135kD protein

3E1 Introduction

The experiments in this section describe experimental approaches that were taken to clone a cDNA encoding the 135kD protein. In one approach, redundant primers were designed from peptide sequence information (Section 3C5) for PCR with a cDNA template. Another approach involved screening the λZAPII library with a 53 base probe designed from one of the longer peptide sequences.

3E2 PCR cloning

A schematic diagram of the PCR cloning method is outlined in Figure 3E2(i). PolyA$^+$ RNA was isolated from WS HeLa cell extracts and reverse transcribed (Section 2B1.10). The cDNA was used as a template in PCR reactions with redundant primers designed from peptide sequence information (Section 2B1.13). As the order of the peptides within the 135kD protein sequence was unknown, pairs of redundant primers in the forward and reverse orientations were made so that in attempting to make interpeptide PCR products, only one of two primer combinations should give a specific product. PCR products were cloned into a vector (Section 2B1.5(v)) and sequenced (Section 2B1.14). Products encoding 135kD sequence could be identified by translating sequences following the primer sequences to check that they matched those predicted from the peptides. These clones could then be used to obtain the remainder of the cDNA clone by further PCR or by screening a cDNA library.

A number of redundant PCR primers were made based upon peptide sequence information (Figure 3E2(ii)). In designing primers, it was important to keep the level of redundancy as low as possible to avoid non-specific priming events. The length was also important as the shorter a primer is, the greater is its ability to hybridise non-specifically to DNA. Restriction enzyme sites were incorporated at the 5' ends of some primers to aid the cloning of PCR products. To increase the chances of priming from correct sequences, the redundancies of the 3' ends of primers were kept to a minimum, i.e. primers did not finish at redundant positions. Partial guess 20mer primers were made initially (P3, P3r, P4 and P4r) but it was found that random priming events occurred, as numerous products were yielded from any one pair in PCR amplifications. Fully redundant primers were designed to avoid highly redundant codons such as those of lysine or serine residues. An examination of the peptide sequences showed that Glasgow 45 was the most useful as it contained no lysine, serine or unknown residues. Two of the lowest redundancy primers, RP4 and RP4/r with a redundancy of 1/256, were designed from this peptide. Other primers of this type, RP5 and RP5r, RP11 and RP11r, RP7 and RP7r, RP10 and RP10r and RP3 and RP3r, had redundancies of 1/1024, 1/1536, 1/512, 1/1024 and 1/256 respectively.

Figure 3E2(i)

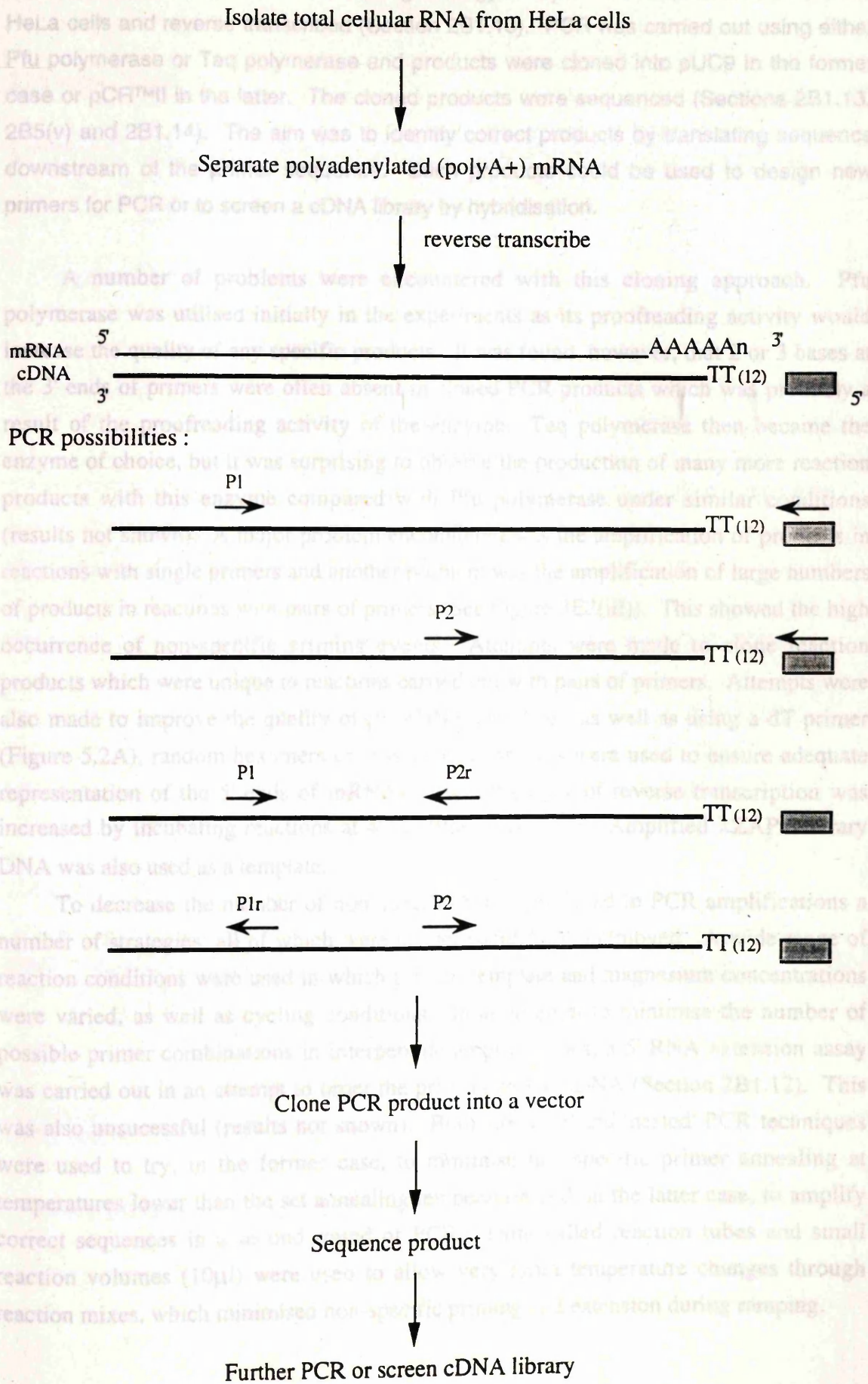

Isolate total cellular RNA from HeLa cells
Separate polyadenylated (polyA+) mRNA
reverse transcribe
mRNA
cDNA
5'
3'
AAAAAn
3'
TT(12)
5'
PCR possibilities :
P1
TT(12)
P2
TT(12)
P1
P2r
TT(12)
P1r
P2
TT(12)
Clone PCR product into a vector
Sequence product
Further PCR or screen cDNA library

Figure 3E2(i) Outline of the PCR cloning strategy. PolyA$^+$ RNA was obtained from WS HeLa cells and reverse transcribed (Section 2B1.10). PCR was carried out using either Pfu polymerase or Taq polymerase and products were cloned into pUC9 in the former case or pCR™II in the latter. The cloned products were sequenced (Sections 2B1.13, 2B5(v) and 2B1.14). The aim was to identify correct products by translating sequence downstream of the primer sequence. Such products could be used to design new primers for PCR or to screen a cDNA library by hybridisation.

A number of problems were encountered with this cloning approach. Pfu polymerase was utilised initially in the experiments as its proofreading activity would increase the quality of any specific products. It was found, however, that 2 or 3 bases at the 3' ends of primers were often absent in cloned PCR products which was probably a result of the proofreading activity of the enzyme. Taq polymerase then became the enzyme of choice, but it was surprising to observe the production of many more reaction products with this enzyme compared with Pfu polymerase under similar conditions (results not shown). A major problem encountered was the amplification of products in reactions with single primers and another problem was the amplification of large numbers of products in reactions with pairs of primers (See Figure 3E2(iii)). This showed the high occurrence of non-specific priming events. Attempts were made to clone reaction products which were unique to reactions carried out with pairs of primers. Attempts were also made to improve the quality of the cDNA template : as well as using a dT primer (Figure 5.2A), random hexamers or reverse PCR primers were used to ensure adequate representation of the 5' ends of mRNAs. The efficiency of reverse transcription was increased by incubating reactions at 42°C rather than 37°C. Amplified λZAPII library DNA was also used as a template.

To decrease the number of non-specific bands produced in PCR amplifications a number of strategies, all of which were unsuccessful, were employed. A wide range of reaction conditions were used in which primer, template and magnesium concentrations were varied, as well as cycling conditions. In an attempt to minimise the number of possible primer combinations in interpeptide amplifications, a 5' RNA extension assay was carried out in an attempt to order the primers on the cDNA (Section 2B1.12). This was also unsucessful (results not shown). Both 'hot start' and 'nested' PCR techniques were used to try, in the former case, to minimise non-specific primer annealing at temperatures lower than the set annealing temperature and, in the latter case, to amplify correct sequences in a second round of PCR. Thin-walled reaction tubes and small reaction volumes (10μl) were used to allow very rapid temperature changes through reaction mixes, which minimised non-specific priming and extension during ramping.

Figure 3E2(ii)

```
Glasgow 52/2

 I  F  E  X  F  V  D  Y  V  A  V  E  Q  L  D  G  D  N  K
ATTTTTGAAAAATTTGTAGATTATGTAGCAGTAGAACAACTTGATGGAGATAATAAA
  C  C  GCCC  C  C  C  C  C  C  C  G  GT A  C  C  C  C  G
  A     GGG     G        G  G  G        T G     G
        TTT     T        T  T  T           C     T

   cgGgatccTTTGTNGATTATGTNGCNGT 3'RP7
             C     C  C

          AAACANCTGATACANCGNCACttaaggc 5' RP7r
            G     A  G

           cgGgatccTATGTNGCNGTNGAGCAGCT 3' RP10
                     C           A  AT

                    ATACANCGNCANCTTGTCGACtTAaggC 5' RP10r
                      G           C  TA

IP1 38mer 5'TTTGTIGATTATGTIGCIGTIGAACAACTIGATGGIGA
                    C           G  G

IP1n 38-mer     5'GATTATGTIGCIGTIGAACAACTIGATGGIGATAATAA
                    C           G  G           C  C

IP1r  38-mer      CTAATACAICGICAICTTGTTGAICTACCICTATTATT5'
                    G           C  C           G  G

IP1nr 38mer AAACAICTAATACAICGICAICTTGTTGAICTACCICT 5'
                    G           C  C


Glasgow 39

 E  P  T  P  G  N  M  S  X  P  X  P  X  L  G  L  D  X  F  N  K
GAACCAACACCAGGAAATATGTCAAAACCAAAACCAAAACTTGGACTTGATAAATTTAATAAA
  G  C  C  C  C  C   AGTCCC  CCCC  CCCCT A  CT A  CCCC  C  C  G
     G  G  G  G      AGCGGG  GGGG  GGGGT G  GT G   GGG
     T  T  T  T        GTTT  TTTT  TTTT  C  T  C   TTT


cgggatcc
GAACCNACNCCNGGNAATATG 3' RP6
  G           C

CTTGGNTGNGGNCCNTTATACctTaagGc 5' RP6r
  C           G
```

```
Glasgow 45
 V  T  F  E  V  F  V  Q  A  D  A  P  I  G  V  A  P  D  Q  K
GTAACATTTGAAGTATTTGTACAAGCAGATGCACCAATTGGAGTAGCACCAGATCAAAAA
  C  C  C  G  C  C  C  G  C  C  C  C  C  C  C  C  C  C  G  G
  G  G        G     G     G     G  G  A  G  G  G  G
  T  T        T     T     T     T  T     T  T  T  T

GTGACCTTTGAGGTGTTTGT 3' RP8
  C  A     A  C
GTNACNTTTcAGGTNTTTGT 3'RP9
     C  A     C
    TTTGAGGTATTTGTGCAGGC 3' P4
           T     T  A
    AAACTTCATAAACAGGTTCG 5' P4r
     G     A  G  T
cgGgatCc
      TTTGAGGTNTTTGTNCAGGC 3' RP4
       C  A     C     A
      AAACTCCANAAACANGTCCGctTAaGgc 5' RP4r
        G  A     G     A
          cGGgaTcCGTNCAGGCNGATGCNCCNAT 3' RP5
                      A     C
                CANGTCCGNCTACGNGGNTActtAaggC 5' RP5r
                      T     G
                 cgggatCcGATGCNCCNATTGGNGTNGC 3' RP11
                            C     C
                                  A
                        CTACGNGGNTATCCNCANCGcttaaggc 5'RP11r
                            G     G
                                  A
                          RP8r 5' CCGCACCGGGGTCTGGTCTT
                                     C     T  A  A
                                           A
                          RP9r 5' CCNCANCGNGGNCTGGTCTT
                                                 A  T

IP2 5'TTTGAAGTITTTGTICAAGCIGATGCICCIATTGGIGTIGCICC 44-mer
      A  G           G                 A
IPn2  44-mer 5'TTTGTICAAGCIGATGCICCIATTGGIGTIGCICCIGATCAAAA
                     G                 A                 C  G

IP2r            AAACAIGTTCGICTACGIGGITAACCICAICGIGGICTAGTTTT
5'
                     C                 T                 G  C
IP2nr AAACTTCAIAAACAIGTTCGICTACGIGGITAACCICAICGIGG 5'
        T  C         C                 T
```

```
Glasgow 52

 N  D  R  F  E  F  P  X  Q  L  P  L  D  E  F  L  Q  K
AATGATCGCTTTGAATTTCCAAAACAACTTCCACTTGATGAATTTCTTCAAAAA
  C  CA A  C  G  C  CCCC  GT A  CT A  C  G  CT A  G  G
        G        A  GGGG   T G  GT G        T G
        T           TTTT     C  T  C          C

AATGATAGATTTGAGTTTCC 3' P3
      C T     A  C
        G

cgggatcc
AATGATAGNTTTGAGTTTCC 3' RP3
  C  CC    C  A  C

TTACTATCTAAGCTTAAGGG 5' P3r
  G  GG A
       G

TTACTATCNAAGCTTAAAGGgcttaAggc 5' RP3r
  G  GG    A  C  G
```

Figure 3E2(ii) The PCR primers designed from the peptide sequences Glasgow 52/2, 39, 45 and 52 are shown. All except the inosine primers are 20mers and some have an additional 8bp at the 5' end incorporating either a BamHI or EcoRI restriction enzyme site; these bases are in lower case lettering except where they correspond to bases in the peptide sequence. The 5' extensions of RP3, RP4 and RP6 are shown on the line above the primer sequence to simplify the layout of the diagram. The sites were used for cloning PCR products into pUC9 when Pfu polymerase was used for the reactions; this was unnecessary when Taq polymerase was used as the products have 5' A overhangs which enable the cloning of products into the pCR™II vector. Primers P3, P3r, P4 and P4r are partially redundant and RP4, RP4r, RP5,RP5r, RP6, RP6r, RP7, RP7r, RP9, RP9r, RP10, RP10r, RP11 and RP11r are fully redundant. Primers RP8 and RP8r were designed to minimise the level of redundancy by taking into account the optimum codon usage in the human genome (Lathe, 1983). The longer inosine primers were designed to minimise non-specific priming events and have inosine residues at positions of four-fold redundancy.

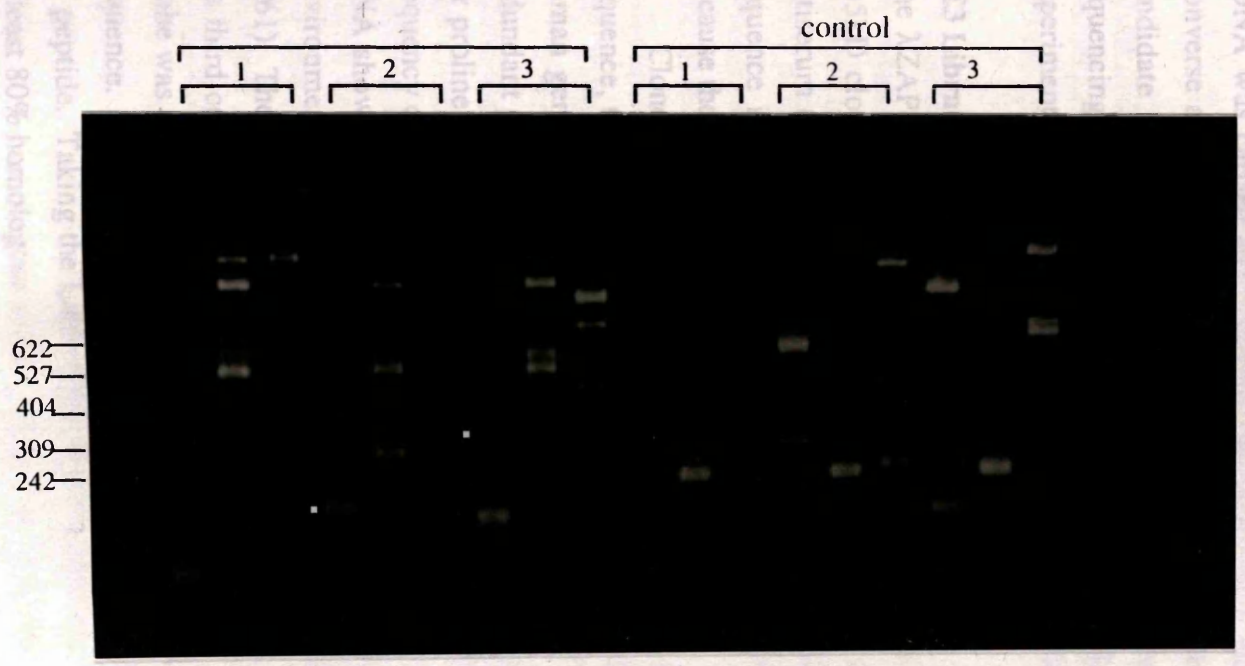


Figure 3E2(iii) An ethidium bromide stained gel showing typical PCR products. PCR reactions were carried out in 10µl reaction volumes in thin walled tubes with amplified cDNA λ ZAP library DNA, 1-5µM primers and 1.5mM MgCl2 in 1X reaction buffer (Section 2B1.13). The DNA was denatured at 99˚C for 10min and the temperature was reduced to 80˚C for the additions of 2.5 units Taq polymerase ('hot-start' PCR). Cycling conditions were 30X[45˚C for 45sec, 72˚C for 2min, 94˚C for 15sec] and 1X[45˚C for 45sec, 72˚C for 5min]. Aliquots (5µl) of each reaction were run on a 1.5% agarose gel which was stained with ethidium bromide. The template in the control tracks was bacteriophage λ DNA. In the centre and right-hand tracks of each group of three tracks, PCR was carried out with the two single primers which were used together to give the products in the first track. It is evident that some bands in the first tracks of each group were single primer artifacts, but some (indicated by white dots) are present in neither the single primer or λ vector controls. These bands were excised, cloned and sequenced.

The primers RP8, RP8r, RP9 and RP9r were designed to amplify the sequence of approximately 60bp encoding the Glasgow 45 peptide. The aim was to make a precise primer hybridising to this region and to use it in a PCR amplification with a second primer hybridising to an arm of the template λZAP DNA. In designing the RP8 and RP8r primers optimum codon usage in the human genome was considered (Lathe, 1985) to minimise the level of redundancy. RP9 and RP9r were fully redundant. Unfortunately, this strategy was also unsuccessful.

Finally, longer primers incorporating inosine at positions of redundancy were designed from peptides Glasgow 45 and Glasgow 52/2. Amplification of WS HeLa cell cDNA was carried out with IP1 and IP2r and a second round with IP1n and IP2nr. Converse amplifications were also performed. These PCR reactions gave a number of candidate bands which were visible by ethidium bromide staining, but cloning and sequencing indicated again that none were correct. However, it later transpired that this experiment had been at least partially successful (see Appendix).

3E3 Library screening

The λZAPII WS HeLa cDNA library was screened in two ways in attempts to isolate a 135kD clone. The library was screened as an expression library with an antipeptide antiserum (Section 3D3) and by DNA hybridisation with a probe designed from peptide sequence information (see below). The former method was unsuccessful, probably because the antiserum was too weak.

Clones have been successfully isolated in the past by designing probes from peptide sequence, taking into account the Lathe rules which define optimum codon usage in the human genome (Lathe, 1985). In this case, a probe was designed based on the least redundant peptide sequence, Glasgow 45 (Figure 3E3). Taking, for example, the codon for proline which is CCN, C would be chosen for the third position as it is used with a frequency of 41% compared to 24% for T and A and 11% for G. Additionally, eukaryotic DNA shows a selective deficiency in CG dinucleotides, as methylation of the C in this environment leads to instability and consequent elimination of CG pairs (Josse *et al.*, 1961). Therefore, in the design of the probe used in these experiments, C was not used in the third codon position when the next codon began with G. It was important that the probe was as long as possible to give the highest chance of efficient binding to the correct sequence. In this case the probe was limited to 53 bases by the the length of the Glasgow 45 peptide. Taking the Lathe rules into account meant that the probe ought to have been at least 80% homologous to the correct sequence (see Section 3E4).

The 53 base probe was labelled at the 5' end with γ^{32}P-ATP and used to screen blots lifted from agar plates onto which the λZAPII cDNA library had been titrated (Sections 2B5.1 and 2B5.3). Initial screening was unsuccessful and at this point the constraints of

time necessitated that these experiments were discontinued. However, this approach later turned out to be successful, as detailed in the Appendix.

Glasgow 45

```
 V  T  F  E  V  F  V  Q  A  D  A  P  I  G  V  A  P  D  Q  K
GTAACATTTGAAGTATTTGTACAAGCAGATGCACCAATTGGAGTAGCACCAGATCAAAAA
 C  C  C  G  C  C  C  G  C  C  C  C  C  C  C  C  C  C  G  G
 G  G        G     G     G     G  G  A  G  G  G  G
 T  T        T     T     T     T  T     T  T  T  T

 5'  TTTGAGGTCTTTGTGCAGGCTGATGCCCCCATTGGTGTGGCCCCTGACCAGAA
```

Figure 3E3 Sequence of the 'guessmer' oligonucleotide used to screen the cDNA λZAP library. The sequence and back translation of Glasgow 45 are shown with the sequence of the 53 base 'guessmer'.

3E4 Discussion

The results described in this Section highlight the difficulties of cloning from peptide sequence information. In particular, the redundancies of the PCR primers meant that high levels of non-specific binding to template sequences occurred, giving rise to large numbers of reaction products during amplification. The presence of incorrect residues in some of the peptide sequences (see Appendix) meant that some primers, particularly those designed from the C-terminal end of Glasgow 45 (RP11, RP11r, RP8r and RP9r), had little, if any, chance of annealing to correct sequences.

It is evident that the most productive and least time consuming approach to isolating a clone was screening a cDNA library with a 'guessmer' probe. Peptide sequences of at least 17-20 residues, uninterrupted by unknown or ambiguous residues, are required for designing a probe long enough for successful use. As mentioned in Section 5E3, given entirely correct peptide sequence information, the homology of a genuine probe can be over 80%. This is lower when microsequence information is not entirely correct; the 'guessmer' based on Glasgow 45 was later found to have a homology of only 70% to the actual DNA sequence.

CHAPTER 4 DISCUSSION

The HSV-1 IE protein Vmw110 is a potent and promiscuous transactivator of gene expression in transfection assays both alone and in synergy with Vmw175. Although it is a non-essential viral protein, both in tissue culture and in mice, viruses which fail to express Vmw110 show a marked cell-type and multiplicity-dependent defect in plaque formation as a result of the failure to initiate viral gene expression (Sections 1D6 and 1D7). In tissue culture, Vmw110 null mutant viruses readily enter a quiescent state from which they can be reactivated by the addition of Vmw110 (Section 1D7). Mouse latency models have shown that Vmw110 plays a role in the efficient establishment of and reactivation from latent infection (Section 1D7). As the condition of the infected cell is likely to be a determining factor in whether or not lytic or latent infection is established (Section 1C3), there must be molecular interactions between the virus and the cell. These observations indicated that any interactions which might occur between Vmw110 and cellular proteins were potentially important in understanding the biology of HSV-1; the aim of the research presented in this Thesis was to identify and characterise any such proteins. Any hypothesis or theory proposing a mechanism of action for Vmw110 needs to account for its non-essential nature in virus infection and its non-specific transactivation properties. The necessity for Vmw175 in the presence of Vmw110 in viral infection also needs to be considered.

4.1 The interaction of Vmw110 with a 135kD cellular protein

The C-terminal 180 residues of Vmw110 were investigated because a deletion in this region (D14 Δ680-720) affected the localisation of Vmw110 to ND10 (nuclear structures containing cellular proteins; Section 1D9.3) and reduced the ability of Vmw110 to transactivate gene expression in transient transfection assays (Section 1D9.5). In addition, a run of serine residues ending at residue 591 might indicate the presence of a possible domain boundary.

When expressed as a GST fusion protein, Vmw110 residues 594-775 (GEXE52) bound a 135kD cellular protein from a number of different whole cell extracts (Section 3A). The fact that the 135kD protein was not visible as a discrete band in the whole cell protein profile indicated that this protein was present in small amounts in the cell and that the interaction between the C-terminal end of Vmw110 and the 135kD protein was strong and specific. GEXE58, containing Vmw110 residues 633-775, did not bind the 135kD protein in this assay so it was concluded that residues 594-633 were necessary for the interaction in this system. Vmw110 residues 594-646 were found to be sufficient for the interaction as GEXE52AvaI bound the 135kD protein (Section 3A6).

As the GST 'pull-down' assay involved the use of isolated domains of Vmw110, it was possible that the Vmw110 residues had not folded correctly and that the interaction

with the 135kD protein was therefore artifactual. The fact that a 135kD protein could be coimmune precipitated in a complex with Vmw110 from infected cell extracts showed that this was unlikely. The reciprocal depletion experiment indicated that the 135kD proteins in the two systems were likely to be the same (Section 3A9). Additional coimmune precipitation experiments have been carried out by other members of the group using infected cell extracts; the Vmw110 protein in a wild type infected cell extract coimmune precipitated with a 135kD cellular protein but the Vmw110 protein of the deletion mutant virus D12 (Δ594-633) did not, confirming the necessity of Vmw110 residues between 594-633 for the interaction with the 135kD protein in infected cell extracts (Meredith *et al.*, 1994; 1995). Although these experiments indicate that C-terminal residues of Vmw110 fold in a manner reflecting that of the intact protein when expressed as GST fusion proteins, it is likely that residues immediately adjacent to GST sequences are unable to behave as they would in the intact protein by virtue of being juxtaposed to foreign residues. This means that the N-terminal boundary of the residues necessary for interacting with the 135kD protein is downstream of residue 594. Even though Vmw110 residues 594-646 are necessary and sufficient for the interaction of Vmw110 with the 135kD protein, these experiments do not exclude the possibility of the involvement of further but non-essential residues of Vmw110 in the interaction.

The Vmw110 sequence between 594-633 is approximately 70% conserved in the equivalent HSV-2 protein but there is little conservation in the equivalent proteins of the other α-herpesviruses. This raises the question of the importance of the interaction in the life-cycle of α-herpesviruses in general. However, the Vmw110 mutant virus D12 (missing Vmw110 residues 594-633) exhibits an impaired growth ability in tissue culture (Meredith *et al.*, 1995), indicating that the interaction is significant in the life cycle of HSV-1. It is possible that this interaction is specific to HSV-1 although motifs in the equivalent proteins of the other α-herpesviruses may interact with the 135kD protein in a different way to Vmw110. It is evident that different viruses disrupt ND10 in different ways (Section 1D9.2) and it is possible that the Vmw110-135kD interaction is connected with this. Therefore, the Vmw110 equivalent of other α-herpesviruses may interact with other cellular proteins which results in the same ultimate effect on ND10; that of their disruption. It would be of interest to investigate in GST 'pull-down' assays or in immune precipitation experiments whether or not the Vmw110 homologues of HSV-2 or other α-herpesviruses or any other viral protein that interacts with ND10 interacts with a cellular protein the same as or similar to the 135kD protein. This would answer the question of whether or not the 135kD protein was involved generally in ND10 disruption during virus infection. If this was the case, then the interaction of this cellular protein with viral proteins may present a target for general anti-viral chemotherapy.

It is possible that a proline-rich sequence between Vmw110 residues 594-633 is responsible for the interaction of Vmw110 with the 135kD protein. The PXXP motif, a copy of which is present in the proline-rich region, is contained within protein domains recognised by SH3 domain proteins (Ren *et al.*, 1993; Yu *et al.*, 1994). The SH3 domain is a 50-60 amino acid domain which is important for the involvement of proteins containing the domain in cell signal transduction. Whilst the 135kD protein does not contain an SH3 domain (see Appendix), it is still possible that the Vmw110:135kD protein interaction is mediated by these residues in Vmw110. There are two mutant Vmw110 proteins, E4 and E9, which contain four amino acid insertions in the proline-rich region (Everett, 1987a). The PXXP domain of E4 is intact but is displaced and this protein has a slightly reduced ability to transactivate gene expression in the absence of Vmw175 in transient transactivation assays (approximately 80% of wild type levels), whilst the PXXP domain of E9 is unaltered and the protein has near wild type transactivating activity. However, in the presence of Vmw175, the ability of E9 Vmw110 to transactivate gene expression is much reduced compared with the level of wild type Vmw110 and Vmw175. It is therefore possible that two GPR repeats in the proline-rich region are important; one of these is lost in E9 Vmw110. Both GPR repeats are present in the E4 insertion mutant protein which shows only a slight reduction in transactivating ability in the presence of Vmw175 (approximately 80% of wild type levels). Whether or not the proline-rich sequence is important for the interaction with the 135kD protein could be more fully investigated by point mutating various residues in this region and using the GST 'pull-down' assay to investigate 135kD binding.

4.2 The interaction of Vmw110 with a 135kD cellular protein from a number of different cell types

It is intriguing that a 135kD protein was pulled down from all the cell types tested, given that some of the cell lines were derived from different species (human, hamster, rat). It would have been less surprising to observe different sized but related proteins. A knowledge of the degree of similarity between these proteins will come by cloning and analysing the relevant cDNAs, although a suitable panel of antibodies to the human 135kD protein may show the degree of cross-reactivity with these proteins. If these proteins were very similar in different species, it could indicate the importance of the role of the 135kD protein in the cell.

The ND7 cell line, a neuronal line originally obtained by the fusion of neuroblastoma cells with primary rat sensory neurons (Wood *et al.*, 1990), was examined to ascertain whether or not the 135kD protein was present in both undifferentiated and differentiated cells. Although these cells are not true neuronal cells, it is possible that the pattern of gene expression in differentiated ND7 cells may reflect that of neurons. It was thought that if the protein was absent from differentiated

cells, this may be an alternative explanation to those already put forward by other investigators (Section 1B3) as to the relative non-permissivity of these cells to virus infection. It may be that in the absence of the 135kD protein, Vmw110 cannot efficiently transactivate gene expression and the virus behaves as a Vmw110 null mutant, thus entering latency more efficiently. The idea of differential gene expression in neurons playing a role in HSV-1 latency has also been proposed by Latchman's group; they suggested that the presence of particular isoforms of Oct-2 in neurons prevents transactivation of IE genes (Section 1B3). Aside from the technical difficulties encountered with this experiment (Section 3A5), the presence of the 135kD protein in both undifferentiated and differentiated ND7 cell extracts could have been a result of the fact that differentiated ND7 cells do not truly reflect the characteristics of neurons regarding gene expression.

One method by which the presence or absence of the 135kD protein in neurons could be ascertained is by staining a section of neuronal ganglia with a suitable 135kD antibody. However, a result showing the presence of the 135kD protein in neuronal cells may not necessarily mean that it is present in similar quantities *in vivo*; if it is involved in an interaction with Vmw110 that is linked to the reactivation of latent virus, its expression may be rapidly induced in the latent to lytic switch. Therefore, it is possible that its expression could arise as a stress or shock response during or following the death of the host but prior to fixing the cells for staining.

It would be informative to observe the effects of HSV-1 infection on a 135kD protein 'knock out' cell line. If HSV-1 plaqued on a 135kD negative cell line with the efficiency of a Vmw110 null mutant on the parental cell line, this would suggest that the interaction between Vmw110 and the 135kD protein is important for the function of Vmw110 in lytic growth. As the alternative to lytic infection is latency, this would provide circumstantial evidence for the necessity of the Vmw110:135kD protein interaction in the reactivation of latent infection. However, solving the role played by Vmw110 in infection, particularly with regard to latency, may not answer the fundamental question of the mechanism by which HSV-1 establishes and reactivates from latent infection; the activity of Vmw110, which may be intricately connected with the 135kD protein, whilst contributing to the efficiency of the process might only complement a mechanism mediated via another viral protein.

4.3 Is the 135kD protein an ND10 component?

Of interest is the fact that the 135kD protein was found to be present in both nuclear and cytoplasmic extracts in GST 'pull-down' assays (Section 3A8). Unfortunately, the r664 antipeptide serum, which was raised to a 135kD peptide (Section 3D3), was too weak to confirm this in immunofluorescence assays. The presence of the 135kD protein in the cytoplasm in quantities too large to suggest that there was leakage of the 135kD protein

from nuclei indicates that this is of functional significance. This suggested that the 135kD protein was not an ND10 protein as it would have been expected to be present predominantly in the nuclear extract. The cytoplasmic location of the 135kD protein may be connected to observations of Everett and Maul (1994) who showed in immunofluorescence experiments that Vmw110 becomes located in the cytoplasm as well as in the nucleus during the course of infection.

4.4 The potential involvement of Vmw110 and the 135kD protein in a transport process

Prior to knowledge of the 135kD cDNA sequence (see Appendix), it was thought that Vmw110 and the 135kD protein were involved in a transport process; it was possible that one was responsible for the transport of the other from the nucleus to the cytoplasm or that both were linked in the cotransportation of other proteins. The nature of such a transport process could only be speculated.

(i) The proposed transport process

The 135kD protein might act at the pretranscriptional level to locate transcription factors translated in the cytoplasm to the nucleus for storage in ND10. Vmw110 consequently would disrupt this arrangement by dispersing ND10 proteins which could result in communication between the nucleus and cytoplasm signalling that the ND10 'stores' were empty. According to this model, the cell would then direct cytoplasmically sequestered transcription factors to the nucleus immediately upon translation which would be favourable for viral gene transcription; they would not enter ND10 due to the presence of Vmw110. This would complement the shut-off of host cell macromolecular synthesis by the virus (Section 1B2.5). It is important to consider how such a proposed process could explain other known properties of Vmw110, such as its synergistic activation of gene expression with Vmw175 in transient transfection assays and its apparent involvement in latency.

(ii) Relating the proposed transport process to other properties of Vmw110

In the above scenario, the synergistic transactivation of gene expression by Vmw110 and Vmw175 in transfection assays could be explained if the gene encoding the 135kD protein was transactivated by Vmw175; the effects of Vmw110 on ND10 disruption would be increased if Vmw110 bound the 135kD protein in a process which allowed the transport of transcription factors into the nucleus but prevented their localisation to ND10. The fact that Vmw175 null mutant viruses are non-viable despite expressing a functional Vmw110 protein could be explained if Vmw110-mediated transactivation depended on a threshold level of the 135kD protein; although Vmw110 transactivates gene expression to a degree by itself in transient transfection assays, this activity may

not be sufficiently high to induce lytic infection in the viral context. This phenomenon could be investigated by examining the levels of gene expression in cells transfected with plasmids encoding Vmw110 only, and Vmw110 and the 135kD protein. In addition, provided there was a low level of IE gene expression during latency (Section 1C3(iv)), the involvement of Vmw110 in reactivation could be explained; the low level of expression of Vmw175 during latency may be increased on a stimulus for reactivation and this would result in increased 135kD protein levels so that the synergistic activation of gene expression of Vmw110 and Vmw175 would come into play.

(iii) Problems

A major drawback of the model explained above is that none of the three ND10 proteins cloned to date have been shown to activate gene expression in transfection assays, which suggests they are not transcription factors (Section 1D9.2) although they could indirectly regulate transcription. In addition, the sequence of the 135kD protein indicates that it is a member of a family of ubiquitin C-terminal carboxy hydrolases (see Appendix) which somewhat negates the hypothesis of its involvement in a transport process with Vmw110. It is an attractive proposition that the 135kD protein is substrate specific in that it acts on a small number of proteins connected somehow with the regulation of gene expression. This being the case, it would have been less surprising if it was located only in the nucleus. Its location throughout the cell suggests that it may be a general deubiquitinating enzyme. Vmw110 may, by virtue of interacting with other proteins, specifically direct its activity.

4.5 The interaction of Vmw110 with ND10

The observation that D12 Vmw110 (Δ594-633) localises to ND10 in immunofluorescence assays raises the question of which residues are responsible for its localisation to these domains. If the 135kD protein was an ND10 protein, it could be that residues other than 594-633 of Vmw110 interact with the 135kD protein for the localisation of Vmw110 to ND10 and that these interactions were undetectable in immune precipitation reactions as the assay conditions were unsuitable. However, the localisation of the 135kD protein in both the nucleus and the cytoplasm, suggesting that it is unlikely to be an ND10 protein, means that C-terminal residues of Vmw110 other than 594-633 are responsible for the initial interaction of Vmw110 with ND10. For example, D14 Vmw110 which lacks residues 680-720 gives diffuse nuclear staining in the viral and plasmid context whilst GEXE52D14 (which includes the same deletion) binds the 135kD protein. An obvious alternative candidate ND10 protein with which Vmw110 C-terminal residues could interact is PML. It would be of value to repeat the Western blot of the GEXE52 'pull-down' experiment in Section 3D2 to ascertain

whether or not the protein apparently detected by the monoclonal antibody 5E10 is PML. It could be that some of the lower molecular weight proteins occasionally seen in the GST 'pull-down' experiments (Section 3A4) were PML isoforms; they may also have been other ND10 proteins.

The immunofluorescence experiments presented in this Thesis show that the r664 serum, raised against a 135kD peptide in rabbits, detects ND10 in the presence of wild type Vmw110. This could be interpreted as meaning that Vmw110 recruits the 135kD protein into ND10. However, D12 Vmw110 gave a similar result, which means either that the 135kD protein interacts with Vmw110 residues outside of 594-633 in this recruitment process or that r664 cross-reacts with Vmw110 (this is not supported by the results of Western blots but is thought to have occurred with another rabbit antiserum, r414 (Section 3D4)). As Vmw110 and the 135kD proteins are large it would not be surprising to find that 135kD protein contacts were made with Vmw110 residues outside 594-633. The use of a specific monoclonal antibody to the 135kD protein will clarify the localisation of the 135kD protein in the presence of Vmw110 and in uninfected cells.

If Vmw110 is responsible for recruiting the 135kD protein into ND10, this could be an important step in the disruption of ND10. The proposed enzymatic activity of the 135kD protein predicted from its sequence may be responsible for ND10 disruption, although it is difficult to envisage how an enzymic activity known to counteract the ubiquitination of proteins targeted for degradation could act to disrupt a multiprotein complex. It is possible that the interaction between Vmw110 and the 135kD protein is unrelated to the disruption of ND10; the fact that D12 Vmw110 has a different disruptive effect on ND10 compared to wild type Vmw110 may be due to a structural alteration in Vmw110 rather than being an effect of the altered Vmw110:135kD protein:protein interaction. This could be tested in an immunofluorescence assay by looking at the effects of wild type Vmw110 on ND10 in a 135kD$^-$ cell line. If the Vmw110:135kD protein:protein interaction is unconnected with the disruption of ND10 by Vmw110, it may be that following ND10 disruption Vmw110 binds the 135kD protein and directs its deubiquitinating activity towards one or more ND10 proteins which are now exposed to ubiquitinating enzymes and targeted for degradation.

As Vmw110 is a large protein, it would not be surprising to find that it is involved in two or more unrelated processes, all of which contribute to its efficient function. In support of this is the observation that the growth of the D12 virus in tissue culture is less efficient than wild type virus but more efficient than the deletion mutant dl1403. The fact that the FXE deletion mutant virus grows almost as inefficiently as dl1403 in tissue culture could be because the RING finger is necessary for the correct folding of more than one functional domain of Vmw110, for example regions 2, 3, and 4 (Section 1D5). Vmw110 C-terminal residues may be required for the initial interaction of Vmw110

with ND10 and a domain dependent on the correct folding of the RING finger may aid in ND10 disruption. This is consistent with the fact that, so far, a cellular protein which interacts with the RING finger has not been identified. The possibility that the Vmw110 RING finger domain interacts with other regions of Vmw110 could be investigated by carrying out GST 'pull-down' experiments with the RING finger domain and various Vmw110 polypeptides. The RING finger dependent activity of Vmw110 required for normal ND10 disruption could therefore be separate from that required for its transactivation properties (Section 1D5) if two functional domains fold around the RING finger. It is equally possible though, that the RING finger could be an exposed functional domain whose individual activity is essential for Vmw110 function but is enhanced by other regions of Vmw110. This would mean that it is important to identify cellular proteins with which this domain interacts in order to fully understand ND10 disruption.

It is unlikely that the localisation property of C-terminal residues of Vmw110 to ND10 and the involvement of those residues in the synergistic activation of gene expression with Vmw175 are unrelated properties because the residues involved are probably too close together to be employed in different functions. However, if ND10 proteins are not transcription factors or indirect activators of gene expression, the connection between ND10 disruption and the increase in gene expression seen in transfection assays is difficult to explain. It is possible that disruption of ND10 is a general virus phenomenon which circumvents a vital cellular defence mechanism. Another explanation is that, as ND10 may be connected with sites of viral replication (Section 1D9.2(iii)), the scaffold around which the proteins are structured may be an essential anchor for viral replication.

4.6 Multimerisation of the C-terminal end of Vmw110

A multimerisation domain has been mapped to the C-terminal region of Vmw110. This has been shown indirectly in immunofluorescence assays, in *in vitro* translation assays and in glutaraldehyde cross-linking experiments (Ciufo *et al.*, 1994) as well as directly in biochemical assays (Section 3B). The experiments presented in this Thesis show that both T7E52 (Vmw110 residues 594-775) and T7E58 (Vmw110 residues 633-775) readily multimerised in all assays tested, although it was not possible to resolve the nature of these multimers. This result clearly separates the residues that are essential for binding the 135kD protein (594-633) from those which are sufficient for multimerisation. As was the case with the GST fusion proteins, it is unlikely that residues in immediate proximity to the vector-encoded MARIR residues at the N-terminal ends of the polypeptides fold correctly which means that the minimal multimerisation interface begins downstream of residue 633.

The data of Ciufo *et al.* (1994) indicates that C-terminal polypeptides of Vmw110 form dimers in *in vitro* translation experiments and that these multimers form only when the polypeptides are cotranslated and not simply mixed together. This suggests that the multimerisation interaction is strong and is unlikely to be easily disrupted, supporting the evidence that the Vmw110 multimerisation and 135kD interaction interfaces are separate.

Whilst essential residues for 135kD binding are not necessary for the localisation of Vmw110 to ND10, it is possible that the Vmw110 multimerisation domain is a requirement. It would be of value to express C-terminal segments of Vmw110 smaller than those encompassed by T7E52 and T7E58 in the T7 expression system to narrow down the multimerisation domain. In conjunction with immunofluorescence experiments using Vmw110 truncation mutants, the multimerisation of Vmw110 as a prerequisite for ND10 localisation could be determined.

4.7 The multiple protein:protein interactions of Vmw110 C-terminal residues

It is difficult to envisage how Vmw110 can multimerise in the presence of the 135kD protein when the domains necessary for both are in such close proximity and particularly when Vmw110 is located at ND10; steric hindrance might be expected to prevent one or the other from occurring. It is possible that Vmw110 binds the 135kD protein with a stoichiometry so that functional complexes contain 3-4 Vmw110 molecules and one 135kD protein molecule. This complex could form prior to Vmw110 interacting with ND10, or Vmw110 multimers may bind ND10 and undergo a conformational change which enables the binding of the 135kD protein; both hypotheses fit in with the idea of Vmw110 recruiting the 135kD protein to ND10. There is also the question of the number of Vmw110 multimers which localise to ND10 during virus infection; this is likely to be more than one because one or two molecules would be unlikely to be detected in immunofluorescence and the amount of Vmw110 produced would mean that Vmw110 would be diffusely spread in the nucleus throughout infection, even at early times. ND10 may contain a number of Vmw110 'docking' sites and be disrupted physically by Vmw110 binding. However, the possible recruitment of the 135kD protein could mean that disruption occurs as a biochemical interaction, a question which should be resolved when the 135kD protein substrate is known. It may also be the case that there is a minimal number of Vmw110 binding sites and that when Vmw110 multimers bind, others are recruited to form higher order Vmw110 complexes which result in the physical disruption of ND10; this would explain the difficulty in determining the precise physical nature of the Vmw110 multimers in the experiments described in this Thesis (Section 3B). It may be that recruitment of the 135kD protein stabilises multimer interactions, either by altering the

conformation of multimers or by inhibiting their degradation in the ubiquitin dependent pathway.

The GST fusion protein GEXE52AvaI, expressing Vmw110 residues 594-646, binds the 135kD protein in 'pull-down' assays. As the data presented in this Thesis suggests that sequences essential for multimerisation exist between residues 633-775, it is unlikely that the GEXE52AvaI protein could multimerise in the normal way. The data of Ciufo *et al.* (1994) indicates that the multimerisation domain lies between residues 617-711 with residues N- and C-terminal of these contributing to the efficiency of multimerisation. Whilst this region overlaps the Vmw110 residues of GEXE52AvaI by 29 residues, the fact that the overlap is so small indicates that these residues may not be sufficient for Vmw110 multimerisation. To be completely sure of this, it is necessary to express Vmw110 residues 594-646 in a T7 system for purification and biochemical analysis. It is likely, therefore, that multimerisation is not a prerequisite for 135kD protein binding so that the stoichiometry of the Vmw110-135kD interaction is 1:1. Due to the large sizes of Vmw110 and the 135kD proteins it is even more difficult to envisage how Vmw110 could multimerise and this complex bind a number of 135kD protein molecules when the binding domains for each property of Vmw110 are so close together. In addition, it is likely that Vmw110 interacts with another cellular protein via its C-terminal end in initially binding to ND10 (Section 4.5). A 1:1 stoichiometry of the Vmw110:135kD protein complex suggests also that the 135kD protein, due to its large size, must contact Vmw110 residues other than those between 594-633 and possibly interact with itself; multiple protein:protein contacts might stabilise the Vmw110:135kD protein complex. It would be of interest to determine the minimal 135kD binding region and to obtain structural information regarding the interaction. The expression of peptides encoding Vmw110 residues 594-646 and the 135kD protein residues which contact these should result in the formation of stable heterodimers.

In addition to its interaction with the 135kD protein and with itself, the C-terminal end of Vmw110 probably interacts with at least one other cellular protein (Section 4.5). The interaction of Vmw110 with a 150kD protein (Section 3A6) has not been observed in coimmune precipitations with Vmw110 which could mean that this interaction is an artifact of the GST 'pull-down' experiments. It is also intriguing that the 150kD protein was not present in all cell extracts. Whether or not it is a precursor of the 135kD protein could possibly be determined in Western blot experiments with a good panel of 135kD antibodies. Vmw110 residues 633-680 were shown to be necessary for binding this protein in BHK cells (Section 3A6). This means that it is probably unrelated to the 135kD protein, unless Vmw110 residues between 594-680 all contribute to the Vmw110:135kD/150kD interaction and, on processing of the proposed precursor protein, the essential residues for the interaction become slightly different. As Vmw110 residues 633-775 can interact with a 150kD cellular protein in GST 'pull-down' assays,

while residues 680-775 do not, it is possible that the Vmw110 multimerisation domain is downstream of residue 680 as it is unlikely that Vmw110 binds other proteins via its multimerisation domain (Section 4.5).

On the other hand, it may be that Vmw110 residues 633-680 contain the multimerisation domain; dimerisation of the GST portion of the fusion proteins (Section 3A3) may prevent the self interaction of these Vmw110 residues so that they are exposed to cellular proteins in the GST 'pull-down' assay. In experiments with intact Vmw110 multimerisation via these residues would prevent the 150kD protein from binding. However, if the multimerisation domain is downstream of Vmw110 residue 680, this would appear to make the potential formation of a Vmw110:135kD protein complex physically more feasible. Once formed, this complex may exclude the binding of the 150kD protein; in the GST 'pull-down' assay the Vmw110:150kD protein:protein interaction may still occur in the presence of the Vmw110:135kD protein:protein interaction because the Vmw110 residues do not multimerise.

4.8 Interactions between viral and cellular proteins

As discussed above, it is likely that Vmw110 interacts with proteins other than the 135kD protein, via residues which require correct folding of the RING finger and also via C-terminal residues other than those necessary for the 135kD interaction or multimerisation. This could be predicted on the basis of Vmw110 being a large protein and on its inability to bind directly to DNA whilst it has profound effects on gene expression. It is also supported genetically by the observation discussed above, that various Vmw110 deletion mutant viruses exhibit a range of reduced growth abilities which are often not as severe as that of dl1403. FXE is the only mutant virus which exhibits a disability as severe as that of dl1403; this could be because the RING finger carries out an essential function facilitated by other regions of the protein or, as discussed above, because mutations in the RING finger have a marked effect on the folding of the entire protein.

Vmw65 also interacts with cellular proteins (Section 1C1.1) and it is unlikely that these interactions and that of Vmw110 with the 135kD protein are the only virus:cell interactions as the life cycle of the virus involves so many gene products. The work presented in this Thesis provides a potentially interesting insight and route of pursuance for understanding the role Vmw110 plays in virus infection. As well as aiding in uncovering the mechanism of one of the fundamental biological properties of the virus, namely the lytic-latent switch, the Vmw110:135kD protein interaction will help in the understanding of the potentially important role played by ND10 in the cell.

APPENDIX

Cloning the cDNA encoding the 135kD protein

All of this work, apart from the RT PCR which was performed by the author, was carried out by R. Everett.

The λ ZAP II WS HeLa cDNA library was screened using the 53 base 'guessmer' probe designed from the sequence of peptide Glasgow 45 (Section 3E3). The method was based on the hybridisation screening method detailed in Section 2B5.3. Two clones, 1142 and 1131, were isolated and sequence analysis showed that they were partially overlapping; sequence encoding peptide Glasgow 45 was found in the overlapping region. Interestingly, three amino acid residues towards the C-terminal end of Glasgow 45 did not correspond to the sequence in the cDNA clones (Figure A). The fact that peptide Glasgow 52 was also contained within clone 1142 was strong evidence that the correct cDNA had been isolated and not that encoding a related protein. The presence of these incorrect residues in Glasgow 45 would have meant that primers designed from the sequence of the C-terminal end of Glasgow 45 (RP5r, RP11, RP11r, RP8r, RP9r; Section 3E2) would have had much less homology than expected to the correct sequence. The production of correct PCR products using these primers would therefore have been very unlikely.

Clone 1131 was also used to screen the PCR products of reactions carried out with the 38 and 44 base inosine-containing primers in a Southern blot experiment. It was found that clone 1131 hybridised to a band which was not visible upon ethidium bromide staining. Following the cloning of the complete cDNA sequence of the 135kD protein and the identification of the positions of the inosine-containing primers, the size of the band recognised by clone 1131 in this experiment was found to be correct. It is possible that correct but invisible products were also produced in other PCR experiments. Consequently, it should have been possible to identify a correct PCR product by cloning and sequencing every product from each reaction. Unfortunately, this would have been too time consuming to be a reasonable approach to obtaining a clone, which was why the method was abandoned in favour of hybridisation screening.

During the cloning procedure described above, the peptide sequences were used regularly to screen the data base of the Washington University Merck Expressed Sequence Tag (EST) Project using the NCBI BLAST E-mail service. The EST Project aims to produce sequences of approximately 300-400 bases at the 5' and 3' ends of about 200,000 cDNA clones over an 18 month period (October '94-March '96). Over 8,000 unedited sequences are entered into the database weekly. The screening procedure resulted in the identification of clone 80922 which contained a sequence encoding peptide Glasgow 39.

Clone 80922, encoding the 3' end of a cDNA was linked to clone 1142 using RT PCR with an exact primer to a sequence at the 3' end of clone 1142 (primer 1782) and an exact primer to a sequence at the 5' end of clone 80922 (primer G39R).

A PCR product of clone 1142 was used to rescreen the λ ZAP II WS HeLa cDNA library. Twelve clones were isolated and sequenced to give a single contiguous cDNA encoding the 135kD protein.

The cDNA encodes a 1102 residue protein of predicted molecular weight 128kD. Sequence analysis has shown that the protein contains two blocks of residues characteristic of the ubiquitin specific protease (USP) family of enzymes. These enzymes are found in a wide range of eukaryotic organisms, including not only humans but also yeast, mouse, *Drosophila* and *C. elegans*. Ubiquitin specific proteases, also known as deubiquitinating enzymes or isopeptidases are thiol proteases (E.C. 3.1.2.15) that recognise and cleave the amide bond at the C-terminal glycine of ubiquitin (Tobias and Varshavsky, 1991; Baker *et al.*, 1992). There is little significant homology of members of the family outside the two regions thought to be involved in catalytic activity.

The ubiquitin proteolytic pathway is a tightly regulated biochemical system in which target proteins are 'marked' for degradation by ubiquitinylation (reviewed by Wilkinson, 1995). The most renowned role in which the pathway is involved is in increasing the rates of intracellular proteolysis in the response of a cell to stress stimuli such as starvation. The result is energy production and the release of amino acids for *de novo* protein synthesis. The pathway is also involved in cell cycle regulation with respect to the selective degradation of specific cyclins (reviewed by Wilkinson, 1995).

USPs are involved in the ubiquitin pathway at four different stages; (i) they cleave ubiquitin from a precursor protein, (ii) they de-ubiquitinylate some proteins targeted for degradation, (iii) they cleave polyubiquitin chains from the peptide remnants of degraded proteins and (iv) they degrade polyubiquitin chains to ubiquitin monomers. The specificity of the pathway is not fully understood; it has been suggested that proteins are randomly ubiquitinated and deubiquitinated (by USPs) but if a protein is damaged, the bound ubiquitin undergoes a conformational change which results in the polyubiquitinylation that targets it for degradation. The fact that at least 15 ubiquitin specific proteases exist in yeast and that there are many more in *Drosophila*, mice and humans suggests that these enzymes may have specific target proteins.

The interaction of Vmw110 with a USP has led to speculations of the mechanism of action of Vmw110. It is possible that in interacting with the 135kD protein, Vmw110 sequesters a protein whose normal function is to remove ubiquitin from a protein targeted for proteolysis. The result of this could be activation of one or more of a number of cellular transcription factors which would explain the potent and promiscuous transactivation of gene expression induced by Vmw110 in transfection

assays (Section 1D5); activation of the transcription factor NFκB occurs in response to external stimuli which result in the specific ubiquitinylation and subsequent degradation of a bound inhibitor, IκB, thus allowing NFκB to move into the nucleus (Palombella *et al.*, 1994). The targeting of the 135kD protein by Vmw110 to ND10 may result in the degradation of one or more ND10 proteins normally sequestered from this pathway; this mechanism could be responsible for the subsequent disintegration of these structures observed in immunofluorescence assays.

Vmw175 induces the expression of a class of ubiquitin encoding genes (Latchman *et al.*, 1987; Kemp *et al.*, 1988) required for efficient ribosomal biogenesis (Finley *et al.*, 1989); their induction leads to an enhancement of both protein synthesis and protein degradation. Sequestration of the 135kD protein by Vmw110 could counteract the Vmw175 induced protein degradation processes by preventing the release of free ubiquitin which would act as a limiting factor in the ubiquitin dependent proteolytic pathway. This would result in a general increase in protein levels and may explain the synergistic activity of Vmw110 and Vmw175 (which requires the Vmw110:135kD interaction domain). This model is valid only if the majority of free ubiquitin is released from ubiquitinated proteins by the 135kD protein; this is feasible in virus infected cells when the majority of cellular gene expression is inhibited but is more difficult to explain in transfection assay systems. Identification of the target of the 135kD protein would be useful in further understanding the role of Vmw110 in virus infection.

Figure A The 135kD protein and cDNA sequences. The protein sequence denoted by the single letter amino acid code is shown above the cDNA sequence. The peptide sequences which were derived from the 135kD protein are shown above the relevant stretch of protein sequence, incorrect or unknown residues (X) being shown in bold. The start and stop positions of selected cDNA clones, marked I----- and -----I respectively, are shown directly below the relevant points in the cDNA sequence. The positions of the primers 1782 and G39R are also shown directly below the cDNA sequence.

Figure A

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AGTACGTGCGCGTCTCCCTGCCGCCGCCGCCCGCCGCGGGCCGCCCCGGGGCCGCCG   60

TCGCCGACGACGCGCGGGAGGAGGAGGAGGAGGCCGCCCCGCCGCCGCCGCCGCCGC  120

CGCCCCGGCTCGCCGCCGCCCGCCCGCCGGGCTCGCAGCCCCGGCCCCCGGCCGCAGGCG  180

                    M  N  H  Q  Q  Q  Q  Q  Q  Q  K  A  G      13
AGGCCCAGGCCGCGGCCGACATGAACCACCAGCAGCAGCAGCAGCAGCAGAAAGCGGGCG  240

E  Q  Q  L  S  E  P  E  D  M  E  M  E  A  G  D  T  D  D  P      33
AGCAGCAGTTGAGCGAGCCCGAGGACATGGAGATGGAAGCGGGAGATACAGATGACCCAC  300
                                    |-----start of clone 1142
P  R  I  T  Q  N  P  V  I  N  G  N  V  A  L  S  D  G  H  N      53
CAAGAATTACTCAGAACCCTGTGATCAATGGGAATGTGGCCCTGAGTGATGGACACAACA  360

T  A  E  E  D  M  E  D  D  T  S  W  R  S  E  A  T  F  Q  F      73
CCGCGGAGGAGGACATGGAGGATGACACCAGTTGGCGCTCCGAGGCAACCTTTCAGTTCA  420

T  V  E  R  F  S  R  L  S  E  S  V  L  S  P  P  C  F  V  R      93
CTGTGGAGCGCTTCAGCAGACTGAGTGAGTCGGTCCTTAGCCCTCCGTGTTTTGTGCGAA  480
                                    start of clone 1131 |-----
N  L  P  W  K  I  M  V  M  P  R  F  Y  P  D  R  P  H  Q  K      113
ATCTGCCATGGAAGATTATGGTGATGCCACGCTTTTATCCAGACAGACCACACCAAAAAA  540

S  V  G  F  F  L  Q  C  N  A  E  S  D  S  T  S  W  S  C  H      133
GCGTAGGATTCTTTCTCCAGTGCAATGCTGAATCTGATTCCACGTCATGGTCTTGCCATG  600
                    (probable) Glasgow 40   K  A  F  D  E  L
A  Q  A  V  L  K  I  I  N  Y  R  D  D  E  K  S  F  S  R  R      153
CACAAGCAGTGCTGAAGATAATAAATTACAGAGATGATGAAAAGTCGTTCAGTCGTCGTA  660
I  X  H  L  F  N  V  K
I  S  H  L  F  F  H  K  E  N  D  W  G  F  S  N  F  M  A  W      173
TTAGTCATTTGTTCTTCCATAAAGAAAATGATTGGGGATTTTCCAATTTTATGGCCTGGA  720
                                 Glasgow 45  K  V  T  F  E  V
S  E  V  T  D  P  E  K  G  F  I  D  D  D  K  V  T  F  E  V      193
GTGAAGTGACCGATCCTGAGAAAGGATTTATAGATGATGACAAAGTTACCTTTGAAGTCT  780
F  V  Q  A  D  A  P  I  G  V  A  P  D  Q  K
F  V  Q  A  D  A  P  H  G  V  A  W  D  S  K  K  H  T  G  Y      213
TTGTACAGGCGGATGCTCCCCATGGAGTTGCGTGGGATTCAAAGAAGCACACAGGCTACG  840

V  G  L  K  N  Q  G  A  T  C  Y  M  N  S  L  L  Q  T  L  F      233
TCGGCTTAAAGAATCAGGGAGCGACTTGTTACATGAACAGCCTGCTACAGACGTTATTTT  900

F  T  N  Q  L  R  K  A  V  Y  M  M  P  T  E  G  D  D  S  S      253
TCACGAATCAGCTACGAAAGGCTGTGTACATGATGCCAACCGAGGGGGATGATTCGTCTA  960

K  S  V  P  L  A  L  Q  R  V  F  Y  E  L  Q  H  S  D  K  P      273
AAAGCGTCCCTTTAGCATTACAAAGAGTGTTCTATGAATTACAGCATAGTGATAAACCTG 1020

V  G  T  K  K  L  T  K  S  F  G  W  E  T  L  D  S  F  M  Q      293
TAGGAACAAAAAAGTTAACAAAGTCATTTGGGTGGGAAACTTTAGATAGCTTCATGCAAC 1080
          end of clone 1311 homology -----|
H  D  V  Q  E  L  C  R  V  L  L  D  N  V  E  N  K  M  K  G      313
ATGATGTTCAGGAGCTTTGTCGAGTGTTGCTCGATAATGTGGAAAATAAGATGAAAGGCA 1140

T  C  V  E  G  T  I  P  K  L  F  R  G  K  M  V  S  Y  I  Q      333
CCTGTGTAGAGGGCACCATACCCAAATTATTCCGCGGCAAAATGGTGTCCTATATCCAGT 1200

C  K  E  V  D  Y  R  S  D  R  R  E  D  Y  Y  D  I  Q  L  S      353
GTAAAGAAGTAGACTATCGGTCTGATAGAAGAGAAGATTATTATGATATCCAGCTAAGTA 1260
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                K N/T I  F  E  X  F  V  D  Y  V  A  V  E  Q  L
I  K  G  K  K  N  I  F  E  S  F  V  D  Y  V  A  V  E  Q  L   373
TCAAAGGAAAGAAAAATATATTTGAATCATTTGTGGATTATGTGGCAGTAGAACAGCTCG 1320
D  G  D  N  K  Glasgow 52/2
D  G  D  N  K  Y  D  A  G  E  H  G  L  Q  E  A  E  K  G  V   393
ATGGGGACAATAAATACGACGCTGGGGAACATGGCTTACAGGAAGCAGAGAAAGGTGTGA 1380

K  F  L  T  L  P  P  V  L  H  L  Q  L  M  R  F  M  Y  D  P   413
AATTCCTAACATTGCCACCAGTGTTACATCTACAACTGATGAGATTTATGTATGACCCTC 1440
      Glasgow 52  K  X  N  D  R  F  E  F  P  X  Q  L  P  L
Q  T  D  Q  N  I  K  I  N  D  R  F  E  F  P  E  Q  L  P  L   433
AGACGGACCAAAATATCAAGATCAATGATAGGTTTGAATTCCCAGAGCAGTTACCACTTG 1500
D  E  F  L  Q  K                 |----------primer 1782---------->
D  E  F  L  Q  K  T  D  P  K  D  P  A  N  Y  I  L  H  A  V   453
ATGAATTTTTGCAAAAAACAGATCCTAAGGACCCTGCAAATTATATTCTTCATGCAGTCC 1560
        end of clone 1142 homology-----|
L  V  H  S  G  D  N  H  G  G  H  Y  V  V  Y  L  N  P  K  G   473
TGGTTCATAGTGGAGATAATCATGGTGGACATTATGTGGTTTATCTAAACCCCAAAGGGG 1620

D  G  K  W  C  K  F  D  D  D  V  V  S  R  C  T  K  E  E  A   493
ATGGCAAATGGTGTAAATTTGATGACGACGTGGTGTCAAGGTGTACTAAAGAGGAAGCAA 1680

I  E  H  N  Y  G  G  H  D  D  D  L  S  V  R  H  C  T  N  A   513
TTGAGCACAATTATGGGGGTCACGATGACGACCTGTCTGTTCGACACTGCACTAATGCTT 1740

Y  M  L  V  Y  I  R  E  S  K  L  S  E  V  L  Q  A  V  T  D   533
ACATGTTAGTCTACATCAGGGAATCAAAACTGAGTGAAGTTTTACAGGCGGTCACCGACC 1800

H  D  I  P  Q  Q  L  V  E  R  L  Q  E  E  K  R  I  E  A  Q   553
ATGATATTCCTCAGCAGTTGGTGGAGCGATTACAAGAAGAGAAAAGGATCGAGGCTCAGA 1860

K  R  K  E  R  Q  E  A  H  L  Y  M  Q  V  Q  I  V  A  E  D   573
AGCGGAAGGAGCGGCAGGAAGCCCATCTCTATATGCAAGTGCAGATAGTCGCAGAGGACC 1920

Q  F  C  G  H  Q  G  N  D  M  Y  D  E  E  K  V  K  Y  T  V   593
AGTTTTGTGGCCACCAAGGGAATGACATGTACGATGAAGAAAAAGTGAAATACACTGTGT 1980

F  K  V  L  K  N  S  S  L  A  E  F  V  Q  S  L  S  Q  T  M   613
TCAAAGTATTGAAGAACTCCTCGCTTGCTGAGTTTGTTCAGAGCCTCTCTCAGACCATGG 2040

G  F  P  Q  D  Q  I  R  L  W  P  M  Q  A  R  S  N  G  T  K   633
GATTTCCACAAGATCAAATTCGATTGTGGCCCATGCAAGCAAGGAGTAATGGAACAAAAC 2100

R  P  A  M  L  D  N  E  A  D  G  N  K  T  M  I  E  L  S  D   653
GACCAGCAATGTTAGATAATGAAGCCGACGGCAATAAAACAATGATTGAGCTCAGTGATA 2160

N  E  N  P  W  T  I  F  L  E  T  V  D  P  E  L  A  A  S  G   673
ATGAAAACCCTTGGACAATATTCCTGGAAACAGTTGATCCCGAGCTGGCTGCTAGTGGAG 2220

A  T  L  P  K  F  D  K  D  H  D  V  M  L  F  L  K  M  Y  D   693
CGACCTTACCCAAGTTTGATAAAGATCATGATGTAATGTTATTTTTGAAGATGTATGATC 2280

P  K  T  R  S  L  N  Y  C  G  H  I  Y  T  P  I  S  C  K  I   713
CCAAAACGCGGAGCTTGAATTACTGTGGGCATATCTACACACCAATATCCTGTAAAATAC 2340

R  D  L  L  P  V  M  C  D  R  A  G  F  I  Q  D  T  S  L  I   733
GTGACTTGCTCCCAGTTATGTGTGACAGAGCAGGATTTATTCAAGATACTAGCCTTATCC 2400

L  Y  E  E  V  K  P  N  L  T  E  R  I  Q  D  Y  D  V  S  L   753
TCTATGAGGAAGTTAAACCGAATTTAACAGAGAGAATTCAGGACTATGACGTGTCTCTTG 2460

D  K  A  L  D  E  L  M  D  G  D  I  I  V  F  Q  K  D  D  P   773
ATAAAGCCCTTGATGAACTAATGGATGGTGACATCATAGTATTTCAGAAGGATGACCCTG 2520
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E  N  D  N  S  E  L  P  T  A  K  E  Y  F  R  D  L  Y  H  R    793
AAAATGATAACAGTGAATTACCCACCGCAAAGGAGTATTTCCGAGATCTCTACCACCGCG 2580

V  D  V  I  F  C  D  K  T  I  P  N  D  P  G  F  V  V  T  L    813
TTGATGTCATTTTCTGTGATAAAACAATCCCTAATGATCCTGGATTTGTGGTTACGTTAT 2640

S  N  R  M  N  Y  F  Q  V  A  K  T  V  A  Q  R  L  N  T  D    833
CAAATAGAATGAATTATTTTCAGGTTGCAAAGACAGTTGCACAGAGGCTCAACACAGATC 2700

P  M  L  L  Q  F  F  K  S  Q  G  Y  R  D  G  P  G  N  P  L    853
CAATGTTGCTGCAGTTTTTCAAGTCTCAAGGTTATAGGGATGGCCCAGGTAATCCTCTTA 2760

R  H  N  Y  E  G  T  L  R  D  L  L  Q  F  F  K  P  R  Q  P    873
GACATAATTATGAAGGTACTTTAAGAGATCTTCTACAGTTCTTCAAGCCTAGACAACCTA 2820
   K  L  Y  Y  Q  Q  L  K  Glasgow 23
K  K  L  Y  Y  Q  Q  L  K  M  K  I  T  D  F  E  N  R  R  S    893
AGAAACTTTACTATCAGCAGCTTAAGATGAAAATCACAGACTTTGAGAACAGGCGAAGTT 2880

F  K  C  I  W  L  N  S  Q  F  R  E  E  E  I  T  L  Y  P  D    913
TTAAATGTATATGGTTAAACAGCCAATTTAGGGAAGAGGAAATAACACTATATCCAGACA 2940

K  H  G  C  V  R  D  L  L  E  E  C  K  K  A  V  E  L  G  E    933
AGCATGGGTGTGTCCGGGACCTGTTAGAAGAATGTAAAAAGGCCGTGGAGCTTGGGGAGA 3000

K  A  S  G  K  L  R  L  L  E  I  V  S  Y  K  I  I  G  V  H    953
AAGCATCAGGGAAACTTAGGCTGCTAGAAATTGTAAGCTACAAAATCATTGGTGTTCATC 3060

Q  E  D  E  L  L  E  C  L  S  P  A  T  S  R  T  F  R  I  E    973
AAGAAGATGAACTATTAGAATGTTTATCTCCTGCAACGAGCCGGACGTTTCGAATAGAGG 3120

E  I  P  L  D  Q  V  D  I  D  K  E  N  E  M  L  V  T  V  A    993
AAATCCCTTTGGACCAGGTGGACATAGACAAAGAGAATGAGATGCTTGTCACAGTGGCGC 3180

H  F  H  K  E  V  F  G  T  F  G  I  P  F  L  L  R  I  H  Q   1013
ATTTCCACAAAGAGGTCTTCGGAACGTTCGGAATCCCGTTTTTGCTGAGGATACACCAGG 3240

G  E  H  F  R  E  V  M  K  R  I  Q  S  L  L  D  I  Q  E  K   1033
GCGAGCATTTTCGAGAAGTGATGAAGCGAATCCAGAGCCTGCTGGACATCCAGGAGAAGG 3300

E  F  E  K  F  K  F  A  I  V  M  T  G  R  H  Q  Y  I  N  E   1053
AGTTTGAGAAGTTTAAATTTGCAATTGTAATGACGGGCCGACACCAGTACATAAATGAAG 3360
                                  end of clone 80922 homology I-----
         Glasgow 39  K  D/SF/S  E  P  T  P  G  N  M  S  X  P
D  E  Y  E  V  N  L  K  D  F  E  P  Q  P  G  N  M  S  H  P   1073
ACGAGTATGAAGTAAATTTGAAAGACTTTGAGCCACAGCCCGGTAATATGTCTCATCCTC 3420
X  P  X  L  G  L  D  X  F  N  K
R  P  W  L  G  L  D  H  F  N  K  A  P  K  R  S  R  Y  T  Y   1093
GGCCTTGGCTAGGGCTCGACCACTTCAACAAAGCCCCAAAGAGGAGTCGCTACACTTACC 3480
            <----------Primer G39R-----------I
L  E  K  A  I  K  I  H  N  -                                  1102
TTGAAAAGGCCATTAAAATCCATAACTGATTTCCAAGCTGGTGTGTTCAAGGCGAGGACG 3540

GTGTGTGGGTGGCCCCTTAACAGCCTAGAACTTTGGTGCACGTGCCCTCTAGCCGAAGTC 3600

TTCAGCAAGAGGATTCGCTGCTGGTGTTAATTTTATTTTATTGAGGCTGTTCAGTTTGGC 3660

TTCTCTGTATCTATTGACTGCCCTTTTTGAGCAAAATGAAGATGTTTTTATAAAGCTT   3718
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