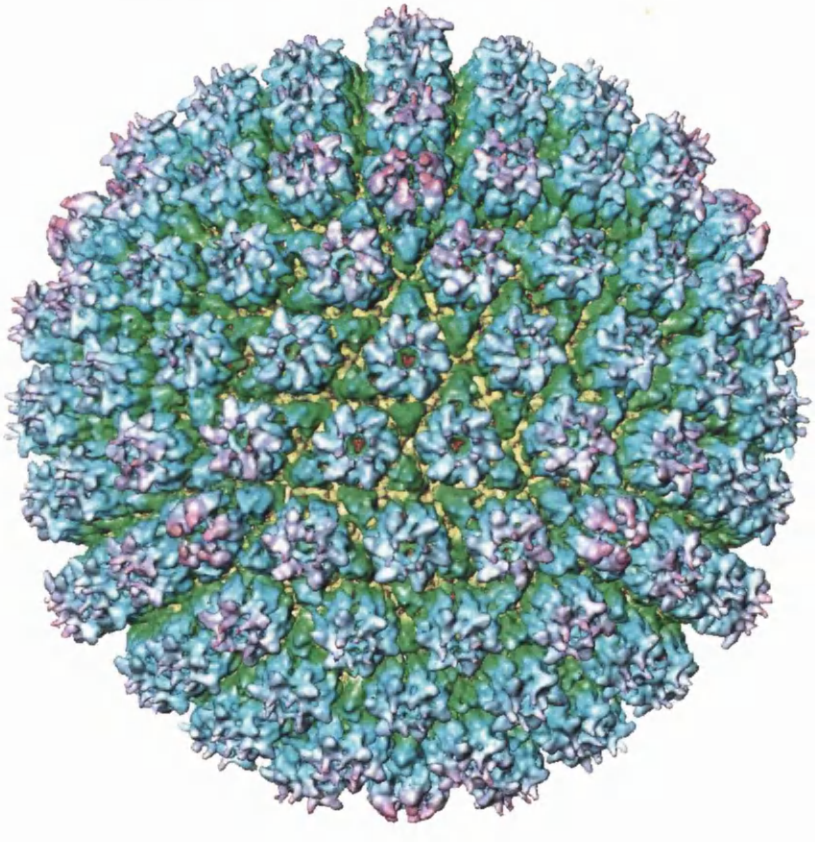
An investigation of the properties and functions of the herpes associated ubiquitin-specific protease, HAUSP

by

Meeta Kathoria



A thesis presented for the degree of Doctor of Philosophy
in
The Faculty of Science at The University of Glasgow

MRC Virology Unit
Church Street
Glasgow
G11 5JR

February 1999

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

SUMMARY

Herpes simplex virus type 1 (HSV-1) is a common human pathogen best known as the causative agent of 'cold sores' around the mouth. It initially infects cells at the periphery, however it often spreads to the sensory neurones where it establishes life-long latent infection and from which it can be reactivated periodically to cause recurrent episodes of disease. The immediate early (IE) protein Vmw110 of HSV-1 stimulates the onset of lytic infection as well as increases the efficiency of reactivation from latency. As such, it has been proposed that Vmw110 plays an important role in the balance between lytic and latent states of infection. The mechanisms by which Vmw110 functions are poorly defined. However, earlier work in which Vmw110 was shown to migrate to discrete nuclear structures called ND10, suggested that it exerts much of its effects through interactions with cellular proteins (Everett & Maul, 1994, Gelman & Silverstein, 1985, Maul *et al.*, 1993). Investigations searching for such interactions resulted in the identification of a novel member of the ubiquitin-specific protease (USP) family named HAUSP (herpes-associated-ubiquitin specific protease) which both strongly and specifically interacted with Vmw110 (Everett *et al.*, 1997, Meredith *et al.*, 1995, Meredith *et al.*, 1994).

Studies described herein were initiated to improve the understanding of the role of HAUSP, both within the cell and for HSV-1 infection. In particular, experiments using a model USP enzyme assay confirmed that HAUSP was an enzymatically active member of the USP family. Furthermore, the presence of specific cysteine and histidine residues were shown to be essential for this activity.

Investigations into the effect of transient expression of HAUSP in eukaryotic cells were also carried out. These studies suggested firstly that levels of intracellular HAUSP may be tightly controlled and secondly that increases in HAUSP expression might be toxic for cells. They also implied localisation of HAUSP to the ND10 domains was limited by protein-protein interactions.

Work was also initiated to search for cellular proteins that interact with HAUSP. This resulted in the identification of strong and specific interactions between: the N-terminal region of HAUSP with cellular proteins of approximately 100kD and 105kD; and sequences in the C-terminal half of HAUSP with a cellular protein of approximately 40kD. Immunoprecipitation analysis supported the interaction of wild type HAUSP with cellular proteins of approximately 40kD and 105kD. It was also revealed that of these cellular

proteins only the approximately 40kD cellular protein (which interacted with the C-terminus of HAUSP) was a substrate for proteasome-dependent degradation.

More direct investigations were also carried out to improve our understanding of the mechanics and functioning of the Vmw110/HAUSP interaction. In particular, a variety of GST 'pull-down' assays were designed and tested to define the region of HAUSP required for this interaction. Results of this work implied that regions of HAUSP spanning between residues 529-576 and 744-861, were necessary for binding to Vmw110. Immunoprecipitation analysis experiments supported the requirement of the 529-576 region. Interestingly, this region is directly downstream of one of the two active site domains of HAUSP.

The effect of the presence of Vmw110 on the normal cellular activities of HAUSP was also tested. Surprisingly, Vmw110 did not appear to reduce the catalytic activity of HAUSP, or hinder HAUSP from interacting with the approximately 40kD cellular protein previously described.

Therefore, the specific functions of HAUSP in uninfected cells and during HSV-1 infection are yet to be fully characterised. However, the results presented in this thesis provide a solid foundation upon which future work leading to a deeper understanding of HAUSP functioning may be based.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to acknowledge Professor Duncan McGeoch for the support he provides to post-graduates students and for the high standard of research facilities supplied within the department.

I would also very much like to thank my supervisor Dr. Roger Everett whom I have come to respect highly as a scientist for both his technical skills and also his scientific ethics.

During my post-graduate studies many members of the department have been of great assistance to me. I am especially grateful to Anne Orr for her guidance. I would also like to thank Michayla Meredith and Jane Parkinson for their technical advice, as well as their support. Patrick Lomonte I would like to thank for his helpful discussions and his wonderful sense of humour. Thanks also to the members of Fraser Rixon's Lab for their generosity.

I am eternally grateful to Jonathan who provided so much encouragement during the writing of this thesis. Thanks also to Darren, Jane, Patrick and Roger for doing such a thorough job (I hope) of proof reading this work.

On a more personal level I would also like to thank my friends in the department for providing such a supportive framework. In particular I would like to thank Richard to whom I am indebted for his understanding, patience and culinary skills. Thanks also to my good friend Sarah for her helping me through these three and a half years by being so supportive.

I am also very grateful to my friends outside the department, especially my past and present flatmates, for maintaining my sanity in the last three years, well maybe?

I am also extremely indebted to the guys from flat 3F for the use of their incredibly comfortable chairs.

Finally, I would like to thank my parents who deserve so much credit for their continual encouragement and unselfish support.

The author was a recipient of a Medical Research Council studentship and unless otherwise stated all the results obtained were by her own efforts.

ABBREVIATIONS

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

A:	adenine, absorbance, alanine, or Amps
Amp:	ampicillin
Amp^r:	ampicillin resistance
APL:	acute promyelocytic leukemia
ATP:	adenosine-5'-triphosphate
β-Gal:	beta-galactosidase
bp:	base pairs
C:	cytosine, carboxy (-terminal end of protein), or cysteine
°C:	degrees celsius
CHAPS:	2-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate
Ci:	Curie
Cm:	chloramphenicol
Cm^r:	chloramphenicol resistance
CMV:	cytomegalovirus
CsCl:	caesium chloride
C-terminal:	carboxy-terminal
(k)D:	(kilo) dalton
DAPI:	4'-6'-diamidino-2-phenylindole
dATP:	2'-deoxyadenosine-5'-triphosphate
ddNTP:	2'-,3'-dideoxyribonucleoside

ddTTP:	2'-,3'-dideoxythymidine-5'-triphosphate
dGTP:	2'-deoxyguanosine-5'-triphosphate
DMSO:	dimethylsulphoxide
DNA:	2'-deoxyribonucleic acid
DNase:	deoxyribonuclease
dNTP:	2'-deoxyribonucleoside
DOTAP:	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N,-trimethylammonium methylsulphate
DTT:	dithiothreitol
dTTP:	2'-deoxythymidine-5'-triphosphate
dUMP:	2'-deoxyuridine-5'-monophosphate
dUTP:	2'-deoxyuridine-5'-triphosphate
dUTPase:	deoxyuridine-triphosphatase
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
(μ)g:	(micro)gram, or glycoprotein
G:	guanine, glycine

GST:	glutathione-S-transferase
HAUSP:	herpes-associated ubiquitin specific protease
HCMV:	human cytomegalovirus
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HHV:	human herpesvirus
hr:	hour(s)
HRP:	horse radish peroxidase
HSV:	herpes simplex virus
IE:	immediate early (alpha)
IPTG:	isopropyl-D-thiogalactoside
IR_L:	long internal repeat
IR_S:	short internal repeat
kb:	kilo base pairs
Km:	kanamycin
Km^r:	kanamycin resistance
L:	Late (gamma), litre
(m/μ)l:	(milli/micro)litre
LAT:	latency associated transcript
LB:	Lauria-Bertani medium
(c/μ/n)m:	(centi/micro/nano)metre
(m/μ/n/p)M:	(milli/micro/nano/pico)molar
MAb:	monoclonal antibody
MHC:	major histocompatibility complex

min:	minute(s)
moi:	multiplicity of infection
(m/μ/n/p)mol:	(milli/micro/nano/pico)moles
N:	nucleoside, asparagine, or amino (-terminal end of protein)
ND10	nuclear domain 10
NP40:	Nonidet P40
OD:	optical density
ORF:	open reading frame
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
pfu:	plaque forming unit
PML	promyelocytic leukaemia protein
PMSF:	phenylmethylsulphonyl fluoride
PODs	PML oncogenic domains
RAR-α:	retinoic acid receptor alpha
rATP:	riboadenosine-5'-triphosphate
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

sec: second(s)

SGB: stacking gel buffer

SV40: simian virus 40

T: thymine, threonine,

TBS: Tris buffered saline

TE: Tris EDTA

Tris: tris (hydroxymethyl) aminomethane

TRITC: tetramethylrhodamine isothiocyanate

ts: temperature sensitive

TWEEN-20: polyoxyethylene-sorbitanmonolaurate

Ub: ubiquitin

UBP: ubiquitin-specific protease

U_L: unique long (region of HSV)

U_S: unique short (region of HSV)

USP: ubquitin-specific protease

U.V.: ultra violet

V: volts

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

VZV: varicella-zoster virus

W: watts

YT: yeast tryptone broth

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				
		Stop Codons	TAA	TAG	TGA			

CHAPTER 1 INTRODUCTION

Research presented in this thesis concerns the interaction of the HSV-1 IE protein Vmw110 with a cellular protein named HAUSP. As will be seen, this project has broad implications for both virus and cellular biology. Therefore, in order to reflect this, an introduction in which the wide ranging topics to which this project relates has been provided.

The first section focuses on general features of Herpesviridae biology and details the life cycle of HSV-1. As Vmw110 is a potent and general transactivator of HSV-1 gene expression, background on the regulation of HSV-1 gene expression is also provided. Following this, details relating to Vmw110 and its role in viral infection are given.

The subsequent sections describe cellular processes with which this project is concerned. In particular, as HAUSP is a novel member of the USP family, relevant background on the ubiquitin system and USP family are supplied. The concluding section concerns the effects of Vmw110 on the cell and includes an up to date understanding of its interaction with HAUSP.

1A HSV-1 biology

1A1 Herpesviridae

HSV-1 is a member of the Herpesviridae, a large family consisting of almost 100 known members derived from a wide range of hosts including horses, cattle, pigs, chickens and humans. In particular, eight distinct human herpesviruses have been identified: 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 herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7) and Kaposi's sarcoma implicated herpesvirus 8 (KSHV, HHV-8). Sequences of the genomes of these viruses can be found in Baer *et al.*, 1984, Cha *et al.*, 1996, Chee *et al.*, 1990, Dargan *et al.*, 1997, Davison & Scott, 1986, Dolan *et al.*, 1998, Gompels *et al.*, 1995, McGeoch *et al.*, 1985, Nicholas, 1996, Renne *et al.*, 1996. Details of these viruses are given in Table 1A1.

1A1.1 Herpesviridae classification

Herpesviruses are classified by the International Committee on the Taxonomy of Viruses as group one double stranded DNA viruses. Their classification is based on the structure of the virion (Dargan, 1986, Rixon, 1993).

1A1.1a Conserved features of herpesvirus virion structure

Their are four main features that are conserved in the structure of herpesvirus virions:

- An electron opaque core which includes a linear double stranded genome of 100-230kb (Epstein, 1962a).
- An icosahedral capsid of approximately 125nm diameter surrounding the core, comprising 162 capsomers made of 150 hexons and 12 pentons (Wildy *et al.*, 1960).
- An amorphous tegument or matrix in which the capsid 'floats' (Roizman & Furlong, 1974).
- An outer envelope derived from the host cell nuclear membrane, that consists of a trilaminar lipid envelope containing viral glycoproteins (Spear & Roizman, 1972).

Human herpesviruses	Herpesviridae sub-family	Associated illness	Genome size (kb)
herpes simplex virus type 1 HSV-1	α	80-95% of oral lesions (common 'cold sores') and 30-50% of genital lesions are caused by HSV-1. Primary infections and subsequent reactivated episodes give similar symptoms. Also in rare cases it causes conjunctivitis, herpetic whitlow, keratitis and encephalitis. Neonatal infections acquired from genitally infected mothers are often life threatening.	152
herpes simplex virus type 2 HSV-2	α	5-20% of oral lesions (common 'cold sores') and 50-70% of genital lesions are caused by HSV-2. Primary infections and subsequent reactivated episodes give similar symptoms. Also in rare cases it causes conjunctivitis, herpetic whitlow, keratitis and encephalitis. Neonatal infections acquired from genitally infected mothers are often life threatening.	155
varicella zoster virus VZV	α	Primary infection: chicken pox- a rash which appears 14-15 days after infection, accompanied by a fever. Subsequent reactivated infection: shingles- lesions appear at the relevant dermatome often accompanied by extreme pain which can persist for months even after the healing of the original lesion.	125
Epstein Barr virus EBV	γ	Primary infection in young children: often asymptomatic. Primary infection in older children and adults: infectious mononucleosis (glandular fever). Associated with Burkett's lymphoma and nasopharyngeal carcinoma.	172
cytomegalovirus HCMV	β	Primary infection: enlargement and fusion of macrophages often occurs and although often asymptomatic it can be fatal (in particular for new-borns). Subsequent reactivated episodes can occur. Infection problematic in immunocompromised individuals, disease symptoms include: gastro-enteritis and retinitis.	>229
human herpesvirus 6 HHV-6	β	Infant rash exanthem subitim.	159
human herpesvirus 7 HHV-7	β	Febrile illnesses.	145
Kaposi's sarcoma herpesvirus KSHV/HHV-8	γ	Associated with Kaposi's sarcoma.	160-170

Table 1A1: Details of human herpesviruses. The subfamily classification, associated illnesses and genome size for each type of human herpesvirus has been provided.

1A1.1b Sequence arrangements in herpesvirus DNAs

The sequence arrangement of the herpesvirus DNAs is one of their most interesting features. Variations in the arrangement of repeats, composed of unit sequences greater than 100bp, allows the herpesviruses to be divided into six groups (shown in Figure 1A1.1b, designated A-F and explained therein). Within these groups it is possible to make further divisions. For example, group D genomes, like that of VZV, have two isomers, differing in the orientation of the unique sequence of the small component of the genome that is flanked by internal inverted repeat sequences. Group E genomes (which include the HSV and HCMV genomes) consist of four different isomers, which show variation in the orientations of the unique domains which flank either side of the internal inverted repeat sequences.

1A1.1c Sub-families of herpesviruses

The members of the Herpesviridae themselves have been further classified into three sub-families α, β and γ on the basis of their biological properties (Roizman *et al.*, 1981). The alpha (α) sub-family are characterised by their short lytic cycle, less than 24hr in tissue culture, and their establishment of latency in sensory neurones. The classification of the beta (β) sub-family is defined by their longer lytic life cycle and slow development of cytopathology in tissue culture. The gamma (γ) group are distinguished by their ability to infect B or T lymphocytes and replicate in lymphoblastoid cells *in vitro*.

1A2 HSV-1 biology

HSV-1, an alpha herpesvirus, was the first human herpesvirus to be discovered and is one of the most intensively investigated of all viruses (reviewed by Roizman & Sears, 1996, Subak-Sharpe & Dargan, 1998, Wagner, 1994). It is a common human pathogen which attains a life-long latent state in sensory neurones after initial infection at the periphery.

HSV-1 is responsible for the common 'cold sore', however it is now been established as the causative agent of 30-50% of genital lesions (Kinghorn, 1993). Although infection with HSV-1 does not, for the most part, cause a life threatening disease, its ability to remain latent in the host for life and cause subsequent episodes of reactivation underlines its evolutionary success. Indeed, this is reflected in its high distribution: 70-90% of people in the developing countries are infected by early adolescence and 60% of adults in developed countries are infected by their thirties.

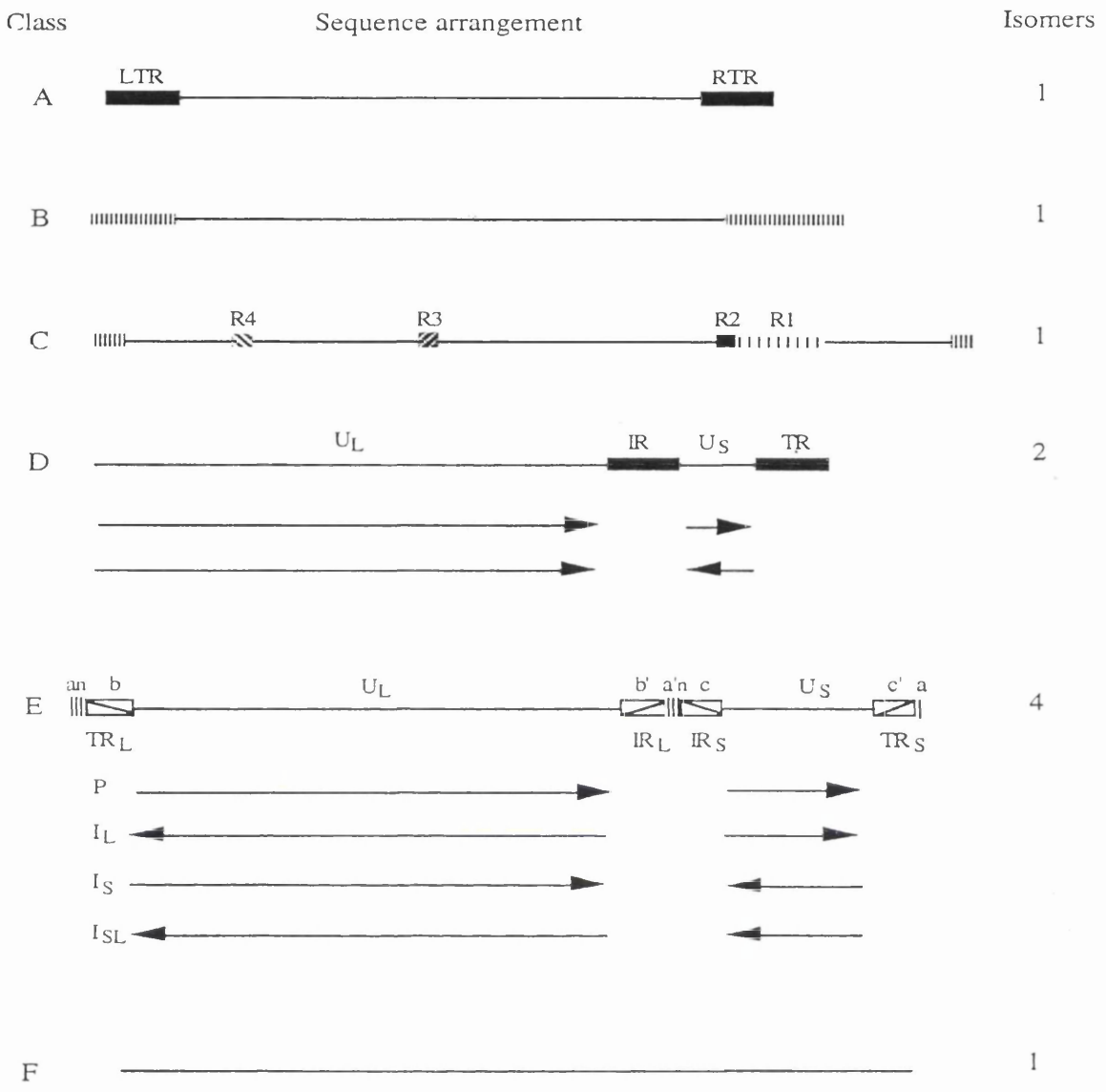


Figure 1A1.1b: A schematic diagram illustrating the six types of genome sequence arrangements found for members of the Herpesviridae family.
The six classes of genome arrangements have been labelled A-F. Genome classes A, B, C, D, E and F are exemplified by channel catfish virus, herpesvirus saimiri, Epstein-Barr virus, varicella -zoster virus, herpes simplex viruses, and tupaia herpesvirus, respectively. 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-R4 for the internal repeats of class C; and internal and terminal repeats (IR and TR) for 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 four combinations in orientation of U_L and U_S shown. Similarly, the short sequence of D type genomes can invert as shown. This figure is a reproduction of one created for the production of the Ph.D. thesis of M.R.Meredith (Meredith, 1996).

Interest in this virus also comes from its use as a model to study translocation of proteins, synaptic connections in the nervous system, membrane structure, gene regulation, as well as many other biological problems, both for general cell biology and for virus research. Its potential use as a vector for gene therapy has also led to a heightened interest in recent years.

1A2.1 HSV-1 genome

The HSV-1 genome consists of a linear double stranded molecule (Becker *et al.*, 1968). The wild type HSV-1 17 Syn^+ strain has a genome size of 152kb (McGeoch *et al.*, 1988, McGeoch *et al.*, 1986, Perry & McGeoch, 1988) with a high G+C content (68%).

It is composed of two covalently linked components consisting of two blocks of unique sequences, the unique long sequence U_L and unique short sequence U_S, interrupted by an internal inverted repeat region and terminated by sequences encoding repeats (see Figure 1A1.1b part E).

The HSV-1 genome encodes at least 76 genes, the long unique sequence U_L encodes 59 of these genes, while the U_S sequence encodes 13 genes. The repeated sequences encode two copies of four genes.

1A2.2 The HSV-1 virion structure and composition

The HSV-1 virion, like all herpesviruses, is composed of an outer envelope, an amorphous tegument and an inner icosahedral capsid, shown in Figure 1A2.2.

1A2.2a The capsid

The large 152kb DNA genome folds itself into a liquid crystalline form within the 100nm capsid. The capsid comprises 162 capsomers, of which 150 are hexameric and 12 are pentameric. The hexamers are located on the faces and edges of the capsid and the pentamers are at the vertices (Baker *et al.*, 1990, Booy *et al.*, 1991, Schrag *et al.*, 1989).

1A2.2b The tegument

Viral structural components which have not been assigned to the envelope or capsid are designated as tegument proteins (Rixon, 1993). The tegument is thought to comprise

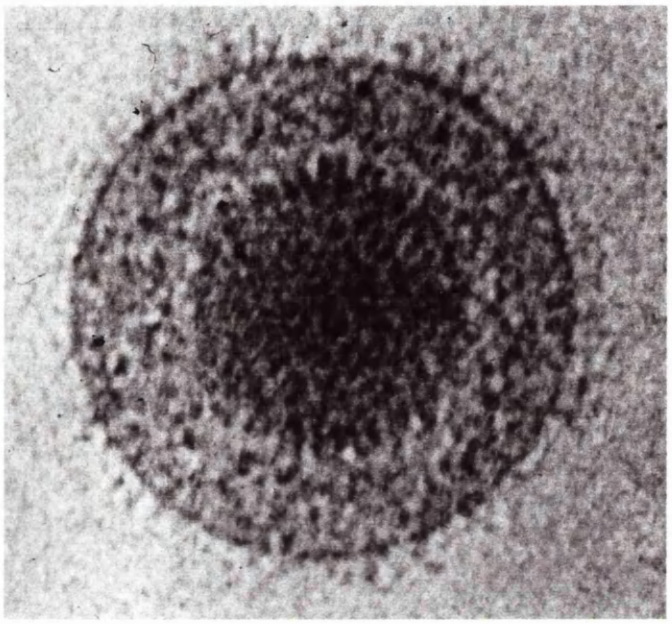


Figure 1A2.2: Electron micrograph of a HSV-1 virion.
The micrograph shows the characteristic features of a herpesvirus virion; the envelope containing the viral glycoproteins, the central inner capsid (~ 125nm in diameter) and the tegument between these structures. The photograph is shown with permission of Dr.F.Rixon.

twenty distinct structural proteins of various functions that are thought to influence the process of infection.

1A2.2c The envelope

The outer surface of the virion is comprised of a trilaminar lipid envelope (Epstein, 1962b) derived from cellular membranes (Armstrong *et al.*, 1961, Morgan *et al.*, 1968). The exact dimensions of the envelope vary depending on the method of visualisation, however its nominal diameter varies between 170-200nm. It contains about eleven viral glycoproteins (reviewed by Spear, 1993), some of which are important for the process of fusion of the virion envelope with that of host cell membranes.

1A2.3 The lytic life cycle

The process by which viruses enter the cell, replicate and are released from the cell is termed the lytic life cycle which comprises a number of stages. The initial stage is attachment to the cell surface receptors, followed by fusion of the envelope with the plasma membrane. This is rapidly followed by transport of the capsid to the nuclear pores where DNA is released into the nucleus. The DNA is then transcribed and replicated, which enables virion production. Finally, the mature virions are released from the cell. The effects of these processes result in cell death.

1A2.3a Entry of HSV-1 into the cell

The first step in the infection is adsorption of the HSV-1 virions to the cell surface (reviewed in Shieh & Spear, 1994, Spear, 1993). From the eleven genes encoding the HSV-1 glycoproteins (gB, gC gD, gE, gG, gH, gI, gJ, gK, gL and gM), no one protein has been found to be solely responsible for attachment to the cell surface. Although, both gB and gC have been shown to be the most prominent glycoproteins involved.

The most important cell receptor identified for the process of attachment of HSV-1 to the cell surface appears to be heparan sulphate. However, it is likely that other receptors are also involved. Furthermore, the factors which define the species and cell type specificity of HSV-1 have not been identified.

After the initial adsorption of the virion to the cell surface the next stage is penetration into the cell, this involves the fusion of the envelope with the cell membrane. Again a number of glycoproteins are involved in this process including: gD which is the most intensively

investigated; gH and gL, which form a heterodimer; and gB (reviewed by Spear, 1993). The cell receptor initially thought to be responsible for penetration of the virus into the cell was the mannose-6-phosphate receptor (Brunetti *et al.*, 1995, Brunetti *et al.*, 1994). However, more recent work suggests other receptors, named herpes entry mediators HveA, HveB, and HveC, are also important for this function (Geraghty *et al.*, 1998, Montgomery *et al.*, 1997, Warner *et al.*, 1998, Whitbeck *et al.*, 1997).

1A2.3b Release of DNA into the nucleus and initiation of infection

After entry into the cell, the capsids are transported to nuclear pores in a process thought to be mediated by the cell cytoskeleton (Kristensson *et al.*, 1986). The parental DNA is then released into the nucleus through the pores in a process that requires a viral function (Batterson *et al.*, 1983, Tognon *et al.*, 1981). It then circularises to form an episome.

Uncoating of the virion on entry to the cell also causes release of tegument proteins such as VP16 (encoded by UL48) and the virion host shut off protein *vhs* (encoded by UL41) that help initiate infection. In particular, *vhs* activates an RNase activity that indiscriminately degrades mRNA, resulting in degradation of cellular mRNA (Kwong & Frenkel, 1987, Kwong *et al.*, 1988, Schek & Bachenheimer, 1985). Although viral mRNA is also targeted, its rate of synthesis is greater than *vhs*-induced degradation. VP16 is an important transactivator that enhances the transcription of the IE class of HSV-1 genes and hence promotes infection where only low numbers of virus particles are present (described in detail in Section 1A2.5c).

1A2.3c HSV-1 gene expression

Transcription of all herpesvirus genes is controlled by the DNA dependent RNA polymerase II and is temporally regulated. This temporal regulation means the HSV-1 genes can be divided into three main kinetic classes classified as: immediate early (IE), early (E) and late (L) genes (Clements *et al.*, 1977); or α, β, and γ genes (Honess & Roizman, 1974, Honess & Roizman, 1975). Late genes can be further subdivided into leaky late and true late genes. Leaky late genes are ones whose expression is induced at low levels before DNA replication, but require DNA replication for the maximal level of transcription to occur. True late genes are ones whose expression is only induced following the onset of replication.

Although basal transcription of HSV-1 genes occurs by the pre-existing transcription apparatus of the cell, viral factors are necessary for regulating the levels of transcription (Ben-Zeev & Becker, 1977, Costanzo *et al.*, 1977). The regulation of HSV-1 gene expression will be described in greater depth later in Section 1A2.5.

Messenger RNAs are post-transcriptionally modified by the normal mechanisms of capping, methylation and polyadenylation. They are then translated on bound and free ribosomes. Some viral proteins are modified post-translationally and examples exist where proteins are cleaved, phosphorylated, sulphated and poly(ADP) ribosylated, (reviewed in Roizman & Sears, 1996).

1A2.3d HSV-1 DNA replication

DNA replication is the next stage in infection, which occurs by the rolling circle mechanism, (reviewed by Roizman & Sears, 1996). Initiation occurs at one of three origins of replication in the HSV-1 genome, by interaction with the ORI binding protein UL9. A helicase/primase complex, made up from UL5, UL8 and UL52, associates with the origin to create an initiation 'bubble' (which requires the hydrolysis of ATP). This initiation 'bubble' then associates with the polymerase UL30/UL42 DNA-binding protein complex. This triggers DNA synthesis to occur continuously along one strand and discontinuously on the other strand. The growth of the DNA fork is maintained by the major DNA-binding protein UL29. This results in the production of head to tail concatemers that accumulate in the nucleus in 'replication compartments' (deBruyn Kops & Knipe, 1994, Quinlan *et al.*, 1984). Other important HSV-1 viral proteins involved in DNA replication include: proteins required for nucleic acid metabolism (thymidine kinase (UL23), ribonucleotide reductase (UL39 and UL40) and dUTPase (UL50)) and proteins which have the potential for participating in a DNA repair function (uracil-DNA glycosylase (UL2) and alkaline exonuclease (UL12)).

1A2.3e Virion assembly

Production of viral DNA enhances the expression of L genes whose products primarily consist of structural proteins. As a consequence of their synthesis, patches form on the inner surface of the nuclear membrane consisting of viral glycoproteins, other membrane associated proteins and the tegument proteins, including VP16 and *vhs*.

The next stage is capsid assembly and DNA packaging (reviewed by Rixon, 1993). The major capsid protein VP5 (UL19) and other proteins encoded by the genes UL18, UL38, UL26, UL26.5 and UL35, assemble into immature capsids within the nucleus. These consist of an outer icosahedral shell, made from VP5 (UL19), VP19C (UL38), VP23 (UL18), and VP26 (UL35), and an internal scaffold made from VP22a (UL26.5) and the product of the UL26 gene.

The UL26 gene product has proteolytic activity which cleaves the preVP22a, which connects the scaffold to the capsid shell, to VP22a, which is no longer connected to the capsid shell, and therefore causes release of the internal scaffold from the capsid shell.

Viral DNA is packaged within the capsid, by cleavage of the concatemeric viral DNA, with the aid of the products of several other genes including UL6, UL12, UL15, UL25, UL28 UL32 and UL33.

Packaging of viral DNA and removal of the internal scaffold are thought to occur simultaneously, resulting in the production of the mature virion. Along with the immature and mature capsids, empty capsids have also been identified. These are thought to be generated from abortive packaging events.

1A2.3f Envelopment of virions and their egress from the infected cell

After packaging of the DNA the capsids are more compact and are enveloped by the patches on the inner nuclear membrane. Using electron microscopy, 'fingers' of nuclear membrane, in which the mature capsids are contained, have been observed to extend into the cytoplasm. The virions then accumulate in the endoplasmic reticulum and transverse the Golgi apparatus and the trans Golgi network to the extracellular space.

1A2.3g Cytopathology

The production of infectious progeny invariably causes cell death after completion of the approximately 18hr lytic cycle. Structural changes seen during infection include the degradation of the nucleolus, and the condensation and margination of host chromatin at the nuclear membrane. This is followed by the duplication and folding of the nuclear membrane. Furthermore, certain mutant viruses can cause infected cells to fuse with neighbouring cells forming polykaryocytes (reviewed by Roizman & Sears, 1996).

1A2.4 HSV-1 Latent state

HSV-1 is not only capable of productive infection in host epithelial cells but can also latently infect sensory neurones (reviewed in Steiner & Kennedy, 1995, Wagner, 1994). It is the ability of HSV-1 to establish, maintain and reactivate, from this latent state that enables it to maintain a life long infection in its host.

1A2.4a Definition

Latent infection is defined as the presence of the viral genome in cells without the production of virus particles, but which has retained the capacity to reactivate and hence resume replication and cause recurrent disease.

1A2.4b Establishment, maintenance and reactivation of HSV-1 latency

HSV-1 latency has been a topic of intensive investigation for many years. However, there is still much controversy and debate surrounding the central issues. Therefore, a review of this field would be outside the scope of this thesis and as such only a brief overview of the various aspects of HSV-1 latency has been provided.

- Establishment

Following entry of HSV-1 to the peripheral epithelial cells, virus particles (most likely the capsids alone) may be attached to the axonal terminals of sensory neurones, usually the trigeminal ganglia, and transported by fast retrograde flow to the cell bodies (Cook & Stevens, 1973, Kristensson *et al.*, 1986). Once in the ganglia, either viral replication occurs with the generation of progeny virus, or latent infection is established. The molecular mechanisms by which one of these options is chosen have yet to be elucidated. However, such a decision is thought to occur early in the interaction between virus and cell, before high levels of expression of IE genes. Cellular factors are thought to play an important role in this decision. If induction of the lytic phase fails, the first step in the establishment of latency is probably the circularisation of the viral DNA, which makes it refractory to degradation. In this manner viral DNA is maintained in the non-dividing, terminally differentiated, neural cells.

- Maintenance

During latent infection the only detectable viral gene expression is that of a major class of transcripts called the latency associated transcripts or LATs (reviewed in Block & Hill, 1997). These transcripts were first detected by northern blotting analysis and *in situ* hybridisation (Deatly *et al.*, 1987, Stevens *et al.*, 1987), subsequent detection by PCR studies have also suggested that these are by far the most abundant transcripts produced during latency (Kosz-Vnenchak *et al.*, 1993, Kramer & Coen, 1995).

- LATs

Transcription of LATs occurs from a promoter located in the repeat sequence bounding the U_L region of the genome, and in the opposite orientation from Vmw110 and ICP34.5. The relative positions within the HSV-1 genome of sequences encoding transcripts of LAT, Vmw110 and ICP34.5 are shown Figure 1A2.4b.

The LATs that are detected during lytic infection are all 2.0kb species, whereas during latency a 2.0kb species as well as a 1.45/1.5kb species can be detected. These are found mainly in the nucleus in an non-polyadenylated state (Wagner, 1994, Wagner *et al.*, 1995). Furthermore, evidence exists that LATs are non-linear lariats, which would explain their stability (Rodahl & Haarr, 1997, Wu *et al.*, 1996). They are abundant, 40,000-100,000 copies can be detected per latently infected neurone. It has been proposed that the 1.45-2.0kb LATs are derived from the processing of a larger 8.3kb precursor (Devi-Rao *et al.*, 1991, Farrell *et al.*, 1991). However, detection of this 8.3kb precursor and 6.0kb splice product by methods other than *in situ* hybridisation has proved difficult.

The biological role of the LATs is debatable. There are three popular theories that describe their significance. The first is that they encode a functional protein that may be important for the reactivation process. However, although open reading frames (ORFs) exist in this region, detailed examination of the DNA sequence does not support a protein coding role, since predicted codon usage is not characteristic of most regions of HSV protein coding DNA. Also, sequences of different HSV-1 strains show frame shifting differences in these ORFs of the LAT region.

The second theory is that as these transcripts are antisense in part to IE1 (the gene encoding Vmw110), they may regulate latency by acting as antisense inhibitors of Vmw110 gene expression. However, recently using a rabbit *in vivo* system it was shown that insertion of

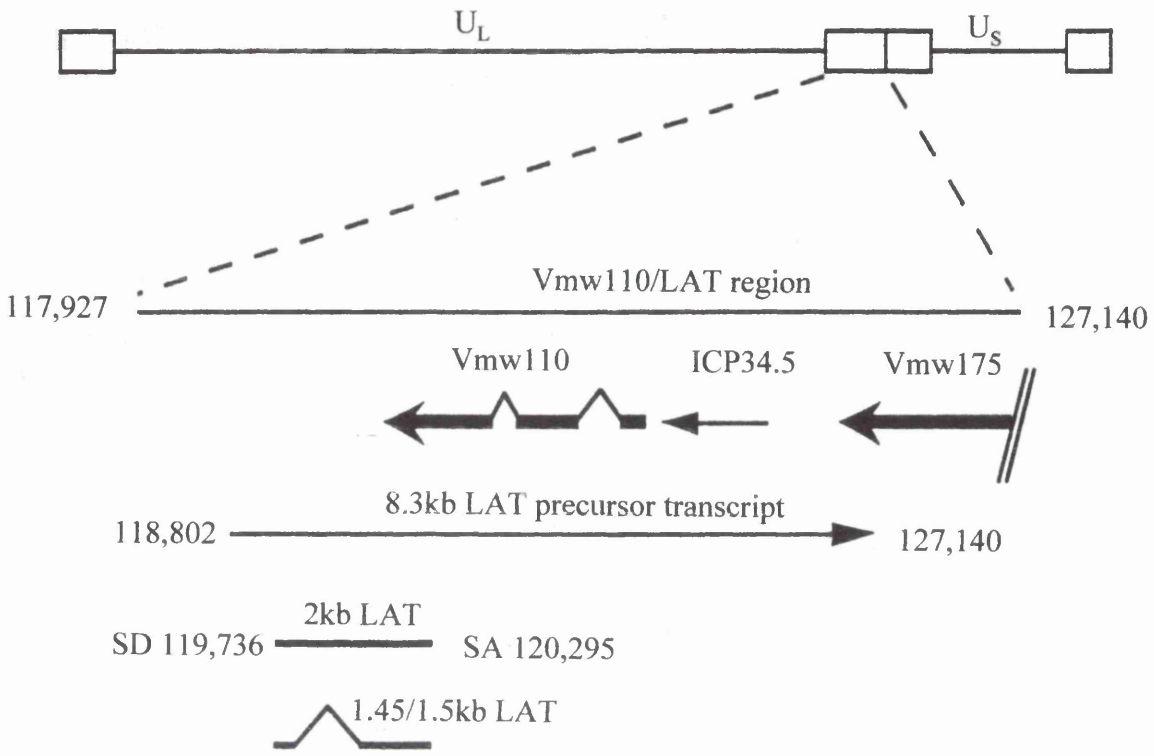


Figure 1A2.4b: Diagram illustrating the relative positions of sequences within the HSV-1 genome encoding the Vmw110, ICP34.5 and LAT transcripts.
A schematic diagram of the HSV-1 genome is provided. The Vmw110/LAT region is present in the repeat region bounding U_L. The horizontal lines drawn underneath represent the relative positions of sequences within the Vmw110/LAT region which encode: the Vmw110 transcript; the ICP34.5 transcript; part of the Vmw175 transcript; the 8.3kb minor LAT transcript; the 2kb major LAT transcript; and the 1.45/1.5kb LAT transcript (only produced during latent infection). The 2kb LAT is derived from the 8.3kb LAT by splicing at the splice donor (SD) and splice acceptor (SA) sites shown. The numbers represent positions in the genome of the HSV-1 17 Syn^+ virus strain. The arrow direction represents the direction in which the sequences are transcribed. The '^' sign represents sequences which are spliced out to produce the appropriate transcript.

the first 1.5kb of LAT, which do not encode sequences antisense to Vmw110, into the glycoprotein C locus of a LAT null mutant restored the normal spontaneous reactivation efficiency (Perng *et al.*, 1996). Therefore belief in the validity of this theory has been reduced.

The last theory is that LATs have no biological significance and could be 'selfish' RNA in the sense that they accumulate with no reason other than for their own survival. However, it seems unlikely that such a high concentration of specific RNA would be produced with no meaning.

- Reactivation

HSV-1 reactivation in humans, with resultant cold sores, can be induced by local stimuli such as: injury to tissues innervated by the neurones carrying the latent infection, or by systemic conditions including: exposure to ultraviolet radiation (sunlight), stress and possibly hormonal irregularities (Hill, 1985). As recurrent infection does not result in permanent sensory loss or any other neurological deficit in the affected dermatomes (Gominak *et al.*, 1990), it is proposed that reactivation does not lead to significant destruction of latently infected neurones, in contrast to the situation of the lytic infection.

1A2.4c *In vivo* models

Our limited understanding of latency has mainly been derived from studies using HSV-1 in experimental animals. Subsequently, human tissue has also been used to try and verify the earlier results. Most animal models stem from a similar general approach (Fraser *et al.*, 1984), comprising of inoculating at a peripheral site (e.g. cornea, pinna of the ear, footpad, etc.), which results in the transportation of the virus particles to the respective sensory ganglia and to the central nervous system where viral replication takes place. Once replication stops the animal tissues may then be studied for various aspects of the latent state. However, when interpreting data from such models it should be remembered that all animal models differ from human infection. Furthermore, different animal models are used by independent research teams and as such the results generated are not directly comparable.

1A2.4d *In vitro* models

An alternative method of studying HSV-1 latency is by use of an *in vitro* latency system (reviewed in Rock, 1993, Stevens, 1989). Examples of such systems are provided in Section 1B2.4, in which the role of Vmw110 in HSV-1 latency is discussed.

1A2.5 Regulation of HSV-1 gene expression

HSV-1 has proven a useful tool to study various aspects of herpesvirus and general gene expression and regulation (reviewed in Everett, 1987b, Roizman & Sears, 1996). HSV-1 gene expression is regulated by both cis and trans-acting factors. The cis-acting factors are present in the HSV-1 genome and trans-acting factors are largely derived from the cell's synthetic machinery, with RNA polymerase II being responsible for the basal transcriptional regulation of HSV-1 genes. However viral trans-acting factors also play a crucial role.

1A2.5a Transcription of HSV-1 genes by RNA polymerase II

RNA polymerase II is present in the nucleoplasm of eukaryotic cells and is responsible for the synthesis of cellular mRNA precursors. Promoters recognised by RNA polymerase II consist of a series of elements or modules, in general: the TATA box (or TATA element), and one or more of a variety of upstream elements such as the CAAT box and the GC box.

The TATA box specifies the start point of transcription and the upstream elements bind regulatory transcription factors. For example, the GC box functions as binding sites for the Sp1 transcription factor. Promoters are usually made of a mixture of these modules, no one element being consistently present.

Transcription by RNA polymerase II is also affected by the presence of enhancer elements. These sites are often located at great distances, usually more than 1000bp up- or downstream, and can function in either orientation. They are thought to bind transcription factor(s), enabling looping of the DNA and interaction with the transcription factors bound to the promoter elements. In this manner gene expression is activated.

The initiation of transcription by RNA polymerase II involves several stages. The first stage is the binding of TFIID, made up from the TATA binding protein (TBP) and TBP-associated factors (TAFs), to the TATA element. The next stages are the binding of TFIIA and TFIIB, then RNA polymerase II, to which TFIIF is bound, which results in the

formation of the minimal transcription initiation complex. This is followed by the binding of TFIIE, TFIIH and TFIIJ to the complex which forms the complete initiation complex. As explained, the basal transcription rate can be modulated by the presence of other regulatory factors bound to promoter or enhancer elements.

1A2.5b HSV-1 cis-acting elements

Most HSV-1 promoters are recognisable as eukaryotic polymerase II promoters with obvious 'TATA' box homologies 20-25 bases upstream of the mRNA cap sites (reviewed in Wagner, 1994). However, the promoters differ in complexity depending on the kinetic class of gene to which they belong.

IE promoters are the most complex, consisting of three components. An IE specific regulatory region, consisting of multiple copies of 'TAATGARAT' consensus sequence, is found at sites distal to and extending up to several hundred bases upstream of the IE mRNA start site. In the proximal promoter region are regulatory sequences similar to 'CAAT' box and cellular transcription factor (such as Sp1) binding sites, which occur at positions analogous to those seen in cellular promoters. Finally, these are followed by the 'TATA' box component.

In general, the E gene promoters are the next most complex, with the same outline as IE promoters, but excluding the IE specific regulatory region.

The L gene promoters are generally the simplest consisting in some cases of only 20-25 bases (for strict late gene promoters), containing only the 'TATA' box and cap site region.

1A2.5c Transcriptional regulation

Transcription of the five IE genes, encoding Vmw12, Vmw63, Vmw68, Vmw110 and Vmw175, is induced by the HSV-1 virion tegument protein VP16, also called αTIF. It is 65kD phosphoprotein that is a component of the tegument. It is composed of two regions of functional significance: the N-terminal which is responsible for DNA and protein interactions, while the C-terminal is responsible for the transactivation function. The mechanism by which it functions has been very well characterised and is reviewed in Flint & Shenk, 1997, Roizman & Sears, 1996, Wagner, 1994.

VP16 does not directly bind to the 'TAATGARAT' sequence, rather its specificity for this sequence is conferred by its association with the cellular, sequence-specific, transcription

factor Oct-1. Furthermore, in order for VP16 to recognise Oct-1 it first needs to interact with another cellular factor named host cell factor (HCF, C1, VCAF, or CFF). Interaction with HCF is thought to cause a conformational change in VP16 that enables it to bind the Oct-1 DNA complex.

Four of the five IE proteins accumulate in the nucleus and promote the efficient expression of E and L genes.

Vmw175, also called ICP4, acts as the most prominent HSV-1 transcriptional activator essential for both E and L gene expression (DeLuca & Schaffer, 1985, Everett, 1984, Gelman & Silverstein, 1985, O'Hare & Hayward, 1985a, Preston, 1979, Quinlan & Knipe, 1985, Watson & Clements, 1980). It also acts as a repressor towards the expression of its own gene, that of Vmw110 and that of transcripts expressed from the LAT promoter (Batchelor & O'Hare, 1990, DeLuca & Schaffer, 1985, Everett & Orr, 1991, O'Hare & Hayward, 1985b). The mechanism of action by which Vmw175 regulates the expression of these genes is not fully understood. However, based on observations of its interactions *in vitro* with the TATA binding protein and the basal transcription factor TFIIB, it has been postulated that Vmw175 may assist in the formation of transcription pre-initiation complexes in viral promoters (Smith *et al.*, 1993).

Vmw110 is a potent and promiscuous activator of the expression of all classes of HSV-1 genes. This activation function appears to be partially dependent on the presence of Vmw175. Details relating to this function of Vmw110 are discussed in greater depth in Section 1B2.2.

Vmw63 has an essential role in the regulation of HSV-1 gene expression, most notably being required for progression of viral infection into the late phase (McCarthy *et al.*, 1989, Rice & Knipe, 1990, Sacks *et al.*, 1985). It is a complex protein which is thought to mediate its regulatory effects at both the transcriptional and post-transcriptional level.

Vmw68 is a regulatory protein important for the optimal production of Vmw110 and a subset of L proteins. Recent work in which an association has been identified, in spatially defined structures, between Vmw68, Vmw175, RNA polymerase II, newly synthesised progeny viral DNA and a nucleolar protein, (EAP or L22), known to be associated with ribosomes and to bind small RNAs, has strengthened support for the role of Vmw68 in L gene expression (Leopardi *et al.*, 1997).

1B Vmw110

1B1 The IE1 gene and gene products

1B1.1 The IE1 gene

The IE1 protein Vmw110, also called ICP0, or IE110, is encoded by the diploid IE1 (RL2 or α0) gene, which is present in the repeated sequences that flank the U_L region of the virus genome (Perry *et al.*, 1986). The IE1 gene is adjacent to sequences encoding the ICP34.5 transcript in the same orientation and partially overlaps sequences which encode the LATs in the anti-sense orientation (Figure 1A2.4b).

The promoter controlling the expression of Vmw110 contains all the common features of an IE promoter, (Section 1A2.5b). Additionally, the promoter also includes a Vmw175 binding site (Faber & Wilcox, 1986, Kristie & Roizman, 1986). The binding of Vmw175 to this site has been implicated in the repression of Vmw110 expression in transfection assays (Everett & Orr, 1991, Gelman & Silverstein, 1987a, Gelman & Silverstein, 1987b, Resnick *et al.*, 1989). However, the relevance of these results is unclear as Vmw110 has been found to accumulate throughout viral infection in tissue culture (Everett & Orr, 1991, Harris-Hamilton & Bachenheimer, 1985, Weinheimer & McKnight, 1987). Results from sequence analysis revealed the gene to be about 3.6kb in size, consisting of three exons and two introns (Perry *et al.*, 1986).

1B1.2 Alternative splicing of IE1 transcripts

The presence of introns is an unusual property for a HSV-1 gene. In fact, Vmw110 is one of only four HSV-1 genes which contain introns and whose pre-mRNAs require splicing in order to produce the final protein product.

Interestingly, alternative splicing of the IE1 pre-mRNA transcript results in the production of at least two protein products. The first, better established protein product is thought to be generated as a result of removal of both introns. However, a second product has also been detected in HSV-1 infection, its abundance varying with cell type (Everett *et al.*, 1993b). The size of this truncated Vmw110 product could best be explained by the retention of the in-frame stop codon in the second intron, which would produce a 262-residue protein. Consistent with this result, it was found that the production of the protein was dependent on the presence of second intron sequences in the viral genome. The

significance of this alternatively spliced truncated Vmw110 protein has not been fully understood, but a potential role has been listed further in the text.

1B1.3 Interesting features of the primary amino acid sequence of Vmw110

The IE1 gene is predicted to encode a protein of 775 residues, a high proportion of which encode alanine, proline, glycine and arginine residues. This is probably the result of the IE1 gene having a high GC content (75.4%).

*Sma*ll clusters of acidic and basic residues span the protein. For the most part these have proved functionally redundant. However, a highly basic region, spanning residues 501-506, has been implicated in nuclear localisation of Vmw110 (Section 1B3.3) and serine-rich domain between residues 554-591, is thought to be the target site for phosphorylation of Vmw110.

A cysteine rich region, between residues 106 and 150, similar to that found in 'metal finger binding domains', was first discovered during characterisation of Vmw110 by Perry *et al.*, 1986. High sequence homology, as well as the conservation of cysteine and histidine residues within this region, has been observed between Vmw110 and the corresponding proteins of other alpha herpesviruses including HSV-2, equine herpes virus type 1, VZV, bovine herpes virus type 1 and pseudorabies virus (Cheung, 1989, Perry *et al.*, 1986, Telford *et al.*, 1992, Wirth *et al.*, 1992). This motif has been identified as a member of the RING finger family. The properties of this motif and its functional significance are discussed in greater detail later.

The only other highly conserved region spans residues 604-767. This region retains 80-85% homology to the equivalent region of the corresponding protein of HSV-2. However, no significant homology has been retained between this region of HSV-1 Vmw110 and the corresponding proteins from other herpesviruses, which implies that this region is important for a function specific to herpes simplex viruses. Interestingly, this region has been shown to be of importance for the multimerisation of Vmw110 and for binding to HAUSP, details of which are discussed later.

1B1.4 Post-translational modifications of Vmw110

The predicted molecular weight of the IE1 gene product is 78,452Da. However, the molecular weight observed for Vmw110 derived from HSV-1 infected cell extracts on an SDS-polyacrylamide gel has been calculated to be 110,000Da (Honess & Roizman, 1974). This discrepancy between the predicted and experimentally observed molecular weights may be explained by post-translational modifications made to the polypeptide. The most well established modification is the phosphorylation of Vmw110, which is predicted to take place on the previously described serine rich region and in part, at least, the UL13 HSV-1 viral protein kinase is thought to be responsible for this (Ackermann *et al.*, 1984, Ogle *et al.*, 1997). In addition, it has been shown that Vmw110 can be nicotidylated *in vitro*.

1B1.5 Multimerisation of Vmw110

Vmw110 purified from the baculovirus expression system demonstrated that it exists as a multimer in solution (Everett *et al.*, 1991). This was later supported by work in which Vmw110 was derived from HeLa cells infected with a recombinant adenovirus which expressed Vmw110 (Chen *et al.*, 1992).

1B2 Role of Vmw110 in HSV-1 infection

1B2.1 Role of Vmw110 in virus growth

The production of HSV-1 mutants with lesions in Vmw110 led to a breakthrough in the understanding of the role of Vmw110 for viral growth (Sacks & Schaffer, 1987, Stow & Stow, 1986). Most significantly it was found that Vmw110 is not essential for growth of HSV-1 in the majority of cell lines.

The first such mutant to be produced was dl1403, which contains a 2kb deletion within both TR_L and IR_L copies of the Vmw110 gene, and encodes a polypeptide consisting of the original N-terminal 105 amino acids, followed by 56 amino acids specified by a reading frame not used by Vmw110 (Stow & Stow, 1986). The effect of deleting the same region from a plasmid encoding Vmw110 resulted in the loss of its transactivation function. Surprisingly, no significant differences were observed between viral polypeptide synthesis, DNA replication, or DNA encapsidation, in cells infected with either: the wild type HSV-1,

or dl1403 virus, at high multiplicities of infection (moi). Furthermore, infection with either virus produced similar particle numbers.

However, at low moi the plaque forming efficiency of dl1403 varied dependent on the cell type, with Vero cells and HFL cells displaying significantly lower plaquing efficiencies compared to BHK cells. Interestingly these differences were not observed at high moi. These results implied firstly that Vmw110 has a cell-type dependent effect, being required to differing degrees in different cell types and secondly a multiplicity dependent effect, being required at low moi.

Similar conclusions were reached by Schaffer's group who isolated two Vmw110 mutant viruses: dlX3.1, which contains a 3.1kb deletion in both IE1 genes, which results in the removal of the majority of the transcriptional regulatory region, as well as the 5' coding sequences, and dlX0.7, which contains a 700bp deletion in the transcriptional regulatory region of both copies of the gene (Sacks & Schaffer, 1987).

Although Vmw110 is not essential for virus growth, the observation that dl1403, dlX0.7 and dlX3.1 mutants had a high particle/pfu ratio implies that Vmw110 is required at low moi to stimulate the onset of lytic infection. This idea was supported by the observation that the dlX3.1 and dlX0.7 viruses form plaques 15-50 fold better on a cell line which contains integrated copies of the IE1 gene.

These results are consistent with later observations that deletion of the IE1 genes caused significant reduction in *de novo* synthesis of infectious virus, following transfection of cells with the mutant HSV-1 DNA (Cai & Schaffer, 1989).

A cellular activity expressed after release from growth arrest has been observed which can compensate for the loss of Vmw110, in both non-neuronal cells (Vero cells) and neuronal cells (NB41A3) (Cai & Schaffer, 1991, Ralph *et al.*, 1994). The observation that release from growth arrest led to the activation of IE, but not E, or L gene expression, may explain how the necessity for Vmw110 is by-passed. This implies that the requirement for Vmw110 is also cell cycle dependent.

Additionally, an activity specified by the osteosarcoma cell line U2OS can substitute functionally for Vmw110 (Yao & Schaffer, 1995). This U20S specified cellular activity can stimulate the plating and replication efficiencies of Vmw110 deletion mutants, and *de novo* synthesis of infectious virus after transfection with Vmw110-null mutant HSV-1

DNA. It has been suggested that the cellular activity specified by U2OS that overcomes the requirement for Vmw110, does so by activating a common pathway. This may involve the fact that normal cell cycle regulation is disrupted in U20S cells.

Similarly, activation by nerve growth factor (NGF) and fibroblast growth factor (FGF) of the PC12 neuronal cell line has been found to enhance HSV-1 gene expression, and replication, of Vmw110 deletion mutants (Jordan *et al.*, 1998). As such, further investigation into the properties of the U20S cell line, and the pathways activated by NGF and FGF in PC12 cells, may lead to a better understanding of the pathways that are influenced by Vmw110.

1B2.2 Role of Vmw110 in transactivation of HSV-1 gene expression

Vmw110's role in HSV-1 infection was first indicated by the discovery that it acts as a potent transactivator of gene expression in transfection assays (Everett, 1984, Gelman & Silverstein, 1985, O'Hare & Hayward, 1985a, Quinlan & Knipe, 1985). These assays involved cotransfecting a plasmid encoding Vmw110, alongside a plasmid encoding a reporter gene under the control of the promoter of choice. Promoter activity was then detected either by the direct quantification of RNA, or in cases where the reporter gene encoded the chloramphenicol acetyl transferase (CAT) protein, the activity of this enzyme was measured.

This established that Vmw110 transactivates expression from all three classes of HSV-1 genes (Everett, 1984, Everett, 1986, Gelman & Silverstein, 1985, Mavromara-Nazos *et al.*, 1986, O'Hare & Hayward, 1985a, O'Hare & Hayward, 1985b, Quinlan & Knipe, 1985, Sekulovich *et al.*, 1988, Shapira *et al.*, 1987). Moreover, its transactivation function was not limited to HSV-1 promoters, rather it appeared to be of a non-specific nature. This was confirmed by the activation of a variety of heterologous promoters including the SV40 early promoter (Everett, 1988b, O'Hare & Hayward, 1985a), the HIV long terminal repeat (Mosca *et al.*, 1987), the rabbit β-globin promoter (Everett, 1985) and the human ε-globin promoter (Everett, 1985).

Interestingly, when plasmids encoding Vmw110 and Vmw175 were cotransfected the level of activation was much greater than the cumulative effect of these activators being transfected separately (Everett, 1984, Gelman & Silverstein, 1986, Quinlan & Knipe, 1985, Shapira *et al.*, 1987).

This work led to the conclusion that Vmw110 is a potent and promiscuous activator of both viral and cellular gene expression and can function either alone or in synergy with Vmw175.

Inspection of the promoters sensitive to Vmw110 did not result in the identification of a consensus site and the only conserved feature was the presence of the basic RNA polymerase II promoter motifs. This implies that Vmw110 is unlikely to act via direct DNA binding. The finding that Vmw110 did not form a complex with DNA in solution supports this idea (Everett *et al.*, 1991). This is consistent with the observation of its non-specific transactivation function. As yet the exact mechanism by which Vmw110 activates gene expression from such a wide variety of promoters is unclear.

1B2.3 Role of truncated Vmw110 in transrepression

The effect of 262 residue truncated Vmw110, described earlier in Section 1B1.2, on HSV-1 gene expression has also been tested (Weber & Wigdahl, 1992). Transfection of a plasmid expressing this truncated Vmw110 protein, resulted in a strong dominant and general inhibitory effect on gene expression, inhibiting activation not only by Vmw110 but also by Vmw175 and VP16.

1B2.4 Role of Vmw110 in HSV-1 latency

- Implications of Vmw110's role in latency based on *in vitro* models

The first indication that Vmw110 was important for processes involved in latency came from *in vitro* latency studies. The model used involved infecting human foetal lung (HFL) cells with HSV-2 at 42°C, a temperature which prevents the onset of lytic infection. Superinfection of these HFL monolayers with wild type HSV-1 virus resulted in the reactivation of the quiescent/latent HSV-2 genomes. However, superinfection with a Vmw110 deletion mutant did not result in the reactivation of the virus (Russell *et al.*, 1987).

As the Vmw110 deletion mutant also incorporated the loss of large sections of LAT, it was unclear from these results whether the failure of the HSV-1 mutant to reactivate latent genomes was due to the loss of Vmw110, or LAT encoding sequences. In addition, it was uncertain whether Vmw110 acted alone or in synergy with other viral protein(s). These questions were in part answered by a later study which found that latent viral genomes

were reactivated by superinfection with recombinant adenoviruses expressing Vmw110 (Harris *et al.*, 1989).

A recent study, using a different *in vitro* latency system, supported and added to these earlier findings. This study used an *in vitro* neuronal model of latency, in which primary sympathetic neuronal cultures were inoculated with HSV-1, genomes which were maintained in a latent/quiescent state could then be reactivated by depriving the cultures of nerve growth factor (NGF) (Wilcox *et al.*, 1997, Wilcox *et al.*, 1987). Using this system, it was shown that Vmw110 deletion mutants reactivated with delayed kinetics and burst sizes compared to those of wild type HSV-1. This system was also used to monitor the efficiency of establishment of latency. This revealed that the efficiency of establishment of latency was decreased by at least 10 fold for Vmw110 mutants as compared to wild type HSV-1. Moreover, it was demonstrated that the ability of a Vmw110 deletion mutant to establish latency and reactivation, could be restored by the provision of a recombinant adenovirus expressing Vmw110. Collectively the results of *in vitro* latency models strongly suggest an important role for Vmw110 in the establishment of the HSV-1 latent state.

- Implications of Vmw110's role in latency based on *in vivo* models

Using *in vivo* latency systems no clear definition of Vmw110's role in HSV-1 latency has been established. This is due to the problems associated with *in vivo* models, such as comparing results from studies using different systems. For example, an investigation using a mouse footpad model implied that Vmw110 was dispensable for the establishment, maintenance and reactivation, from the latent state (Clements & Stow, 1989). This is in contradiction to a study using the mouse ocular model, which demonstrated that the three Vmw110 deletion mutants, dL1403, dLX0.7 and dLX3.1, which could replicate in the eye and ganglia (although with reduced efficiency), varied in their ability to establish and reactivate from the latent state (Leib *et al.*, 1989). They concluded that Vmw110 does play a role in the establishment and reactivation of latency.

The question of the role of LAT encoding sequences, which were in part deleted from these Vmw110 deletion mutants, was partially answered by a subsequent study (Cai *et al.*, 1993). This study showed that the ability of mutant viruses to produce graded levels of transactivating activity correlated well with the ability of these viruses to replicate in mouse eyes and ganglia, during the establishment of latency. They also demonstrated that

insertion of a single copy of the Vmw110 gene into the genome of the Vmw110 and LAT double mutant led to restoration in the ability of the virus to replicate in eyes and ganglia, and reactivate from latency. This suggests a distinct role of Vmw110 from LAT in the establishment and reactivation processes of latency.

1B3 Vmw110 functional domains

Mutational analysis combined with functional studies of Vmw110 have revealed the presence of four main functional regions (Figure 1B3). These consist of the N-terminal RING finger domain, a nuclear localisation domain, a C-terminal interaction domain and a C-terminal multimerisation domain. A description of these domains and their importance for HSV-1 viral growth, regulation of gene expression and latency is provided below, with the exception of the C-terminal interaction domain. This latter domain represents the region of Vmw110 required for interaction with HAUSP. Details concerning this domain are included in Sections 1E4 and 1E5, in which the interaction of Vmw110 with HAUSP is discussed. The significance of these domains for the effects of Vmw110 on the cell are detailed later in this introduction (Sections 1D1.4 and 1D2).

1B3.1 RING finger domain

Vmw110 is a member of the RING finger family of proteins, defined by the presence of a cysteine/histidine-rich, zinc chelating domain, known as the RING finger domain. This motif lies between residues 116-156 (Freemont *et al.*, 1991, Perry *et al.*, 1986) and can be described as: Cys-x_2-Cys-$x_{(9\text{-}39)}$-Cys-$x_{(1\text{-}3)}$-His-$x_{(2\text{-}3)}$-Cys-x_2-Cys-$x_{(4\text{-}48)}$-Cys-x_2-Cys, where x can be any amino acid. The structures of two members of the RING finger family: the immediate early EHV-1 protein from equine herpesvirus and the proto-oncoprotein PML, have been solved by 'H^1'nuclear magnetic resonance (NMR) methods (Barlow *et al.*, 1994, Borden *et al.*, 1995, Everett *et al.*, 1993a).

RING finger family

Proteins containing the conserved RING finger are derived from an evolutionarily wide spectrum of origin including plants, fungi, vertebrates and viruses (Freemont *et al.*, 1991). This broad conservation suggests a fundamental biological significance for this domain. However, no single molecular function has been ascribed to the RING finger, and proteins which contain this motif are extremely varied in their biological roles. Examples exist of RING finger proteins being required for regulation of gene expression, inhibition of

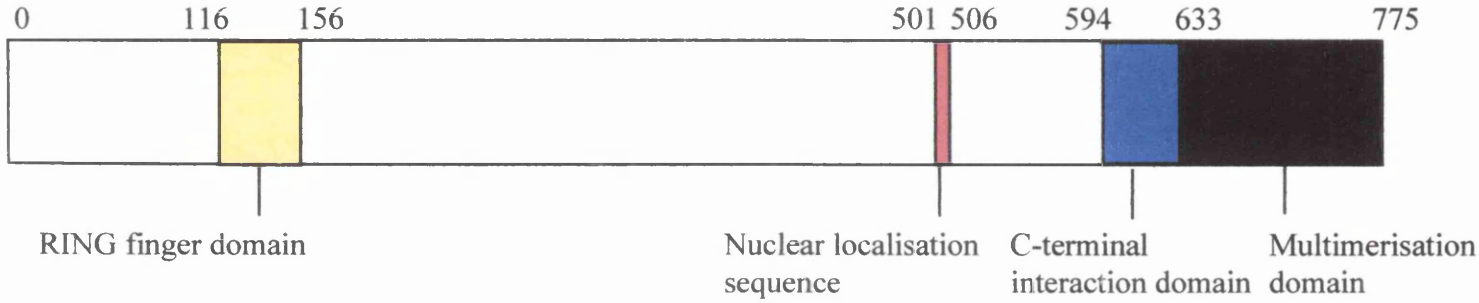


Figure 1B3: The location of the functional domains within the Vmw110 encoding amino acid sequence.
The yellow box represents sequences encoding the RING finger region. The red box represents a highly basic sequence most likely coding for nuclear localisation. The blue box shows sequences encoding a C-terminal interaction domain. The black box represents sequences encoding the multimerisation domain. The positions of these domains within the primary amino acid sequence of Vmw110 is indicated by the numbers provided above. The identification and relevance of these domains has been discussed in the text in Sections 1B3, 1E4 and 1E5.

apoptosis and DNA repair (Freemont, 1993). Nonetheless, evidence is now accumulating that the RING finger is associated with protein-protein interactions (Saurin *et al.*, 1996).

- Role of RING finger domain for transactivation function of Vmw110

Plasmids encoding deletion or insertion mutants of the Vmw110 RING finger domain, were tested in transfection studies. The results of these assays demonstrated that the RING finger domain was essential for the activation of HSV-1 genes by Vmw110 (Cai & Schaffer, 1989, Chen *et al.*, 1991, Everett, 1987a, Everett, 1988a). Furthermore, recent mutational analysis studies have resulted in the identification of precise residues within the RING finger domain responsible for this effect (Everett *et al.*, 1995, Lium & Silverstein, 1997).

- Role of RING finger domain in viral growth

The construction and characterisation of HSV-1 mutants demonstrated that removal of the RING finger was as deleterious for viral growth as complete deletion of the IE-1 gene (Cai & Schaffer, 1989, Everett, 1989). Furthermore, residues identified as being essential for the transactivation function of Vmw110 were also shown to be necessary for viral growth (Everett *et al.*, 1995, Lium & Silverstein, 1997).

- Role of RING finger domain in HSV-1 latency

Studies using the *in vitro* latency system (Section 1B2.4) suggested that the RING finger domain is essential for the role of Vmw110 in reactivation from latency (Harris *et al.*, 1989). In particular, these investigations showed that reactivation of HSV-2 from a quiescent state could not be induced by superinfection with a mutant of HSV-1 from which the RING finger domain had been deleted, unlike its wild type counterpart, even though its replicating efficiency was unchanged.

1B3.2 Multimerisation domain

Physical studies of Vmw110 have suggested that it forms multimers in solution (Chen *et al.*, 1992, Everett *et al.*, 1991), (Section 1B1.5). An extensive characterisation of this domain was carried out using four independent experimental systems: colocalisation in DNA-transfected cells; cross-linking; immunoprecipitation analysis; and far western-blotting studies, using *in vitro* translated polypeptides, and *Escherichia coli* (*E.coli*)

glutathione S-transferase (GST) fusion proteins (Ciufo *et al.*, 1994). These experiments highlighted a region between residues 617-712 as being required for fully efficient multimerisation, with sequences approximately 60 residues either side also contributing.

A subsequent study confirmed these findings and further defined residues required for multimerisation to lie between 633-775 (Meredith *et al.*, 1995). In particular, a purified C-terminal fragment of Vmw110 encoding residues 633-775 was shown to form a multimeric species. These results were confirmed by comparison of the sedimentation behaviour of wild type and C-terminal deletion mutants of Vmw110 expressed in virus-infected cells.

- Role of multimerisation domain for the transactivation function of Vmw110

Mutants of the Vmw110 multimerisation domain were tested in transient transfection and infection studies to investigate the significance of this region for the transactivation function of Vmw110. Collectively, these studies demonstrated that the multimerisation domain was important for the ability of Vmw110 to transactivate HSV-1 promoters, more so in the presence of Vmw175 than in its absence (Chen *et al.*, 1991, Chen & Silverstein, 1992, Everett, 1987a, Everett, 1988a). However, the level of requirement of the multimerisation domain varied depending on promoter type, cell type and transfection conditions.

- Role of the multimerisation domain in viral growth

The construction and characterisation of HSV-1 mutants demonstrated that deletion of the Vmw110 C-terminal residues 680-720 or 723-767, required for multimerisation, was almost as deleterious for viral growth as complete deletion of the IE-1 gene (Everett, 1989).

- Role of the multimerisation domain in HSV-1 latency

Deletion of the sequence encoding residues 680-720 of Vmw110 from HSV-1 did not alter the ability of superinfection by the virus to reactivate quiescent HSV-2 genomes in an *in vitro* latency system (Harris *et al.*, 1989). However, the whole multimerisation domain had not been removed in this mutant, therefore the true significance of Vmw110 multimerisation for the reactivation process is yet to be determined.

1B3.3 Nuclear localisation sequence (NLS)

A highly basic region encoded by residues 501-506, which is similar to the SV40 large T antigen nuclear localisation motif, has been implicated in nuclear localisation of Vmw110. This is based on the observation that deletion of this region results in the protein being located in the cytoplasm (Everett, 1988a). This was supported by a subsequent study in which insertion of a short oligopeptide VRPRKRR at residue 500 of a Vmw110 mutant in which residues 500-506 had been deleted, resulted in the restoration of its karyophilic (nuclear localised) phenotype (Mullen *et al.*, 1994). However, this basic motif alone is not sufficient for nuclear localisation and residues on its C-terminal side are also required (Everett, 1988a).

- Role of the NLS for the transactivation function of Vmw110

Deletion of the NLS motif caused reduction in the ability of Vmw110 to transactivate HSV-1 promoters in the presence of Vmw175. However, an insertion into this motif had no such effect (Everett, 1988a).

- Role of the NLS in viral growth

The HSV-1 mutant, in which residues 475-548 (inclusive of the NLS) had been deleted, caused a moderate reduction in the plaquing efficiency of the virus in tissue culture and the viral polypeptide synthesis (Everett, 1989). This mutation also affected transport of Vmw110 into the nucleus in virus infected cells (R. Everett personal communication).

- Role of the NLS in HSV-1 latency

A HSV-1 NLS mutant virus has not been tested in the *in vitro* latency system. As such the importance of this region for the role of Vmw110 in reactivation from latency is not known.

1C Ubiquitin system

Ubiquitin (Ub) is a 76-residue protein that exists in cells, either free or covalently linked to other proteins. At first ubiquitin was identified as an essential component of an ATP-dependent proteolytic system, in which it functioned by conjugating to proteins and thus targeting them for proteolytic processing. However more recently, novel roles that do not involve this degradation pathway have been revealed.

Indeed, the number of cellular processes in which ubiquitin-dependent pathways are involved have avalanched in recent years. These include: stress response, apoptosis, signal transduction, cell differentiation, the cell cycle, embryogenesis, DNA repair, transmembrane transport, vesicular transport and functions of the nervous system (reviewed in Hochstrasser, 1995, Hochstrasser, 1996, Peters *et al.*, 1998, Varshavsky, 1997, Wilkinson, 1995).

1C1 Organisation and enzymology of the ubiquitin system

1C1.1 Enzymes involved in the conjugation of ubiquitin to an acceptor protein

An elaborate enzymatic system is present in eukaryotic cells that enables covalent linkage of ubiquitin to an acceptor protein, in order to produce proteins tagged with either a single ubiquitin moiety, or a multi-ubiquitin chain. The ubiquitin is covalently linked to the acceptor protein through an amide (isopeptide) bond, between the C-terminal carboxyl group of the Gly 76 residue of ubiquitin, and the ε-amino group of a Lys residue in the acceptor protein (Ciechanover, 1994, Hershko, 1996, Hochstrasser, 1996, Jentsch, 1992, Varshavsky, 1996, Wilkinson & Hochstrasser, 1998).

The activation of ubiquitin and its transfer to substrate involve a linked sequence of enzymatic reactions (Figure 1C1.1). The first step is the conjugation of ubiquitin to an E1 Ub-activating enzyme, which requires the hydrolysis of ATP. This results in the formation of a high-energy thioester bond, between Gly 76 of ubiquitin and a specific Cys residue of E1 (Hershko, 1996, Hochstrasser, 1996, Jentsch, 1992).

Ubiquitin then forms a thioester bond with a second protein, an E2 ubiquitin-conjugating enzyme, in a transesterification reaction. The E2 enzyme then catalyses isopeptide bond formation between ubiquitin and a Lys residue on an acceptor protein. This step often

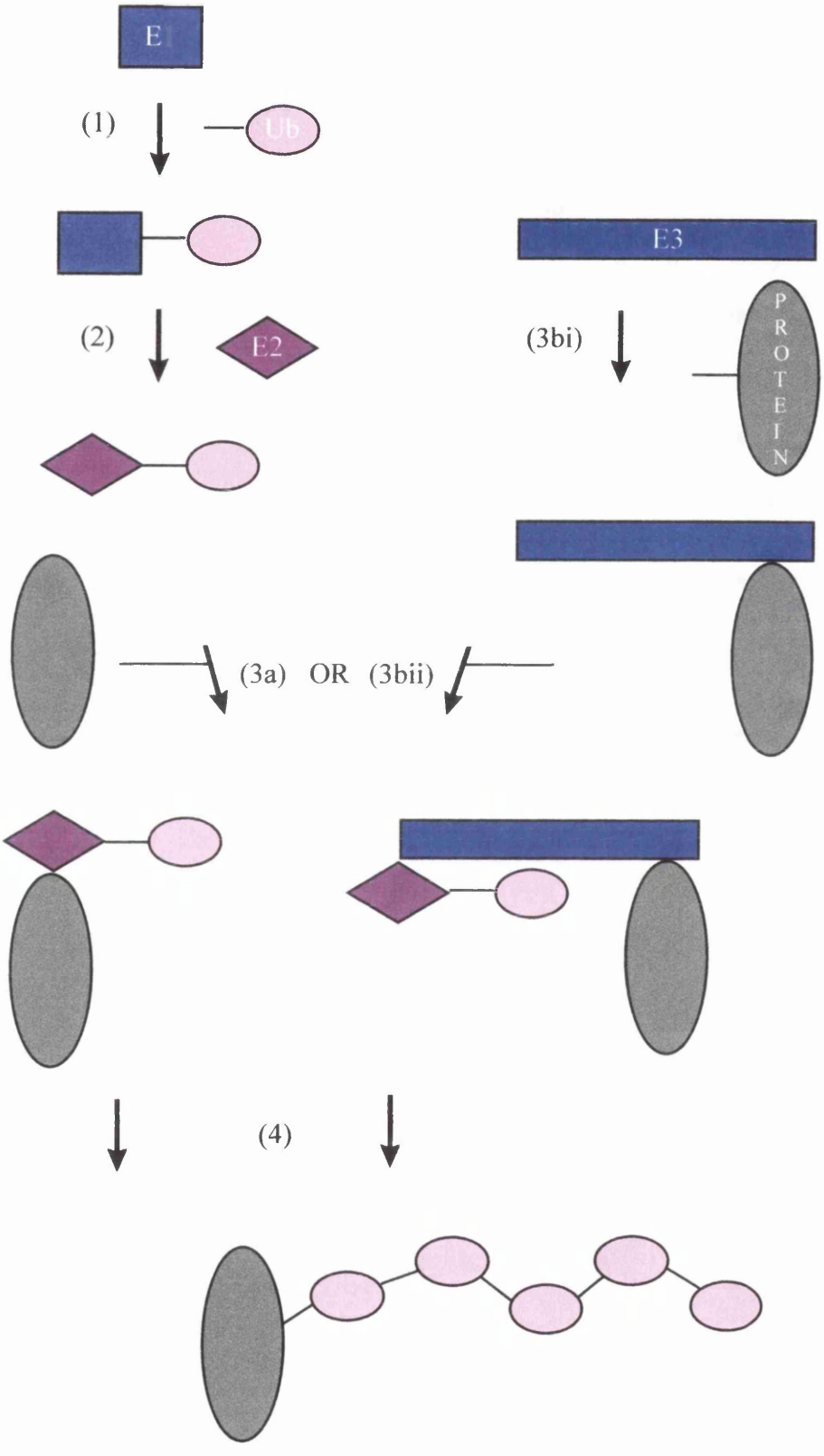
Figure 1C1.1: Main enzymatic steps in conjugation of Ub to a protein substrate.
(1)- ATP dependent activation of Ub with the formation of a high-energy thiolester bond with an Ub-activating enzyme E1.
(2)- Transfer of activated Ub to an Ub-conjugating enzyme E2.
The transfer of Ub from the E2 enzyme to a protein substrate can either occur:

- Independently of any other factors :

(3a)- Binding of the protein substrate to the E2-Ub.
(4)- Ligation of Ub to a lysine residue of the protein substrate, followed by the formation of a multiubiquitin chain.

- Or with the assistance of an E3 Ub-protein ligase.

(3bi)- Binding of the protein substrate to a specific E3.
(3bii)- Formation of an intermediary complex between E3, the protein substrate and E2-Ub.
(4)- Ligation of Ub to a lysine residue of the protein substrate, followed by the formation of a multiubiquitin chain.


E1
(1)
Ub
E3
(2)
E2
(3bi)
PROTEIN
(3a) OR (3bii)
(4)

requires the presence of an additional factor, an E3 or ubiquitin-protein ligase (Hochstrasser, 1996, Jentsch, 1992, Varshavsky, 1996). There are two alternative methods by which the E3 enzymes appear to work. The first mechanism involves the transfer of ubiquitin from the E2 thiol group to a thiol group on the E3, ubiquitin can then be transferred to the Lys residue of the acceptor protein. The second mechanism involves E3 acting as an adapter, positioning the acceptor protein in a preferential position for ubiquitin transfer from the Ub-thioester-linked E2.

1C1.2 Enzymes involved in the cleavage of ubiquitin

Enzymes also exist which can cleave ubiquitin from ubiquitin-protein fusions, and/or isopeptide-bond linked ubiquitin-protein conjugates. These are termed deubiquitinating enzymes (DUBs). They are a large, heterogeneous group of specialised thiol proteases, which can be classified into two sub-families (Section 1C3.1). As HAUSP is a member of the USP family, which is one of the two sub-types of DUB enzymes, details relating to these enzymes are provided in Section 1C3.

1C2 Ubiquitin-degradation system

The best defined role for ubiquitin modification of proteins is in facilitating the degradation of proteins by a complex protease called the 26S proteasome (Hochstrasser, 1995, Hochstrasser, 1996, Wilkinson, 1995).

The 26S proteasome complex is a large supramolecular complex, composed of a core proteinase known as the 20S proteasome, and a pair of regulatory complexes, which are most likely equivalent to a separable multisubunit protein known as PA700 (for 700kD proteasome activator). The exact composition of these regulatory complexes and their functions is not fully understood, but it is hypothesised that several versions of these components exist and are interchangeable, which would result in the presence of several types of 26S proteasome *in vivo*.

Ubiquitin-dependent degradation of proteins consists of several stages. The first stage involves the targeting of ubiquitin tagged proteins to the 26S proteasome. This is thought to involve the recognition and binding of the ubiquitin multi-chain to a component of the PA700 regulatory complex. The next stage is the unfolding of the protein, which probably also requires a component of the PA700 regulatory complex, as well as the hydrolysis of ATP. Once the protein is unfolded, it can be fed into the 20S proteinase cylinder, which is

lined with many varied proteases which degrade the input protein into peptide fragments which are released through fenestrations in the wall of the proteasome.

1C3 Ubiquitin-specific proteases

1C3.1 Classification of deubiquitinating enzymes

Enzymes responsible for the proteolytic processing of ubiquitin at the C-terminal Gly 76 residue have been termed: isopeptidases (Matsui *et al.*, 1982), ubiquitin carboxyl-terminal hydrolases (Pickart & Rose, 1985, Rose, 1988), ubiquitin thiolesterases (Rose & Warms, 1983), ubiquitin-specific proteases (Tobias & Varshavsky, 1991) and deubiquitinating enzymes (Papa & Hochstrasser, 1993). However, for the purposes of this thesis the acronym DUB enzymes will be used.

Two distinct families of DUBs exist. The first family is a relatively small group of proteins, with significant similarity to the neurone specific human protein PGP 9.5 (UCH-L1) (Wilkinson *et al.*, 1989). This family comprises proteins of relatively small molecular weight (<40kD). They are responsible for cleavage of ubiquitin from peptides and small adducts.

The second family is composed of a much larger group of thiol proteases. They are termed ubiquitin-specific proteases (USP or UBP). These proteins are generally much larger, varying in size from 50kD to 250kD. They are generally responsible for cleaving ubiquitin from a wide range of substrates *in vivo*. HAUSP is a member of this family and as such the properties of these proteins are discussed in greater detail below.

1C3.2 Conserved features of USPs

These proteins all contain several short consensus sequences that are likely to comprise the catalytic domains (Baker *et al.*, 1992, Papa & Hochstrasser, 1993). In particular, the most highly conserved features are Cys and His boxes (Figure 1C3.2), however, additional short sequences also show some conservation (Papa & Hochstrasser, 1993, Wilkinson *et al.*, 1995). An interesting observation is that motifs conserved amongst the USPs do not have high sequence similarity to the regions surrounding the conserved Cys and His residues of the first family of DUBs. This suggest the two families arose as a result of convergent evolution as opposed to divergent evolution.

Cysteine box consensus pattern:

LIVMFY-x(3)-AGC-NA-x-**C**-FY-LIVMC-NS-SC-x-LIVM-Q

Histidine box consensus pattern:

Y-x-L-x-SAG-LIVMT-x(2)-**H**-x-G-x(4,5)-G-**H**-Y

Figure 1C3.2: Sequences of the cysteine box and the histidine box conserved motifs of USPs. The letter 'x' represents any amino acid. The C (cysteine) and H (histidine) residues highlighted in bold are predicted to be required for catalytic activity.

1C3.3 USPs are a large and heterogeneous group

Sequence information indicates that the USP family is very large. Indeed, in yeast 16 proteins that potentially encode for USPs have been identified, which is greater than the number of yeast E2 ubiquitin-conjugating enzymes identified (Hochstrasser, 1996).

Yeast USP mutants often do not show major phenotypic abnormalities. This suggested that either these proteins control functions which when deleted can not be detected by the standard phenotypic assays used, or that there is much redundancy amongst these proteins (Baker *et al.*, 1992, Papa & Hochstrasser, 1993).

1C3.4 Substrates of USPs

- Ubiquitin precursor proteins

Ubiquitin genes always encode ubiquitin as part of a fusion protein. The primary translation products of one class of ubiquitin genes consist of a 'linear' peptide bond linked ubiquitin chain made of 3 to 52 ubiquitin molecules (Ozkaynak *et al.*, 1984, Swindle *et al.*, 1988). In the cases of the other genes encoding ubiquitin, a ubiquitin precursor is synthesised, in which ubiquitin is fused to the N-terminal of any of several proteins or peptides (Finley *et al.*, 1989).

- Polyubiquitin chains

Multi-ubiquitin chains are found on proteins destined for proteolysis by the 26S proteasome.

1C3.5 Potential roles of USPs in the ubiquitin system

- Processing of ubiquitin precursors

As mentioned, ubiquitin is synthesised within a fusion protein. Such proteins require the release of free ubiquitin monomers by cleavage of ubiquitin at its C-terminus. This function may be performed by USPs.

- Proof-reading of protein ubiquitination

A second role for USPs is in the regulation of ubiquitin-dependent processes. For example, a USP could inhibit a ubiquitin-dependent process by removal of ubiquitin from either

mono or polyubiquitinated protein substrates before they are committed to their fate, e.g. degradation by the proteasome. This mechanism could be a proof-reading step to ensure only appropriately targeted proteins are degraded (Cox *et al.*, 1986, Ellison & Hochstrasser, 1991, Wilkinson & Mayer, 1986). This regulatory mechanism, involving the conjugation/deconjugation of ubiquitin to proteins, is analogous to that of the phosphorylation/dephosphorylation regulatory mechanism.

- Recycling of ubiquitin from polyubiquitinated proteins following commitment to degradation

As ubiquitin is a stable protein once polyubiquitinated proteins have been targeted for degradation by the proteasome, USPs are required for release of ubiquitin from peptide remnants. The free ubiquitin can then be recycled. It is not clear as yet at which stage in the degradative cycle ubiquitin release occurs.

- Maintaining free ubiquitin levels and keeping the proteasome free of ubiquitin chains

Along with the mechanisms discussed above, other ways by which USPs may function to stimulate protein ubiquitination and/or degradation also exist. For example, long ubiquitin chains are sometimes conjugated to inappropriately targeted proteins and failure to remove such chains could result in the depletion of cellular ubiquitin levels. Additionally, proteasomes and certain enzymes of the ubiquitin conjugation system e.g. E3a, are known to bind to ubiquitin chains (Deveraux *et al.*, 1994, Reiss *et al.*, 1989). Such chains which have either been generated *de novo*, or as a result of protein proteolysis, need to be removed in order to prevent these chains inhibiting the proteasome, or other ubiquitin system enzymes.

1C3.6 Examples of USPs in cellular regulation

Although sequence analysis has identified the presence of many genes which encode USPs only a few of these have been analysed and their regulatory functions understood. Provided below are a few examples of these showing the diversity of function that this group of enzymes displays.

- The role of the *Drosophila fat facet* gene product in development

The product of the *Drosophila fat facet* (*faf*) gene has been shown to have deubiquitinase activity. Interestingly, the *faf* gene is required for normal eye development, in particular it is thought to regulate a cell communication pathway essential very early in facet development which limits the number of photoreceptor cells in each facet of the compound eye to eight (Huang *et al.*, 1995).

It is of interest that mutations in the Cys or His active site boxes resulted in a similar phenotype to that observed for *Drosophila faf*-null mutants. This suggests that the deubiquitinating activity of Faf is central to its biological role, specifically modulating the degradation rate of a key regulatory factor of facet development. This was supported by the observation that mutations in various alleles of the 20S proteasome subunit suppressed the defect seen in the *faf* mutants. Hence, Faf is thought to have a proof reading function, deconjugating ubiquitin from a regulatory protein and thus preventing its degradation by the proteasome. Indeed, recent work suggests Faf works by negatively regulating the RTK/Ras/MAPK signalling pathway during *Drosophila* development (Isaksson *et al.*, 1997).

- The role of yeast Ubp3 in silencing

Ubp3, a deubiquitinase expressed in yeast, is found to strongly bind to Sir4, a protein which is important in the transcriptional silencing of genes near the telomere and in the silent mating type loci of yeast (Moazed & Johnson, 1996).

The exact role of Sir4 in silencing is thought to involve its interactions with the origin recognition complex, Sir2 and Sir3 proteins, which result in the formation of a silencing complex. These complexes assemble at a particular subset of chromosome sites and are thought to alter the state of chromatin at these sites and prevent the encoded genes from being transcribed. As deletion of Ubp3 resulted in the significant improvement in the silencing of genes, either inserted near the telomere or at one of the silent mating type loci, it suggested that Ubp3 is an inhibitor of silencing. However, the exact mechanism by which the interaction of Sir4 and Ubp3 interferes with silencing is as yet unclear.

- The role of *Drosophila* D-Ubp-64E in position effect variegation

Another example of a USP functioning in transcriptional silencing is that of the D-Ubp-64E deubiquitinase of *Drosophila.* A mutant of D-Ubp-64E was shown to act as an enhancer of position effect variegation (PEV). Furthermore, additional copies of the D-Ubp-64E gene suppressed PEV (Henchoz *et al.*, 1996). PEV is a form of transcriptional silencing in which chromosome rearrangements occur that place euchromatic genes next to a heterochromatin boundary.

- The role of human Tre2 deubiquitinase in growth control

The human *tre2* gene encodes a deubiquitinase enzyme. It was first proposed to have a role in growth control based on the observation that a mutation in its Cys active site box resulted in oncogenic properties (Papa & Hochstrasser, 1993). There are two mechanisms by which *tre2* may be functioning in growth control either by enhancing the degradation of a positive regulator of cell proliferation or by preventing the degradation of a tumour suppressor by deconjugating its polyubiquitinated intermediates.

- The murine DUB family is a novel family of cytokine inducible deubiquitinating enzymes

The first member of this family to be identified was DUB-1. It is an erythroid cell-specific IE gene. Its expression is induced in the presence of the cytokines: interleukin 3, interleukin 5 and growth maturation-cytokine stimulating factor, requiring the βc common subunit of the cytokine receptor (Zhu *et al.*, 1996a, Zhu *et al.*, 1996b). Once expressed it becomes rapidly degraded in a proteasome-dependent manner.

A role of this gene in regulation of growth control was suggested by the observation that constitutive expression of this gene caused cell cycle arrest in the G_1 phase. Furthermore, cells which constitutively expressed an active site mutant of the gene were not blocked in the G_1 phase of the cell cycle.

Other DUB encoding genes are located in the same region on mouse chromosome 7 (Zhu *et al.*, 1997). DUB-2, the only other member to be analysed in detail, was also found to be an IE cytokine-inducible gene, induced by interleukin 2. It has high sequence homology to DUB-1 (88% identical), varying only in a C-terminal hypervariable region. Therefore, it has been proposed that different cytokines induce the expression of specific DUB genes, in

turn the deubiquitinase enzyme generated modulates the ubiquitination state of a specific regulatory factor resulting in a cytokine specific response.

- Role of mammalian isopeptidase T in the metabolism of degradation intermediates

The mammalian isopeptidase T (isoT) USP has substrate specificity for unanchored ubiquitin chains, i.e. ubiquitin oligomers with a free C-terminal (Wilkinson, 1995). Such chains may be produced as a result of *de novo* synthesis by E2 enzymes, or as a result of action by the 26S proteasome. As the 26S proteasome is found to have strong affinity for ubiquitin chains, it is thought the presence of an excess of these unanchored ubiquitin chains may inhibit the 26S proteasome. This theory is supported by the observation that yeast wild type cells overexpressing such chains have defects in their protein proteolysis. As such, the result of the action of isoT may be to prevent such inhibition. Indeed, *in vitro* studies have supported such a role for isoT (Hadari *et al.*, 1992). A yeast homologue called Ubp14 has recently been identified that is thought to share a similar biological property: a Ubp14 null mutant was shown to have defects in the degradation of a number of distinct proteins and also accumulate an excess of unanchored ubiquitin chains (Amerik *et al.*, 1997).

- Role of yeast Doa4 in metabolism of degradation intermediates

The yeast Doa4 USP has been implicated to have diverse physiological functions (Papa & Hochstrasser, 1993). Mutant *doa4* yeast cells have a variety of phenotypic abnormalities, which include failure to sporulate (Papa & Hochstrasser, 1993) and failure to accurately co-ordinate replication from different parts of the genome (Singer *et al.*, 1996).

Doa4 is thought to function by cleaving ubiquitin substrate remnants still bound to the 26S proteasome. Evidence of such a role comes from the observation that cells in which *doa4* is mutated have an excess of small ubiquitinated species which are slightly bigger than the unanchored ubiquitin chains cleaved by isoT. Furthermore, Doa4 has been found in purified preparations of the 26S proteasome (Wilkinson & Hochstrasser, 1998). It is thought that an accumulation of such ubiquitin remnants in the absence of functional Doa4 would also lead to inhibition of the proteasome.

1D Effects of Vmw110 on the cell

1D1 Redistribution of ND10 domains

The localisation of Vmw110 to discrete regions within the nucleus, when expressed from transfected plasmids, was first observed by Gelman and Silverstein in 1985 (Gelman & Silverstein, 1985). However, it was not until later that Maul and co-workers observed that Vmw110 colocalised with discrete nuclear structures, known as ND10 domains, early in HSV-1 infection. In addition, they observed that at later times of infection Vmw110 was responsible for the dispersion of the ND10 proteins PML and Sp100 (Everett & Maul, 1994, Maul & Everett, 1994).

It is thought that the interactions between Vmw110 and host ND10 domains are the key by which Vmw110 exerts its effect. Relevant aspects of ND10 domain biology are discussed below, however, a broad review of the subject can be found in Sternsdorf *et al.*, 1997a.

1D1.1 Introduction to ND10 domains

ND10 domains were first identified by use of a variety of autoimmune sera and monoclonal antibodies (Ascoli & Maul, 1991, Koken *et al.*, 1994, Weis *et al.*, 1994, Xie *et al.*, 1993). Interestingly, the ND10 constituents are thought to be associated with the highly organised three dimensional framework which consists of proteins and ribonucleoproteins, known as the nuclear matrix (Ascoli & Maul, 1991, Dyck *et al.*, 1994, Koken *et al.*, 1994, Weis *et al.*, 1994).

Examination of the larger ND10 domains using either light microscopy at high magnification, or electron microscopy, reveal a 'doughnut-like' structure of these domains consisting of a dense fibrillar ring which encompasses a central core (Dyck *et al.*, 1994, Weis *et al.*, 1994). These structures are quite large, approximately 0.3μm in diameter. The number per cell varies considerably depending on cell type and other factors, but in general 10-20 can be located. Hence, they were named ND10 (Ascoli & Maul, 1991), but alternate names have also been given including PML oncogenic domains (PODs) (Dyck *et al.*, 1994), or Kr-bodies (Lamond & Carmo-Fonseca, 1993).

A growing number of constituents have been identified as components of these domains these include Sp100 (Ascoli & Maul, 1991), PML (Dyck *et al.*, 1994), PIC-1 (Boddy *et al.*, 1996), HAUSP (Everett *et al.*, 1997), NDP55 (Stuurman *et al.*, 1992), LYSP100/Sp140

(Bloch *et al.*, 1996, Dent *et al.*, 1996), Rfp (Cao *et al.*, 1998) and Int-6 (Desbois *et al.*, 1996). Not all these components are always found in the ND10 domains and some only transiently associate with the domains. Details of the ND10 constituents Sp100, PML and PIC-1, which have direct relevance for the purposes of this introduction, are discussed in greater depth below.

1D1.1a Sp100

The Sp100 protein was the first ND10 constituent to be identified. This discovery came as a result of studies using autoimmune sera produced from patients with primary biliary cirrhosis (Ascoli & Maul, 1991). Furthermore, Sp100 was found to firmly bind the nuclear matrix (Xie *et al.*, 1993). Since then it has been characterised both biochemically and by cDNA cloning (Szostecki *et al.*, 1990, Szostecki *et al.*, 1987). A number of alternatively spliced isoforms of the protein have also been shown to exist including SpAlt-HMG, Sp140 and LYSP100B.

An illustration of the structural and functional domains of these proteins has been provided in Figure 1D1.1a. Features of interest include:

- A sequence with striking similarity to parts of the peptide-binding groove of MHC class 1 molecules which has been designated MHC-like.
- A region named HSR which represents a highly amplified region in the equivalent gene of some mouse populations.
- A transactivating domain which was identified through transfection studies in which Sp100 fragments fused to a DNA binding domain were cotransfected with a reporter plasmid.
- A region overlapping the transactivation domain which was identified as having retained weak homology to several transcriptional regulatory proteins, including the HIV-1 Nef protein.
- A HNPP box which represents a region with high sequence similarity to an interferon-inducible human nuclear phosphoprotein HNPP1/2.
- A HMG domain which represents an almost complete high mobility group 1 protein sequence which have been shown to directly bind to DNA.

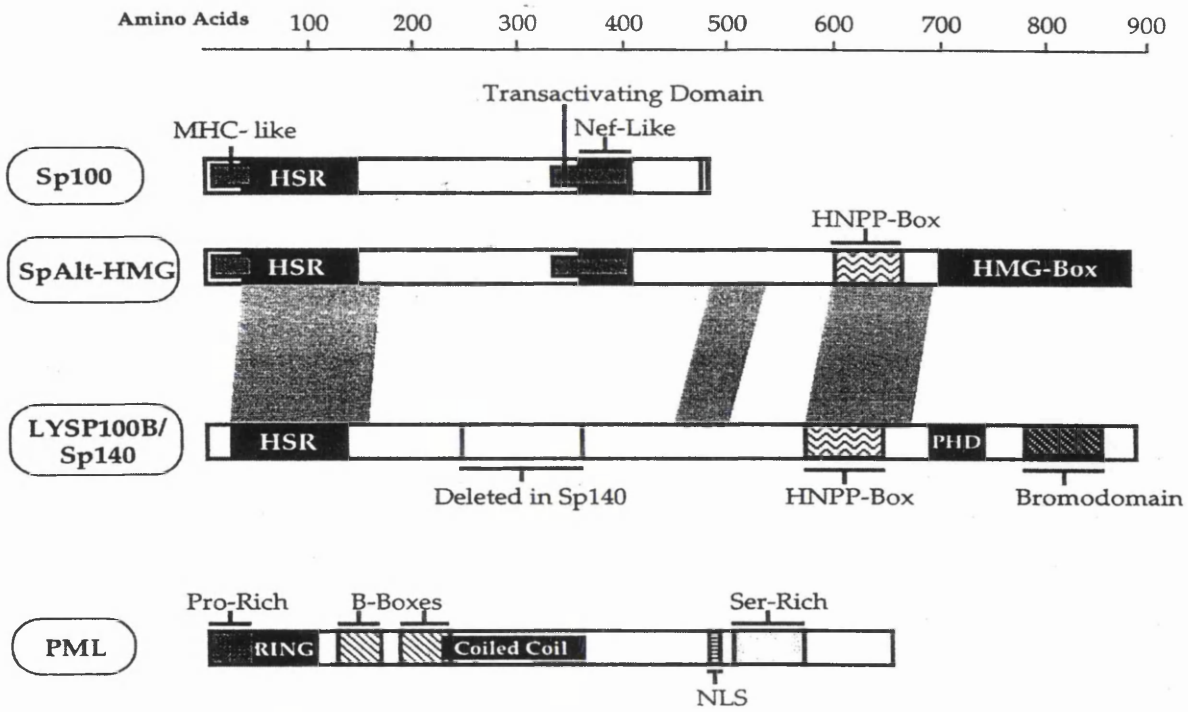


Figure 1D1.1a: Structural and functional domains of Sp100 isoforms and PML.
Various isoforms of Sp100 are illustrated including the originally described Sp100 protein, SpAlt-HMG, LYSP100B and Sp140. SpAlt-HMG contains the entire Sp100 sequence except the last four amino acids. An additional splice variant called Sp100-B is identical with SpAlt-HMG up to amino acid 684 and has four additional amino acids at the C-terminus. Regions with high sequence similarity between SpAlt-HMG and LYSP100B are indicated. For PML one representative splice variant is shown. Abbreviations: MHC-major histocompatibility complex type I; HSR-HSR domain; HNPP-human nuclear phosphoprotein; HMG-high mobility group; PHD-PHD finger domain; RING-Ring finger domain; NLS-nuclear localisation sequence.
This diagram has been adapted from one illustrated in Sternsdorf *et al*, 1997.

Interestingly, a recent study has suggested a role for Sp100 in transcriptional silencing (Seeler *et al.*, 1998). In particular, it was demonstrated that the Sp100 binds to members of the heterochromatin protein 1 (HP1) family of non-histone chromosomal proteins. These are involved with transcriptional silencing phenomena known as position effect variegation as well as in the proper functioning of the centromere during mitosis (Elgin, 1996, Kellum & Alberts, 1995). Overexpression of Sp100 led to accumulation of HP1 in these domains. Furthermore, they showed that a splice variant of Sp100, which the authors named SP100-HMG, concentrated with HP1 in ND10 domains and when bound to a promoter Sp100, SP100-HMG and HP1 behaved as transcriptional repressors in transfected mammalian cells. As such, it suggests a mechanism by which ND10 may be involved with the control and maintenance of chromatin or heterochromatin architecture.

1D1.1b PML

PML was the second component of ND10 domains to be identified. It exactly colocalises with Sp100 and is firmly attached to the nuclear matrix. As PML is involved in the development of acute promyelocytic leukaemia (APL), the discovery of its localisation to ND10 domains prompted much interest in these domains. APL is caused by a chromosomal translocation which results in the production of a fusion protein between the N-terminus of PML and the retinoic acid receptor alpha (RAR-α) (de The *et al.*, 1991, Goddard *et al.*, 1991, Kakizuka *et al.*, 1991, Kastner *et al.*, 1992, Pandolfi *et al.*, 1992). The consequence of the expression of this fusion protein is the disruption of the normal distribution of the ND10 proteins, from 10-20 discrete structures per nucleus, to a much greater number which are distributed both in the cytoplasm and nucleus. It is possible to treat this illness using retinoic acid, this results in the proteasome-dependent destruction of the PML-RARα fusion protein, which leads to the restoration of the ND10 distribution and normal differentiation of the tumour cells (Dyck *et al.*, 1994, Koken *et al.*, 1994, Weis *et al.*, 1994, Yoshida *et al.*, 1996).

Sequence analysis has revealed that the PML gene synthesises a large number of alternatively spliced transcripts (Fagioli *et al.*, 1992) which result in the formation of PML proteins that range in size from 47-160kD (Chelbi-Alix *et al.*, 1995, Grotzinger *et al.*, 1996). However, the difference in function of these various forms of PML has yet to be understood.

PML belongs to a family of proteins which share an amino-terminal tripartite domain,

called the RING-B-Box-coiled-coil motif (RBCC), characterised by a RING finger, one or two additional cysteine-rich regions known as B-box domains and a coiled-coil region (Freemont *et al.*, 1991, Lovering *et al.*, 1993, Reddy *et al.*, 1992). The positions of these domains and that of the serine rich domain and the nuclear localisation sequence are shown in Figure 1D1.1a.

Extensive structure/function analysis on PML has demonstrated that the RING finger is both important for the formation of ND10 domains and for mediating PML-PML oligomeric interactions (Boddy *et al.*, 1997). However, a complete RBCC motif is necessary and sufficient for localisation of PML to ND10 domains (Barlow *et al.*, 1994, Borden *et al.*, 1996, Le *et al.*, 1996). Furthermore, the two B-boxes and the coiled-coil domain have been shown to be important for interaction with another member of the RBCC family, the Ret finger protein (Rfp) (Cao *et al.*, 1998). This is consistent with the finding that Rfp is a transient member of ND10 domains. Finally, the serine rich domain is speculated to have a regulatory function, which is consistent with the fact that in the PML/RARA fusion protein this region is truncated whereas the RBCC motif has been retained (de The *et al.*, 1991, Fagioli *et al.*, 1992, Kakizuka *et al.*, 1991).

In addition to Rfp, PML has also been shown to interact with a novel ubiquitin-like protein, the PML-interacting clone, or PIC-1 (Boddy *et al.*, 1996). This protein is also a component of the ND10 domain and is discussed in greater detail later in the text. Interestingly, recent studies have shown that PML is actually covalently modified by PIC-1, which results in the formation of several high molecular weight conjugates of PML (Kamitani *et al.*, 1998, Muller *et al.*, 1998, Sternsdorf *et al.*, 1997b). It has been suggested that the modification of PML by PIC-1 plays an essential part in the formation of ND10 domains (Muller *et al.*, 1998).

The exact function of PML has yet to be characterised, though several functional studies suggest growth and transformation suppressing properties of overexpressed PML in APL cells (Ahn *et al.*, 1995), as well as other cell lines (Koken *et al.*, 1995, Le *et al.*, 1996, Mu *et al.*, 1994). This is consistent with findings that overexpression of PML in *neu* oncogene expressing NIH/3T3 transformed cell lines caused a marked reduction in the *in vivo* and *in vitro* growth rates, as well as a reversion of the transformed phenotype (Koken *et al.*, 1995, Liu *et al.*, 1995). This role of PML in the control of cell growth and tumourgenesis was further supported by a recent study in which the effects of eliminating PML expression

were observed in mice (Delva *et al.*, 1998). However, work by other groups suggests that this growth rate regulating function of PML is not observed for all cell lines and under all experimental conditions (Sternsdorf *et al.*, 1997a). PML has also been suggested to enhance the effects of various steroid hormone receptors (Guiochon-Mantel *et al.*, 1995) and to act as a transcriptional repressor (Mu *et al.*, 1994). Therefore, collectively this work suggests a role for PML in transcriptional regulation.

1D1.1c PIC-1

The association of PIC-1 with ND10 domains primarily arose from the discovery of its strong and specific interaction with PML (Boddy *et al.*, 1996). Antisera raised against PIC-1 gave a staining pattern which partially overlapped that of the pattern produced by antisera against PML. Interestingly, cDNAs identical to that encoding PIC-1 were discovered almost simultaneously by four other laboratory groups designated: SUMO-1 (small ubiquitin-related modifier) (Mahajan *et al.*, 1997), GMP1 (GAP modifying protein 1) (Matunis *et al.*, 1996), Sentrin (O'Kura *et al.*, 1996) and UBL1 (ubiquitin-like 1) (Shen *et al.*, 1996).

The PIC-1 protein belongs to the ubiquitin family of proteins, a heterogeneous group of proteins which share the ubiquitin-like domain (UbH-domain). The lysine residues, which are conserved in ubiquitin and which are required for the normal polyubiquitination, have not been conserved in the UbH-domain of PIC-1. Therefore it is unlikely that PIC-1 is involved in the normal ubiquitin-dependent pathway of protein degradation.

However, it has been suggested that like ubiquitin PIC-1 acts as a post-translational modifier of proteins. Indeed, recent studies have demonstrated that PIC-1 covalently binds several proteins, including the mammalian guanosine triphosphate (GTP)ase-activating protein RanGAP1, I kappa B alpha and PML (Desterro *et al.*, 1998, Everett *et al.*, 1998a, Kamitani *et al.*, 1998, Mahajan *et al.*, 1997, Matunis *et al.*, 1996).

A yeast homologue of PIC-1 has been identified named SMT3 (retaining 73% homology), which has been described as a high copy suppressor of mutations in the mitosis fidelity protein MIF2 (Meluh & Koshland, 1995), a protein which is involved in mitosis during anaphase (Brown *et al.*, 1993).

1D1.2 Factors that alter ND10 domains

ND10 domains are normally associated with the nuclear matrix, which itself is insoluble and non-extractable. However, the domains themselves are dynamic in nature, being modulated by a number of factors. These factors can result in changes in size, number, morphology and subcellular distribution of the ND10 domains, and sometimes even in complete loss of the punctate staining pattern. Factors which effect ND10 morphology and composition include: interferon treatment, stress, the cell cycle, and virus infection.

1D1.2a Interferon treatment alters ND10 composition

Interferons type I (α,β) and type II (γ) have been shown to strongly enhance Sp100 and PML gene expression both at the RNA and protein level (Chelbi-Alix *et al.*, 1995, Grotzinger *et al.*, 1996, Guldner *et al.*, 1992, Lavau *et al.*, 1995) with very similar kinetics (Grotzinger *et al.*, 1996). In addition, interferon treatment also causes the expression of different isoforms of both Sp100 and PML, that are not produced in untreated cells.

1D1.2b ND10 distribution is cell cycle regulated

The average number and size of ND10 domains changes during progress through the cell cycle (Koken *et al.*, 1995). At the beginning of the cell cycle when the cell is in growth arrest (G_0) a low number of ND10 domains can be observed, which steadily increases as the cell progresses through G_1 and then into the S phase when the highest number of ND10 domains may be observed, as well as a diffuse nuclear form of PML (Koken *et al.*, 1995, Terris *et al.*, 1995). It is interesting to note that ND10 domains are found juxtaposed to replication domains in the mid to late S phase of the cell cycle (Grande *et al.*, 1996). Furthermore, during mitosis PML and Sp100 dissociate from each other, PML forming large aggregates at the periphery of the mitotic cell and Sp100 becomes diffusely distributed (Sternsdorf *et al.*, 1997a).

1D1.2c ND10 domains are redistributed after stress

Stress factors such as heat shock, heavy metals or other environmental stimuli are known to affect the morphology of ND10 domains. For example, heat shock and cadmium ion exposure leads to the redistribution of the ND10 domains into hundreds of small dots, forming a 'microspeckled' pattern (Maul *et al.*, 1995). In contrast to this amino acid

starvation leads to an increase in size, but a reduction in the number of ND10 domains (Kamei, 1996).

1D1.2d Viral factors influence ND10 domains

Work in the past few years has demonstrated that a number of DNA, as well as RNA, virus infections result in the modification of ND10 domains. Whether this is a consequence of the virus hijacking the host cell machinery or a defence reaction on the part of the cell to the invasion is unclear. However, the conservation of this virus-host interaction in a wide variety of virus infections suggests it has some fundamental role to play.

DNA viruses:

- Herpesviridae

In particular, the IE gene products of HSV, CMV and EBV interact with the ND10 domains. In the case of HSV-1 the IE protein Vmw110 colocalises with ND10 domains at early times in infection and at later stages of infection is responsible for the complete loss of ND10 staining (Everett & Maul, 1994, Maul & Everett, 1994). A similar scenario is seen for CMV in which ND10 staining is also lost as a result of infection. The IE1 protein is found to colocalise with ND10 domains early in infection and at later times cause their redistribution. In addition, the IE2 immediate early protein is located adjacent to these domains during early times of infection. Interestingly, a recent study has suggested that IE transcripts, found adjacent to ND10 domains in HCMV infection, are funnelled into the spliceosome assembly factor SC35 domain by IE2, which also recruits some components of the basal transcription machinery (Ishov *et al.*, 1997).

The Epstein-Barr virus also expresses a protein EBNA-5 early in infection which colocalises with ND10 domains. However, in this case EBNA-5 is homogenously distributed throughout the nucleus within the earliest phase of infection and only associates with the ND10 domains after the first day of infection. This association does not alter the morphology of the ND10 domains. Furthermore, this protein has been found to directly bind to the tumour suppressor proteins p53 and pRb and results in their partial colocalisation with the ND10 domains (Jiang *et al.*, 1991, Szekely *et al.*, 1993).

- Adenovirus

Adenovirus infection also results in the redistribution of ND10 domains. In particular, the E4ORF3 protein has been shown to redistribute PML, and in part, also the Sp100 components of the ND10 domains, into fibrous track-like structures (Carvalho *et al.*, 1995). The E1A oncoprotein also colocalises with these PML-containing fibres for which the conserved Rb-binding motif is required.

RNA viruses:

- Human T cell leukaemia virus (HTLV)

This reterovirus is responsible for causing adult T cell leukaemia. It has been found that the Tax protein of the virus, which is thought to be one of the key factors in provoking transformation of infected cells (Nerenberg *et al.*, 1987), directly binds to a transient member of ND10 Int-6 and relocates it to the cytoplasm (Desbois *et al.*, 1996). Hence, it has been suggested that this may be an underlying mechanism for the transforming function of Tax.

- Influenza A

This virus has been shown to increase the number and intensity of ND10 foci in infected HeLa cells, when probing against Sp100 (Guldner *et al.*, 1992). This effect is similar to that of interferon treatment. However, as only infected cells are affected it suggests this is a virus-induced effect.

- Arenavirus

Infection with arenavirus lymphocytic choriomeningitis causes the redistribution of PML (Borden *et al.*, 1998). This effect is provoked by the viral Z protein, which is also a member of the RING finger family of proteins.

1D1.3 ND10 domains may be sites of early transcription and genome replication of DNA viruses

An interesting feature conserved amongst at least four of the ND10 interacting DNA viruses investigated so far (SV40, adenovirus, HCMV and HSV-1), is the deposition of viral DNA adjacent to the ND10 domains (Ishov & Maul, 1996, Maul *et al.*, 1996). In the case of SV40 virus infection replication begins adjacent to the ND10 domains. This is not

true of adenovirus and HSV-1 infections, in which ND10 domains are redistributed before replication. However, when either E4ORF3-null adenovirus mutant or Vmw110-null HSV-1 mutant infections were carried out, in which the ND10 domains were not dispersed, the replication compartments were observed adjacent to these nuclear domains. Indeed, a recent study has suggested that viral DNA is first deposited adjacent to ND10 domains and that subsequently prereplicative sites are formed containing ICP8 which are precursors of the replication compartments in which viral DNA is synthesised (Lukonis *et al.*, 1997).

Viral gene expression was not required for the transport of the parental genomes to the periphery of the ND10 domains for all four of the viruses mentioned. Collectively these studies suggest that early stages of transcription and replication may be occurring at these sites.

1D1.4 Contribution of the functional domains of Vmw110 for redistribution of ND10 domains

1D1.4a RING finger

There is some conflict as to the precise role of this domain in ND10 redistribution. The most popular theory is that the RING finger is not required for localisation of Vmw110 to ND10 domains, rather it is required for their dispersion. This is based on the observation that infection with mutants of HSV-1, from which the RING finger domain of Vmw110 is deleted, results in the accumulation of Vmw110 in punctate domains, even at late times of infection (Everett & Maul, 1994, Maul & Everett, 1994, O'Rourke *et al.*, 1998). Similar observations were made from cells transfected with plasmids encoding RING finger deleted Vmw110.

An alternative view is that this domain is required for the localisation of Vmw110 to the ND10 domains. This is based on the observation that transfection of cells with plasmids encoding mutants of Vmw110, in which the conserved cysteine and histidine residues of the RING finger had been substituted, resulted in the transfected proteins producing a more nuclear diffuse staining pattern (Lium & Silverstein, 1997, O'Rourke *et al.*, 1998). However, it is likely that these mutations cause drastic conformational changes to the whole protein and it is these changes which result in the observed loss of interaction with the ND10 domains.

1D1.4b Multimerisation domain

The C-terminal residues that are involved in formation of Vmw110 multimers are required for interaction with ND10 domains. This is based on the observation that transfection of plasmids encoding Vmw110 C-terminal deletion mutants, or Vmw110 C-terminal truncation mutants, resulted in the transfected proteins producing a predominately nuclear diffuse staining pattern (Chen *et al.*, 1991, Everett, 1988a, Mullen *et al.*, 1994).

However, the C-terminal does not appear to be sufficient for localisation of Vmw110 to ND10. This was first suggested by the observation that fusion proteins, consisting of the C-terminus of Vmw110 and β-Galactosidase (β-Gal), only conferred either diffuse or micropunctate staining in cells transfected with plasmids encoding these proteins (Everett & Maul, 1994). Additionally, a study which involved the use of hybrids of the N-terminus of Vmw175 and the C-terminus of Vmw110 in transfection assays showed that the C-terminus of Vmw110 was not sufficient for the translocation of the fusion protein to the ND10 domains (Mullen *et al.*, 1994). Therefore, it seems likely that more than one domain of Vmw110 is involved with its interaction with ND10 domains.

1D1.4c Nuclear localisation sequence

Transfection of a Vmw110 mutant, in which a region inclusive of the NLS was deleted, resulted in diffuse cytoplasmic fluorescence, and for a proportion of the cells PML could also be observed relocated to the cytoplasm (Everett & Maul, 1994). As such, it was suggested PML cycles between the nucleus and cytoplasm.

1D2 Vmw110 is required for the proteasome-dependent degradation of several picylated cellular proteins including PML, during HSV-1 infection

Disruption of ND10 domains during HSV-1 infection correlates with the loss of several high molecular weight isoforms of PML (Everett *et al.*, 1998a). The isoforms of PML that are depleted probably correspond to those of PML-PIC1 conjugates. This is dependent both on Vmw110 and active proteasomes. As such, it has been suggested that Vmw110 is responsible for the targeting of PML conjugates to the proteasome for degradation and that this results in the disruption of ND10 domains observed during HSV-1 infection. The RING finger domain of Vmw110 was essential for this process, loss of which resulted in the stabilisation of the high molecular weight PML isoforms. Vmw110 was also shown to

be responsible for the elimination of several other conjugated proteins which were produced as a result of transfection of a plasmid expressing PIC-1.

1D3 Interactions of Vmw110 with cellular proteins

In an attempt to investigate the molecular mechanism by which Vmw110 functions, several groups set out to identify cellular proteins that interact with Vmw110. Using a combination of GST 'pull-down' and coimmunoprecipitation assays Everett and co-workers identified a novel cellular protein, named HAUSP, that strongly and specifically bound to Vmw110 (Everett *et al.*, 1997, Meredith *et al.*, 1995, Meredith *et al.*, 1994). The role of this interaction for HSV-1 infection has been studied for the purposes of this thesis. As such, details established at the start of this investigation relating to this interaction are described in Section 1E.

The yeast two hybrid system has also been used to identify cellular proteins that interact with Vmw110. The result of these studies suggested that Vmw110 interacts with Cyclin D3, through sequences in its second exon, and elongation factor 1δ, through sequences in its C-terminal (Kawaguchi *et al.*, 1997a, Kawaguchi *et al.*, 1997b).

1E Vmw110 strongly and specifically interacts with a USP dubbed HAUSP

1E1 Evidence for interaction between Vmw110 and HAUSP

HAUSP was first identified as a 135kD protein that coimmunoprecipitated alongside Vmw110 (Meredith *et al.*, 1994). In particular, Vmw110 was immunoprecipitated from infected cell extracts as a fast sedimenting complex composed of itself and this 135kD cellular protein. These results were supported by further immunoprecipitation experiments in which exogenously purified Vmw110 was added to lysates of a number of different cell types, including neuronal (neuroblastoma ND7) and non-neuronal (BHK and HeLa) cell lines.

The GST 'pull-down' technique was also used to support the authenticity of this interaction. A GST fusion protein encoding the C-terminal residues 594-775 of Vmw110 also interacted with a 135kD cellular protein. Depletion binding experiments verified that the same Vmw110-associated protein was identified using these different experimental strategies. These experiments showed that prior depletion of the 135kD cellular protein from the cell extract, by Vmw110 immunoprecipitation, resulted in a dramatic decrease in the amount of the 135kD protein which bound to the GST fusion protein encoding residues 594-775 of Vmw110 and vice versa.

1E2 Cloning and sequence analysis of cDNA encoding HAUSP

Peptide sequences of the 135kD protein were derived by purification of the protein from large scale GST 'pull-down' assays using a GST fusion protein encoding Vmw110 residues 594-775. These sequences were used to screen a HeLa cDNA library and resulted in the isolation of cDNAs encoding the Vmw110-associated protein (Everett *et al.*, 1997). Analysis of the cDNA sequence revealed an ORF of 1102 residues which was predicted to encode a protein of 128kD. Screening of the NCBI nr database demonstrated that this Vmw110-associated protein contained only two regions of high sequence homology with other proteins. These conserved regions matched the sequence requirements for the USP family (Baker *et al.*, 1992, Papa & Hochstrasser, 1993). For this reason the protein was renamed HAUSP (herpesvirus-associated ubiquitin specific protease).

Furthermore, two potential yeast homologues have been identified, the *Saccharomyces cerevisiae* protein, GenBank accession number SC995X6, (which has 59% similarity and

34% identity) and the *Schizosaccharomyces pombe* protein, GenBank accession number Q09879, (which has 57% similarity and 35% identity). The apparent identification of HAUSP homologues in yeast and mammalian cells suggests that this protein has been conserved throughout eukaryotic evolution, which implies that it has a fundamental role for cell biology.

As part of the requirements of this thesis further sequence analysis of the cDNA sequence has been carried out, in order to identify regions potentially important for protein-protein interactions, and are included in Section 3C2.

1E3 HAUSP is expressed in a wide variety of cells and several alternative spliced isoforms may exist

Results of screening of the NCBI dbest database of expressed sequence tags, with HAUSP encoding cDNA, identified several precisely matched entries with cDNAs which were derived from human brain, liver, placenta, lung and melanocyte cells. This suggested that HAUSP is expressed in a variety of cell types.

The possibility of several alternatively spliced isoforms of HAUSP existing was suggested from northern blotting analysis which revealed the presence of two transcripts. Both transcripts were found in low abundance which suggests that levels of HAUSP expression are tightly controlled. The existence of HAUSP isoforms was supported by immunoprecipitation experiments using anti-HAUSP rabbit serum which reproducibly precipitated, in addition to the major HAUSP protein, a protein of slightly higher molecular weight than the original HAUSP band.

1E4 Definition of Vmw110 sequences required for interaction with HAUSP

In order to identify the region of Vmw110 required for interaction with HAUSP, GST 'pull-down' assays were performed using GST fusion proteins containing selected segments of Vmw110 and their ability to 'pull-down' HAUSP assessed (Meredith *et al.*, 1994). This work demonstrated that a GST fusion protein encoding Vmw110 sequences 594-775 was able to 'pull-down' HAUSP, but a GST fusion protein encoding Vmw110 residues 633-775 was unsuccessful. This implied that the Vmw110 region between 594-633 was required for interaction with HAUSP.

Two further approaches were used to define the region of Vmw110 required for interaction with HAUSP (Meredith *et al.*, 1995). They involved investigating the effect of deleting specific regions of Vmw110 on its ability to coimmunoprecipitate HAUSP from cell lysates. The first approach consisted of using a mutant of HSV-1 in which residues 594-633 of Vmw110 had been deleted. The result of this work showed that the expressed Vmw110 mutant protein, was unable to coimmunoprecipitate HAUSP. The second approach involved using Vmw110 proteins which had been purified from the baculovirus expression system, in which either residues 594-633, 680-720, or those encoding the RING finger, had been deleted. The results of this work supported the previous findings that Vmw110 residues 594-633 are required for interaction with HAUSP.

Furthermore the mutant of Vmw110, in which residues 594-633 had been deleted, was still able to multimerise, which suggests that sequences required for multimerisation and those required for binding to HAUSP are separate.

Interestingly, residues 600-633 of Vmw110 have been conserved in the equivalent protein of HSV-2 (retaining 70% homology). However, this is not the case for the corresponding proteins derived from other herpesviruses. Therefore, Vmw110 residues important for interaction with HAUSP probably have a function specific to HSV.

During the final preparations of this thesis work was published which further defined the region of Vmw110 required to interact with HAUSP (Everett *et al.*, 1999). This study demonstrated that residues essential for binding to HAUSP *in vitro* lie between residue 618 and residue 632. Moreover, substitution of specific residues within this crucial region resulted in the identification of charged residues that were essential for HAUSP binding *in vitro*. Coimmunoprecipitation experiments were also carried out using cell extracts derived from cells infected with mutants of HSV-1 which carried the above mentioned site specific mutations in the Vmw110 encoding region. Results from these experiments supported those observed using the GST 'pull-down' technique. It is also worth noting that the effects of substituting these Vmw110 residues on the role of Vmw110, for transactivation, viral growth, and redistribution of ND10, were similar to those described below when the effects of removing residues 594-633 of Vmw110 were tested.

1E5 Role of the HAUSP interaction domain in HSV-1 infection

1E5.1 Role of the HAUSP interaction domain for the transactivation function of Vmw110

Transfection studies were carried out using a mutant of Vmw110 in which residues required for interaction with HAUSP had been deleted. The results showed that removal of this region caused a significant reduction in the levels of gene expression activation observed in synergy with Vmw175 (Everett, 1988a, Everett *et al.*, 1999).

1E5.2 Role of the HAUSP interaction domain in viral growth

Infection of cells with the HSV-1 mutant, in which the HAUSP interaction domain had been deleted, resulted in a reduction of viral growth, as compared with wild type HSV-1 infection (Everett *et al.*, 1999, Meredith *et al.*, 1995). However, the reduction to viral growth was not as severe as observed with mutants in which either the RING finger domain, or the complete C-terminal region of Vmw110 had been deleted.

1E5.3 Role of HAUSP interaction in the redistribution of ND10 domains

Results from indirect immunofluorescence experiments, using anti-HAUSP rabbit serum, show that HAUSP is predominantly a nuclear protein, present in a minority of ND10 (Everett *et al.*, 1997). Interestingly, infection of cells with HSV-1 leads to an increased association of HAUSP with ND10 domains at early times of infection. This is best observed in cells infected with the HSV-1 RING finger deletion mutant in which the PML containing domains are not disrupted. In these cells Vmw110, HAUSP and PML are seen to colocalise in the ND10 domains. As such, HAUSP is suggested to be a transient member of ND10 for whom interaction with ND10 domains may be dependent on cell status.

To test the significance of the HAUSP interaction domain of Vmw110 for the redistribution of ND10 domains immunofluorescence studies were performed on cells infected with a HSV-1 mutant in which this region had been deleted (Everett *et al.*, 1999, Meredith *et al.*, 1995). Examination of these cells showed that introduction of this mutation to Vmw110 did not alter the ability of Vmw110 to colocalise with ND10 domains. However, in contrast to results using wild type HSV-1, colocalisation of HAUSP

with PML was no greater in infected cells than that seen in uninfected cells. Furthermore, although in most cases infection with this HSV-1 mutant resulted in the complete disruption of ND10 domains, as seen using wild type HSV-1, a significant number of cells were observed in which a few ND10 domains were retained. A further difference which was observed from that of wild type infection was that in some cases the Vmw110 was retained in large cytoplasmic foci at later times of infection. However, results from such experiments varied depending on incubation time, cell type and from cell to cell which means it was hard to compare the relative kinetics of ND10 disruption with a high level of accuracy.

1F Aims of the work presented in this thesis:

Vmw110 is an IE protein of HSV-1 that is thought to be an important factor for both the lytic and latent stages of infection. HAUSP is a novel member of the USP family that was identified to strongly and specifically interact with Vmw110. The studies described in this thesis were initiated to gain a better understanding of the roles of HAUSP within the cell and also for HSV-1 infection.

The cellular role of HAUSP was investigated using three strategies; firstly to investigate the potential deubiquitinase activity of the protein using an *in vivo* bacterial assay; secondly to observe the effect of transient expression of HAUSP in eukaryotic cells and thirdly to search for cellular proteins that interact with HAUSP, using GST fusion proteins encoding residues of HAUSP as bait in GST 'pull-down' assays.

The role of the interaction of HAUSP with Vmw110 for HSV-1 infection was investigated using two further strategies. The first being to define the region of HAUSP required for interaction with Vmw110 using a combination of GST 'pull-down' and immunoprecipitation assays. Mutants of HAUSP from which the critical region had been removed could then be produced and tested in functional assays. Secondly, the effect of Vmw110 binding to HAUSP on its normal cellular activities was investigated.

MATERIALS AND METHODS CHAPTER 2

2A Materials

2A1 Plasmids

The following plasmids were provided by the acknowledged authors:

pGEX2T: This vector is designed for inducible, high-level bacterial expression of proteins or protein fragments as fusions with *Schistosoma japonicum* glutathione S-transferase (GST). Expression of GST fusion proteins is under the control of the IPTG-inducible tac promoter and an internal *lac* I^q gene which ensures that expression is inducible when transfected in certain strains of *E.coli*. It was obtained commercially from Pharmacia.

pGEX2TN3: (Meredith *et al.*, 1994) This was derived from pGEX2T by insertion of a DNA fragment containing *Nco*I-*Eco*RI-*Hind*III restriction sites between the *Bam*HI and *Eco*RI restriction sites downstream of the GST coding sequence, thus removing the original *Bam*HI, *Sma*I and *Eco*RI restriction sites.

pGEX2TNMCR: (Meredith *et al.*, 1994) This was derived from pGEX2TN3 by insertion of the entire pUC19 multiple cloning site between the *Eco*RI and *Hind*III restriction sites.

pGEXE52: (Meredith *et al.*, 1994) This plasmid encodes GST sequences fused to codons 594-775 of Vmw110. The normal translational termination signal for Vmw110 is present.

pGEXE58: (Meredith *et al.*, 1994) This plasmid is similar to pGEXE52 and encodes GST sequences fused to codons 633-775 of Vmw110.

pT7E52: (Meredith *et al.*, 1995) This plasmid expresses Vmw110 codons 594-775 as a non-fusion protein from the T7 promoter, in a plasmid based on pET8C.

pT7E58: (Meredith *et al.*, 1995) This plasmid expresses Vmw110 codons 633-775 as a non-fusion protein from the T7 promoter, in a plasmid based on pET8C.

p111: (Everett, 1987a) This encodes the entire Vmw110 coding region under the control of its natural promoter in a pUC9 vector.

pT7110: (Everett *et al.*, 1991) This plasmid contains a cDNA version of the entire coding sequence of Vmw110 under the control of a T7 promoter.

pT7111: (R.Everett unpublished) This plasmid is a derivative of pT7110 which has been truncated in the 3' region to remove irrelevant sequences. Both pT7111 and pT7110 express full length Vmw110 in *E.coli.*

pGEX4222: (R.Everett unpublished) This plasmid expresses GST sequences fused to residues 26-1102 of HAUSP.

pT7135: (Everett *et al.*, 1997) This plasmid expresses HAUSP under the control of an IPTG-inducible T7 promoter, in a pBR322 Amp^r replicon. It was derived in several stages. The first stage was the linkage of appropriate fragments from overlapping cDNAs. After the introduction of an *Nde*I restriction site at the presumed initiating ATG codon by PCR mutagenesis the complete HAUSP coding sequence was inserted into the T7 expression plasmid pET3a (a pBR322 Amp^r replicon, obtained commercially from Novagen) using the *Nde*I restriction site to place the initiating ATG at the optimal position for expression.

pT7135His: (M.Kathoria unpublished) This plasmid expresses the entire HAUSP coding sequence as a fusion protein with a His tag at the N-terminus under the control of the T7 promoter. It was derived by ligation of the *Nde*I-*Hind*III fragment of pT7135 into the *Nde*I-*Hind*III restriction sites of the pET28a vector (obtained commercially from Novagen).

pET24a135: (R.Everett unpublished) This plasmid expresses the entire HAUSP coding sequence under the control of an IPTG-inducible T7-driven promoter, in a pBR322 Km^r replicon and contains a F1 origin so that infection with helper phage would result in virions containing single-stranded DNA that corresponds to the coding strand. It was derived by ligation of the *Nde*I-*Hind*III fragment of pT7135 into the *Nde*I-*Hind*III restriction sites of the pET24a vector (obtained commercially from Novagen).

pAC-M-β-gal: (Baker *et al.*, 1992) This plasmid expresses the ubiquitin-methionine-β-galactosidase (Ub-M-β-gal) fusion protein under the control of a yeast promoter in a pACYC184 Cm^r replicon and was kindly provided by R.T. Baker.

pAC-R-β-gal: (Baker *et al.*, 1992) This plasmid expresses the ubiquitin-arginine-β-galactosidase (Ub-R-β-gal) fusion protein under the control of a yeast promoter in a pACYC184 Cm^r replicon and was kindly provided by R.T. Baker.

pRB105: (Baker *et al.*, 1992) This plasmid expresses the yeast UBP2 USP enzyme from an IPTG-inducible tac promoter in a pKK-based plasmid (pBR322 Amp^r replicon) and was kindly provided by R.T.Baker.

pRB307: (Baker *et al.*, 1992) This plasmid expresses the Ub-GST fusion protein under the control of an IPTG-inducible tac promoter in a pKK-based plasmid (pBR322 Amp^r replicon) and was kindly provided by R.T.Baker.

pACYCHAUSP: (Everett *et al.*, 1998a) This plasmid expresses HAUSP under the control of a T7 driven IPTG-inducible promoter in a pACYC184 Cm^r replicon.

pACYCUBP2: (Everett *et al.*, 1998a) This plasmid expresses yeast UBP2 under the control of a yeast promoter in a pACYC184 Cm^r replicon.

pJ7Ω: (Morgenstern & Land, 1990) This plasmid enables easy insertion and subsequent expression of exogenous genes in a wide variety of mammalian cells. It comprises a mammalian transcription unit composed of a simian CMV IE94 promoter flanked 3' by a polylinker, an intron and a transcriptional termination signal which is linked to a pBR322 derived backbone.

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 intestine phosphatase, *E.coli* DNA polymerase I Klenow fragment and proteinase K from Boehringer Mannheim. PCR was carried out using Thermus aquaticus DNA polymerase (Taq polymerase) obtained from Boehringer Mannheim. T4 DNA ligase for use in site-directed mutagenesis was purchased from New England Biolabs.

2A3 Synthetic oligonucleotides

Oligonucleotides were either ordered directly from Cruachem or they were synthesised on site using a Biosearch model 8600 DNA synthesiser or a Cruachem PS250 automated synthesiser by Dr. J.McLauchlan, Mr. R.Adams, Mr. R.Reid, Mr. A.Orr, or Mr. D.McNab.

2A4 Bacteria (*E.coli* strains)

The *E.coli* strain DH5α (F'/*end*A1 *hsd*R17 (r_k^-mk+) *sup*E44 *thi*l *rec*A1 *gyr*A (Nal^r) *rel*A1 Δ(*lac*ZYA-*arg*F) U169 (ϕ80*dlac*Δ(*lac*Z)M15) was used for maintenance and propagation of plasmid DNA.

Strain BL21 (DE3) pLysS (F^- *ompT* $r^-_Bm^-_B$) was used for the expression of proteins whose expression is under control of the T7 promoter: 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 Cm^r. Protein expression from pGEX plasmids utilised this BL21 *E.coli* strain.

The *E.coli* strain NovaBlue (DE3) (*end*A1 *hsd*R17($r_{k12}^-m_{k12}^+$) *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac*[F'*pro*A^+B^+ *lacI*^{q}*ZΔM15::Tn10*] (DE3) was used for the standard deubiquitinase assays. *E.coli* bacteria were used for the deubiquitinase assays as they lack the ubiquitin degradation pathway and thus there would be no problems of background deubiquitinase activity. There are several reasons for the suitability of this strain over other *E.coli* strains. Firstly, NovaBlue bacteria produce a truncated endogenous β-gal and as such it is possible to distinguish this from an exogenously expressed full length β-gal construct. Also, NovaBlue (DE3) bacteria carry the integrated lysogenic λ bacteriophage DE3 leading to high-levels of expression from T7-driven plasmids, as well as the *lacI*^{q} repressor which provides tighter control over basal expression of IPTG-inducible promoters than the wild type repressor in other strains.

The CJ236 *E.coli* strain F' *cat* (=pCJ105:M13^SCm^r)/*dut ung*$^{-1}$ *thi-1 relA spoT1 mcrA* was used to aid synthesis of uracil-enriched single-stranded plasmid DNA which is the first stage in the production of site specific mutants. The CJ236 strain encodes a *dut*$^{-1}$ mutation that leads to a deficiency in the dUTPase enzyme (that normally converts dUTP to dUMP). This causes a build up of the dUTP intracellular pool, leading to the increased incorporation of dUTP in place of dTTP in DNA. The *ung*$^{-1}$ mutation in the CJ236 strain also enhances the uracil enrichment of DNA. This mutation leads to a deficiency of the wild type uracil-N-glycosylase enzyme that would normally remove uracil residues which have been incorrectly incorporated in DNA.

2A5 Bacterial culture media

The DH5α strain was grown in Lauria-Bertani medium (LB) (10g NaCl, 10g Bactopeptone, 5g yeast extract in 1L water, pH7.5) 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 LB. Where necessary, media and agar plates were supplemented with antibiotics: 70μg/ml Amp for bacteria harbouring plasmids with Amp^r, 25μg/ml Cm for strains harbouring the pLysS plasmid and 30μg/ml Km where the bacteria harboured plasmids with Km^r.

2A6 Viruses

Wild type herpes simplex virus strain 17 Syn^+ was used for the production of wild type Vmw110 (Brown *et al.*, 1973). The D12 HSV-1 virus was used for the production of a Vmw110 mutant in which residues essential for interaction with HAUSP were not present (Meredith *et al.*, 1995).

CJ236 R408 helper phage (obtained commercially from New England Biolabs) was used during the site-directed mutagenesis of plasmid DNA for the preparation of single-stranded plasmid DNA. The CJ236 R408 helper phage was used to infect CJ236 bacteria into which plasmid DNA had been transformed. This helper phage provided the necessary replicative enzymes and phage coat proteins to enable replication and encapsidation of single-stranded DNA from the F1 origin site, into phage particles which accumulated in the culture supernatant.

2A7 Cells and tissue culture media

All cell culture media were obtained from Gibco.

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% new born calf serum, 2.5% foetal calf serum (FCS) and 100units/ml penicillin and 100μg/ml streptomycin.

Hep2 cells (obtained from the cytology department of the Institute of Virology), an epithelial cell line, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 100units/ml penicillin and 100μg/ml streptomycin.

2A8 Antisera and monoclonal antibodies

The anti-Vmw110 rabbit polyclonal antiserum r95 was obtained from Dr. R.Everett. It was prepared after immunisation with a bacterially expressed protein which encompasses Vmw110 residues 105-211 (Everett *et al.*, 1993b).

The anti-Vmw110 mouse monoclonal antibodies (MAbs) 11060 and 10503 were obtained from Dr. A.Cross. They were raised against the purified baculovirus-expressed HSV-1 Vmw110 polypeptide (Everett *et al.*, 1993b). The MAb 11060 recognises an epitope between residues 20-105, whereas the MAb 10503 recognises an epitope between residues 633-775.

The anti-HAUSP rabbit polyclonal antisera r201 and r206 were obtained from Dr. A.Cross. They were generated by immunisation of rabbits with branched chain peptides, r206 was raised against a peptide including residues 26-41 of HAUSP (R.Everett unpublished) and r201 was raised against a branched chain peptide including residues 1087-1102 of HAUSP (Everett *et al.*, 1997).

The anti-HAUSP MAb antiserum 16613 was obtained from Dr. A.Cross. It was raised against a purified GST fusion protein which encodes HAUSP residues 1-193 (R.Everett unpublished).

The anti-pp65 MAb was purchased commercially from Capricorn Products Inc.

The anti-PML MAb 5E10 was kindly provided by Professor Roel Van Driel (Amsterdam) (Stuurman *et al.*, 1992).

The anti-PML rabbit polyclonal antiserum r8 was kindly provided by Dr. Paul Freemont, ICRF (Imperial Cancer Research Fund) London (Boddy *et al.*, 1996).

The anti-β-gal rabbit polyclonal antiserum r12741 was kindly provided by H.Marsden (MRC Virology Institute).

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

2A9 Radiochemicals

Radiochemicals were purchased from Amersham at the following specific activities:

α^{35}S dATP 1000-1500Ci/mmol (12.5μCi/μl)

^{35}S L-methionine 800Ci/mmol (10μCi/μl)

2A10 Solutions

Recipes of general solutions have been listed below, the details of all other solutions have been included either in the text or at the end of the relevant section.

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

10X Loading buffer for agarose gels and non-denaturing polyacrylamide gels: 1X TBE, 1% SDS, 50% glycerol, 1mg/ml bromophenol blue

PBS(A): 170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH7.2

PBS-complete: PBS(A) plus $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$ at 1g/L

STET: 8% sucrose, 5% Triton-X100, 50mM EDTA (pH8), 50mM Tris.HCl (pH8)

20X TBE : 2.5M Tris, 0.8M boric acid, 54mM EDTA

TE: 10mM Tris.HCl (pH7.5), 1mM EDTA pH8

2A11 Chemicals and reagents

All chemicals and reagents were purchased from BDH Chemicals UK or Sigma Chemical Company, unless otherwise stated below or in relevant sections:

Amersham Life Science: rainbow markers

Beecham Research: ampicillin

Bio-Rad: ammonium persulphate (APS), gelatin, N,N,N′,N′-tetramethylethylenediamine (TEMED), coomassie brilliant blue, gelatin

Boehringer Mannheim: protease inhibitors, λDNA, DOTAP liposomal transfection reagent

Boston Biochem Inc: β-lactone-lactacystin

CalBiochem: MG132, lactone-lactacystin

Difco: agar, bactotryptone, yeast extract

Fisons: ammonium hydroxide, acetone

Fluka: formamide, formaldehyde

Gibco BRL: IPTG

Marvel: dried skimmed milk

Melford Laboratories Ltd: caesium chloride

National Diagnostics: 30% acrylamide (2.5% cross-linker)

Pharmacia: rATP, dNTPs, ddNTPs

Prolabo: boric acid, butanol, chloroform, ethanol, glacial acetic acid, glycerol, hydrochloric acid, isopropanol methanol

Scotlab: acrylamide: N, N′-methylene-bis-acrylamide 19:1

UKC Chemical Laboratories: Citifluor

2B Methods

2B1 Nucleic acid manipulation and cloning procedures

2B1.1 Restriction enzyme digestion of DNA

Restriction enzyme digestion of DNA was carried out at 37°C (or the temperature specified by the supplier), for 2hr in 20µl reaction volumes of 1X buffer (supplied with the enzyme), using 1 unit of enzyme per 0.5µg DNA to be digested per hour. The buffer system used most frequently was the Boehringer Mannheim A, B, H, L, M system and at all other times New England Biolabs NEBuffer 1, 2, 3, 4 system was used. Usually, 0.5µg DNA was digested for diagnostic analysis and 5-20µg for isolation of specific restriction fragments. Samples (5µl) of plasmid DNA prepared by the miniprep method (see Section 2B1.7) were digested in the presence of 50µg/ml RNase A.

2B1.2 End repair

To enable efficient ligation it was often necessary to modify the ends of the digested DNA fragments. Protocols describing how such modifications were made have been detailed below.

On completion of the end repair procedure the modified DNA was purified by a method involving several stages. The first stage was the heat inactivation of enzymes for 10min at 70 ° C. A phenol extraction was then performed on the sample. This involved the addition of an equal volume of TE-saturated phenol to the sample followed by vortexing for 10sec. The aqueous and phenol phases were then separated by centrifugation at 13,000rpm for 2min at RT. The upper aqueous phase containing the DNA was then transferred to a fresh tube. A chloroform extraction was then performed on the sample in a similar manner to the phenol extraction except that chloroform was used in the place of phenol. The DNA was then precipitated using the ethanol precipitation method. This involved the addition of 2.5 volumes of 100% ethanol and 1/20th the volume of NaCl to the sample which was then left at -20 ° C for 30min. The DNA was pelleted by centrifugation at 13,000rpm for 5min at 4 ° C and the DNA pellets were washed in 80% ethanol and resuspended in water before use in ligation reactions or storage at -20 ° C.

2B1.2a Blunt ending of DNA fragments

Fragments generated by enzymes which produce 5' overhangs sometimes required modification (i.e. blunt ended) to enable ligation. DNA was blunt ended by addition of dNTPs (at 50μM per dNTP) and 2 units of DNA polymerase I large Klenow fragment to the digestion mixture. This reaction was then left for 20-30min at 37°C.

2B1.2b 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 often necessary to prevent re-circularisation of the vector fragment upon ligation. This was done by incubation of the digestion mixture with 1 unit of calf intestinal alkaline phosphatase in the appropriate buffer for 20-30min at 37°C.

2B1.3 Purification of synthetic oligonucleotides

Synthetic oligonucleotides were produced by the phosphoramidite method (Section 2A3) using 200μM 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), and the solution pushed through the column in 200μl aliquots with 20min incubation between each fresh addition. After incubation with the final aliquot, the 1.5ml solution was pushed back and forth 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 lysophilisation, the oligonucleotide was dissolved in 200μl water. An equal volume of formamide plus 10μl formamide dyes were added before boiling for 5min prior to electrophoresis on a 12% denaturing polyacrylamide gel (Section 2B1.4d).

Oligonucleotides were purified by passive elution from gel slices excised from the gel following visualisation of the DNA from U.V. (ultra violet) transillumination; the oligonucleotides could be visualised at 254nm as dark shadows against a fluorescing TLC (thin layer chromatography) plate. Gel slices were crushed and the DNA eluted into 400μl 1X TE 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 (13,000rpm). The pellet was resuspended in 100µl of water, ethanol precipitated again and the final DNA pellet was washed in 80% ethanol, lysophilised and resuspended in water. The DNA concentration was determined by measuring the absorbance at 260nm on a Beckman DU-62 spectrophotometer, assuming that for short oligonucleotides (less than 30 nucleotides) 1OD A_{260} = 20µg/ml.

2B1.4 Electrophoretic separation and purification of DNA fragments

2B1.4a Non-denaturing agarose gels

DNA fragments produced by restriction enzyme digestion or PCR were resolved by non-denaturing 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. Appropriate size markers were always run on the gels: *Hpa*II digested pBR322 gave fragments in the range 27-622bp, while λ DNA digested with *Hind*III gave fragments of 0.55-23.1kb. For separation of fragments over 100bp, 0.5-2% agarose gels made up in 1X TBE were used and run 12V/cm in 1X TBE buffer. Following electrophoresis, the gels were stained in 1µg/ml EtBr (ethidium bromide) solution followed by rinsing the gel three times with water. DNA was then visualised under U.V. light (normally short wave, but long wave was used for preparative gels). Photography was carried out using The Imager (Appligene).

2B1.4b DNA purification from agarose gels

Agarose blocks containing appropriate DNA fragments were excised from gels under long-wave U.V. using a U.V. Products Inc. transilluminator and the DNA recovered using a commercial kit, GENECLEANII (BIO101)1 Inc., La Jolla, CA. The kit contains a silica matrix which binds DNA in the presence of high concentrations of sodium iodide (Vogelstein & Gillespie, 1979).

The 1X TBE gel slice was weighed to estimate its volume and cut into approximately 2mm cubes to facilitate gel dissolution in 4.5 volumes NaI solution and 0.5 volumes TBE modifier. This sample was incubated at 55 °C until the gel slice was 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 twice in 500µl 'NEW' wash and allowed to air dry for 5min at RT. The DNA was then eluted

into 10µl water by mixing the washed pellet with water and incubating the sample at 55 ° C for 5min. The matrix was pelleted and the supernatant containing the DNA transferred to a fresh tube. A second elution of DNA from the silica matrix was then carried out and the 10µl supernatant mixed with the first elution and stored at -20 ° C.

2B1.4c Non-denaturing polyacrylamide gels

5-8% Vertical non-denaturing polyacrylamide (acrylamide:N,N'-methylene-bisacrylamide 19:1) gels containing 1X TBE, were used to resolve DNA fragments between 50-200bp. Polymerisation was initiated by adding 0.01volumes 10% APS and 0.001volumes TEMED. Samples were loaded following addition of the relevant amount of gel loading buffer and run in 1X TBE in a kit manufactured in house at up to 16V/cm. DNA fragments were visualised using the EtBr staining method described in Section 2B1.4a.

2B1.4d Denaturing polyacrylamide gels

Vertical denaturing polyacrylamide (acrylamide:N,N'-methylene-bis-acrylamide 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 initiated by adding 0.01volumes 10% APS and 0.001volumes TEMED.

DNA sequencing gels (0.35mm thick and 0.35cm long) contained 6% or 8% acrylamide. The samples were boiled for 2min with formamide dyes prior to loading and run at 40W in 1X TBE for varying lengths of time depending upon the sequence to be visualised. Gels were then vacuum dried on a Bio-Rad S83 gel dryer and exposed to Kodak X-OMAT S film for 1-7 days, before developing the film in a Kodak X-OMAT ME-3 processor.

Gels for purifying oligonucleotides contained 12% acrylamide (1.5mm thick X 25cm long). The samples boiled in formamide dyes for 2min prior to loading and run at 250V in 1X TBE, in a kit manufactured in house, until the bromophenol blue was approximately ¾ way down the gel.

2B1.5 DNA ligation

Vector and insert DNA fragments with appropriate compatible termini (purified by the GENECLEANII kit) were ligated in a 1:2-10 ratio in a 20µl reaction volume of 1X Ligase buffer, 1mM rATP and 2 units of T4 DNA ligase overnight at RT and if necessary could be

stored at -20 °C. A 6µl aliquot of the ligation mix was then transformed into competent *E.coli* (Section 2B1.6).

Solutions for DNA ligation :

5X Ligase buffer: 250mM Tris.HCl (pH7.6), 50mM $MgCl_2$, 5mM DTT, 5mM ATP, 25% PEG (Poly ethylene glycol) 8000

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. DH5α bacteria were streaked onto a nutrient agar plate and a fresh colony used to set up a 1ml overnight culture (grown in LB). The overnight culture was inoculated into 100ml LB and grown at 37 °C until the OD_{450nm} was about 0.3. The culture was cooled on ice and centrifuged at 2,600 rpm at 4 °C for 10min. The pellet was gently resuspended in 5ml total 0.1M $MgCl_2$ (cooled to 4 °C). The sample was then spun again at 2,600rpm for 5min at 4 °C and the pellet resuspended in 5ml total of 0.1M $CaCl_2$ (cooled to 4 °C). This sample was then left on ice for 45min and then spun at 2,600rpm for 5min at 4 °C. The pellet was then resuspended in 5ml 0.1M MOPS (3-[N-Monopholino propane sulphonic acid]) pH6.5. Aliquots of 100µl, 200µl and 300µl amounts were frozen quickly in liquid nitrogen and then stored at -70 °C.

An aliquot of 10ng of plasmid DNA or 6µl of a ligation reaction were transformed into a 100µl aliquot of thawed competent DH5α bacteria and left to equilibrate for 30min on ice. The sample was then heat shocked at 37 °C for 1min and 500µl 2YT added and left for 45-60min shaking at 37 °C. A 300µl aliquot of the transformation sample was then plated out onto LB agar plates containing the appropriate antibiotics. The plates were incubated overnight at 37 °C.

2B1.7 Miniprep plasmid DNA preparation

The boiling miniprep method was used to produce plasmid DNA on a small scale. Fresh single colonies were picked and inoculated into 2.5ml of 2YT, and the cultures grown overnight at 37 °C in a shaking incubator. Aliquots of 1.5ml of the cultures were centrifuged for 20sec in a microfuge (13,000rpm) and the pellet resuspended in 200µl STET. The bacterial cells were then lysed by addition of 5µl lysozyme (fresh 10mg/ml

solution in STET) and samples boiled for 50sec. Cell debris were pelleted by centrifugation for 10min (13,000rpm) and the supernatant was removed and made up to 150µl with STET. Plasmid DNA was precipitated by the addition of 135µl isopropanol (0.9 volumes) and pelleted by centrifugation for 5min. The pellets were then washed with 80% ethanol, air dried for 5min at RT and resuspended in 20µl water and stored at -20 ° C.

2B1.8 Large scale plasmid DNA preparation

E.coli containing the desired plasmid were streaked (from an older agar plate) or spread (from an aliquot of newly transformed competent cells, Section 2B1.6) onto a LB agar plate containing the appropriate antibiotics (Section 2A5) and then incubated at 37°C overnight.

2B1.8a 'Maxi-boiling' method of large scale DNA preparation: for use in transfections

Plasmid DNA was prepared from bacteria according to the 'maxi-boiling' method of Holmes & Quigley, 1981 and the supercoiled DNA purified on a caesium chloride gradient. A single colony was picked from a fresh plate on which the plasmid transformed bacterial cells were grown and used to inoculate 350ml LB containing antibiotics as appropriate, which was incubated in a shaking incubator at 37°C overnight. Bacterial cultures were centrifuged at 5,000rpm for 10min at RT in a Sorvall GS3 rotor, and the pellets resuspended in 20ml STET plus 2.5ml freshly prepared 10mg/ml lysozyme in STET. The mixture was brought to the boil over a bunsen flame and placed in a boiling water bath for 45sec, before pelleting cell debris by centrifugation in a Sorvall SS34 rotor at 18,000rpm for 50min at RT. Nucleic acids were precipitated from the supernatant by the addition of 0.9 volumes of isopropanol, and pelleted by centrifugation in a Beckman GPR centrifuge at 3,000rpm for 5min at RT. The pellet was resuspended in 5.5ml 1X TE (to give a volume Xml), then X+0.7g caesium chloride and 0.2ml 10mg/ml EtBr were added. The samples were incubated on ice for 20min prior to centrifugation at 3,000rpm for 10min at 4°C. The supernatant was transferred to a Dupont 03945 crimp-seal centrifuge tube using a syringe and then centrifuged at 45,000rpm, 15°C for 16-18hr in a Sorvall TV865 vertical rotor. The lower band (which contains supercoiled plasmid DNA) was removed using a syringe, taking care to avoid contamination with chromosomal DNA from the upper band. The EtBr was removed from the plasmid solution by 2-3 extractions with TE saturated butan-1-ol, and the caesium chloride was removed by dialysing against 1X TE at RT for 2hr. Following this, the solution was treated with 100µg/ml RNase A at 65°C for

1hr, then 100µg/ml proteinase K plus 0.1% SDS at 37°C for 1hr. The plasmid DNA was then cleaned by phenol:chloroform extraction, and concentrated by ethanol precipitation. The DNA was then pelleted by centrifugation at 3,000rpm for 15min at RT and resuspended in 400µl 1X TE and then ethanol precipitated again. The final pellet was washed in 80% ethanol and resuspended in an appropriate volume of 1X TE before storage at -20°C. The plasmid concentration was determined by measuring the absorbance at 260nm on a Beckman DU-62 spectrophotometer, assuming 1OD A_{260} = 50µg/ml.

2B1.8b Alternative-alkaline lysis method of large scale plasmid DNA preparation

A single colony was picked from a fresh plate on which the plasmid transformed bacterial cells were grown and used to inoculate 100ml LB (containing antibiotics as appropriate). This sample was incubated in a shaking incubator at 37°C overnight. Bacterial cultures were centrifuged at 5,000rpm for 10min at RT in a Sorvall GS3 rotor, and the pellets resuspended in 1ml Glucose/Tris/EDTA solution (50mM Glucose, 25mM Tris, 10mM EDTA) and transferred to a 10ml snap cap tube. The cells were then lysed by addition of 250µl lysozyme (25µg/ml prepared in Glucose/Tris/EDTA solution) and mixed by inversion. This sample was left to incubate for 10min at RT. A freshly prepared aliquot (2.5ml) of 0.2M NaOH/1% SDS was then added, mixed by inversion until the solution became homogenous and cleared, and left for 10min at 4°C. The cell debris was precipitated by addition of 1.9ml of 3M potassium acetate solution and mixed by inversion until the viscosity was reduced and a large precipitate formed. The sample was then left at 4°C for 10min. The bacterial chromosomal DNA and proteins were then pelleted by centrifugation at 9,500rpm in a Sorvall SS-34 rotor at 4°C. The supernatant was then decanted to a fresh centrifuge tube. The plasmid DNA was then precipitated by addition of 0.6 volumes of isopropanol, mixed by inversion and left for 10min at RT. The plasmid DNA was pelleted by centrifugation at 9,500rpm for 10min at RT. The supernatant was then discarded and the pellet washed with 80% ethanol. The DNA was then dissolved in 400µl water and transferred to a fresh eppendorf tube. Contamination by RNA was treated by addition of 1µl RNaseA (10mg/ml) and left at 60°C for 30min. Protein contamination was then treated by extraction first with an equal volume of TE-saturated phenol and followed by an extraction with an equal volume of chloroform and the DNA concentrated by ethanol precipitation. The DNA pellet was then washed with 80% ethanol and resuspended in 200µl water and stored at -20°C.

2B1.9 Sequencing of DNA

DNA was sequenced either using the didexoy nucleotide chain termination method as described below, or by an on site ABI Prism 377 automated DNA Sequencer which was operated by Lesley Taylor from the Institute of Virology.

Dideoxy sequencing of DNA:

Plasmid DNA was sequenced by the dideoxy nucleotide chain termination method as described by Sanger *et al.*, 1977, details of the buffers used for this procedure have been provided after the protocol description. Purified plasmid DNA (about 2µg), or 10µl miniprep DNA, was denatured at RT for 10min in a 20µl reaction volume containing 0.4M sodium hydroxide. The denatured DNA was then precipitated by the addition of 6µl 3M sodium acetate pH4.5, 14µl water and 120µl ethanol, and left on ice for 10min. The DNA was then pelleted by centrifugation in a microfuge at 13,000rpm, for 10min at RT. The pellet was then washed with 80% ethanol and resuspended in 8µl water. The primer was annealed to the DNA template at 37°C for 20min in a 10µl reaction volume consisting of 1X Sequencing buffer and 5pmol of primer (sequences of primers used for the purposes of this thesis have been listed in Table 2B1.9).

Meanwhile, the labelling mix was prepared which, for four sequencing reactions, consisted of 1.5µl ^{35}S dATP and 4.5µl 11.8µM dATP. Using this labelling mixture, reaction mixes were made for each nucleotide which for four sequencing reactions consisted of 9µl of the appropriate Nseq solution and 1.5µl of labelling mix. An aliquot (2µl) of each reaction mix was dispensed into one of four round bottomed wells labelled T, G, C and A on a 96 well microtitre plate (Nunc).

After the annealing reaction was completed, 2units of *E.coli* DNA polymerase I large Klenow fragment were added to the annealed template. Aliquots (2µl) of these DNA samples were then dispensed into the 4 wells containing the T, G, C and A nucleotide reaction mixes. The plate was then 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 and incubating the samples at 37°C for 30min. The reactions were stopped by the addition of 2µl formamide dyes and could be stored at -20°C. The samples were boiled for 2min prior to loading on a denaturing polyacrylamide gel (Section 2B1.4d).

Solutions used for dideoxy sequencing of DNA :

i- Dideoxy sequencing buffers:
d-N-0 solutions
dT-0: 1µl 5mM dTTP, 20µl 5mM dCTP, 20µl 5mM 7-deaza dGTP, 50µl 10X TE, 370µl water
dC-0: 20µl 5mM dTTP, 1µl 5mM dCTP, 20µl 5mM 7-deaza dGTP, 50µl 10X TE, 370µl water
dG-0: 20µl 5mM dTTP, 20µl 5mM dCTP, 1µl 5mM 7-deaza dGTP, 50µl 10X TE, 370µl water
dA-0: 20µl 5mM dTTP, 20µl 5mM dCTP, 20µl 5mM 7-deaza dGTP, 50µl 10X TE, 540µl water
ii- 10X TE: 100mM Tris.HCl pH8.0, 1mM EDTA
iii- Sequencing mixes (Nseq solutions):
T sequencing mix: 500µl dT-0 solution, 500µl 600µM ddTTP
C sequencing mix: 500µl dC-0 solution, 105µl 140µM ddCTP, 395µl water
G sequencing mix: 500µl dG-0 solution, 155µl 200µM ddGTP, 345µl water
A sequencing mix: 500µl dA-0 solution, 250µl 140µM ddATP, 250µl water
iv- 10X Sequencing buffer: 100mM Tris.HCl, 100mM $MgCl_2$, pH8
v- Chase mix: All four dNTPs, each at 0.25mM

Sequencing Primer	Oligonucleotide sequence
1664	CATTTGTTCTTCCATAAAG
1782	GGTGTGAAATTCCTAACATTG
2141	CCATGTCCTCGGGCTCGCTC
424NTRUNC	ATCTCCACTATGAACCAG
GEX2T	GCAGGGCTGGCAAGCCAC
H+8	CTGAGTGAAGTTTTACA
KEKE	CTGAACAAACTCAGCAAGCG
Xba STOP	AATTAATCTAGATTAATT

Table 2B1.9: Oligonucleotide sequences of sequencing primers

2B1.10 Site-directed mutagenesis:

Site-directed mutagenesis was conducted by a method based on the use of uracil-modified DNA (Kunkel, 1985, Kunkel *et al.*, 1987), the buffers used for this procedure have been described after the protocol description. This method involves preparing a template of single-stranded plasmid DNA (for the purposes of this thesis pET24a135 was used) in which a proportion of the thymidine bases have been replaced by uracil. A mutagenic primer is then annealed to this template and the complementary strand synthesised, (however this strand contains thymidine in place of uracil). This double stranded template is then transformed into competent *E.coli* cells in which the uracil-enriched single-stranded template is degraded and replaced by a thymidine-enriched strand, using the mutant strand

as a template. In this manner a double stranded plasmid could be synthesised in which a site-specific mutation had been incorporated.

2B1.10a Preparation of uracil-enriched single-stranded DNA

An overnight culture of CJ236 bacteria was set up using 10µl of frozen stock in 8ml of 2YT (in the presence of Cm, Km and 100µg/ml uridine) and left shaking at 37°C. An aliquot of 4ml of the overnight culture was used to inoculate 200ml 2YT containing 100µg/ml uridine in 2 litre baffle flask and left to grow for 30min at 37°C. Helper phage CJ236 R408 (1.2 $X10^{10}$ pfu/ml) was added and the sample incubated for a further 9.5hrs. The culture was centrifuged at 9,000rpm for 10min in a GSA rotor. The supernatant was transferred to a fresh centrifuge bottle and recentrifuged at 9,000rpm for 10min. The supernatant was transferred to a sterile GSA bottle and if necessary could be stored at 4°C. The phage was precipitated by addition of 0.25 volumes of 20% PEG 6000/2.5M NaCl mixed by inversion and left at 4°C for 30min. The phage was pelleted by centrifugation at 12,000rpm for 15min in a GSA rotor. The supernatant was decanted and 5ml of this used to resuspend the phage pellet (which often is smeared down the side of the bottle). The resuspended phage was transferred to a 30ml Oakridge tube and centrifuged at 7,000rpm for 15min in a SS34 rotor. All the supernatant was removed and the pellet resuspended in 2ml of 1X TE. A phenol extraction was performed on the sample using an equal volume of TE-saturated phenol and mixed end-over-end for 1hr interspersed by 3 periods of vigorous vortexing. The sample was then centrifuged at 2,600rpm for 5min at RT to separate the phases and the upper aqueous phase was transferred to a fresh tube. To the tube containing the phenol and interphase 1ml of 1X TE was added, this sample was vortexed and centrifuged at 2,600rpm for 5min and the aqueous phase removed and pooled with the first extraction. This sample was then extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1) and then once with an equal volume of chloroform/isoamyl alcohol (24:1). The DNA was precipitated by the addition of 80µl/ml 4M NaCl/50mM EDTA and 2.5 volumes ethanol, mix by inversion and incubation at -20°C for a minimum of 2hr. The DNA was pelleted by centrifugation at 7,000rpm for 20min in a GSA rotor. The supernatant was removed and the sample recentrifuged for 20sec to remove any remaining supernatant. The single-stranded DNA was dissolved in 0.3ml water, ensuring complete dissolution of DNA by rolling the water around the sides of the tube and transfering the sample to a fresh eppendorf tube. The DNA was reprecipitated by addition of 24µl 4M NaCl/50mM EDTA and 750µl ethanol. The sample

was inverted and incubated at -20°C for 2hr minimum. The DNA was pelleted by centrifugation at 13,000rpm for 10min. The supernatant was removed and the sample recentrifuged for 20sec to remove any remaining supernatant. The purified single-stranded DNA was then dissolved in 100µl water, ensuring complete dissolution by rolling the water around the sides of the eppendorf tube. The DNA concentration was determined by measuring the absorbance at 260nm on a Beckman DU-62 spectrophotometer, assuming that for single-stranded DNA 1OD A_{260} = 33.3µg/ml.

2B10.b Design and production of a mutagenic primer

The second stage of this procedure was the design and production of an oligonucleotide (the mutagenic primer). The oligonucleotides were designed to encode the appropriate mutation required and the sequence surrounding the mutation. The primers were also designed to incorporate a silent base change that would result in either the introduction or removal of a restriction site. This result allowed rapid screening of mutants by restriction digests. The primers were then synthesised and purified as in Sections 2A3 and 2B1.3. Mutagenic primers that were synthesised for the purposes of this thesis have been listed in Table 2B1.10.

2B1.10c Addition of a phosphate to the 5' termini of a mutagenic primer

It was necessary to add a phosphate group to the 5' ends of the mutagenic primers to facilitate the subsequent synthesis of a complementary strand of DNA. The reactions were carried out in 30µl of 1X Kinasing buffer containing 100ng of mutagenic primer, 1mM rATP and using 5 units of T4 polynucleotide kinase. The sample was then left for 45min at 37°C. To adjust the oligonucleotide to a 1ng/µl concentration, 70µl water was added and the reaction stopped by heat inactivation at 70°C for 10min and stored at -20°C.

2B1.10d Annealing of the mutagenic primer to the uracil-enriched single-stranded DNA and synthesis of the complementary strand

The annealing reaction was carried out in 10µl of 1X Annealing buffer containing 200ng uracil-enriched single-stranded DNA and 2ng of the 5' phosphorylated mutagenic primer. The sample was placed at 65°C for 3min to remove any secondary structures that would inhibit the annealing and then cooled slowly to RT by placing the tube in a vessel containing the 65°C heated water and allowing the water to cool naturally to RT. The

sample was centrifuged for 5sec at 13,000rpm to spin down any liquid which had condensed on the sides of the tube.

The synthesis of the complementary strand was carried out by addition of 1µl 10X Synthesis buffer, 6 units of T4 DNA ligase and 1 unit of T7 polymerase to the annealed template/primer. This sample was incubated on ice for 5min, then at 25°C for 5min and finally at 37°C for 90min. The synthesis reaction was stopped by addition of 10µl Stop solution and an aliquot of the DNA (5µl) transformed into competent DH5α *E.coli* bacteria (Section 2B1.6). The fresh bacterial colonies grown from these transformations were used to prepare plasmid DNA via the miniprep method (Section 2B1.7). The DNA was then checked by restriction analysis (Section 2B1.1) and/or sequencing (Section 2B1.9) to ensure correct incorporation of the mutation.

Site-directed mutagenesis buffers:

i- 5X Kinase buffer: 350mM Tris.HCl (pH7.5), 50mM $MgCl_2$, 25mM DTT
ii- 10X Annealing buffer: 200mM Tris pH7.4, 20mM $MgCl_2$, 500mM NaCl
iii- 10X Synthesis buffer: 4mM dNTP, 175mM Tris.HCl (pH7.4), 37.5mM $MgCl_2$, 15mM DTT
iv- Stop solution: 10mM Tris, 10mM EDTA pH8

Mutant	Mutagenic primer sequence (reverse and complementary)
C223S	AAG|AAT|CAG|GGC|GCC|ACT|AGT|TAC|ATG|AAC K N Q G A T **S** Y M N
H456L	GTC|CTG|GTT|C**TT**|**TCC**|GGA|GAT|AAT V L V **L** S G D N
H464L	CAT|GGT|GGA|C**TT**|TAC|GTA|GTT|TAT|CTA H G G **L** Y V V Y L
E/E546/547P/P	TTG|GTG|GAG|CGC|TTA|CAA|**CCA**|**CCG**|AAA|AGG|ATC|GAG L V E R L Q **P** **P** K R I E
K/E556/557P/P	GCT|CAG|AAG|CGG|**CCG**|**CCG**|CGG|CAG|GAA|GCC A Q K R **P** **P** R Q E A
H562L	CGG|CAG|GAA|GC**G**|**CT**T|CTC|TAT|ATG|CAA R Q E A **L** L Y M Q

Table 2B1.10: DNA and translated codon sequences of mutagenic primers. Mutated bases and codons have been highlighted in bold. The introduction of a restriction site is indicated by underlining of the relevant sequence.

2B2 Deubiquitinase assays

The assay involved the use of a model USP substrate Ub-X-β-gal, in which ubiquitin was N-terminally linked to X-β-galactosidase (X being either methionine or arginine). Competent *E.coli* strain NovaBlue (DE3) cells were prepared using the method outlined in

Section 2B4.2 and were transfected with either the pAC-M-β-gal or pAC-R-β-gal plasmid (Section 2A1) and grown on LB agar plates containing Cm. Colonies from these plates were picked and used to make competent cells (Section 2B4.2). These cells were then transfected with either a T7-driven IPTG-inducible HAUSP expression plasmid or pRB105 and grown on LB agar plates containing the appropriate antibiotics. Single colonies were then picked from fresh plates of NovaBlue (DE3) parent, NovaBlue (DE3)/pAC-X-β-gal, NovaBlue (DE3)/pAC-X-β-gal/pT7-HAUSP, NovaBlue (DE3)/pAC-X-β-gal/pRB105 and used to inoculate 10ml 2YT. These cells were grown in a shaking incubator for 2-3hrs at 37°C until the OD_{450nm} was about 0.3. Two aliquots (4ml) of each culture were taken, one aliquot left uninduced whilst the other was induced with 4µl 100mM IPTG. The cultures were left to grow for a further 3hr at 37°C, then bacterial cell extracts were harvested by centrifugation of the cultures at 2,600rpm for 15min at 4°C and pellets resuspended in 800µl PBS(A). Aliquots (5µl) of these total protein extracts were run on 6% SDS-PAGE gels (Section 2B4.5) and analysed by western blotting (Section 2B4.7) using rabbit polyclonal antibody r12741 at 1/10,000 dilution.

2B3 Tissue culture

2B3.1 Growth of cells

Mammalian derived cells were passaged in sterile, $175cm^3$ plastic flasks (Nunc) in the appropriate media (Section 2A7), and incubated at 37°C in a humidified incubator under 5% CO_2. Confluent monolayers were harvested by washing first with versene (0.6mM EDTA in PBS(A), 0.002% phenol red) and then trypsin/versene (1:2) (supplied by the Institute of Virology Media Services) and then resuspended in 10ml of the appropriate media. For continual passage, Hep2 and WS HeLa cells were split in a 1:10 ratio every 3-4 days.

2B3.2 Liposomal mediated transfection for immunofluorescence experiments

Cells were seeded on 13mm glass coverslips in 24 well Linbro plates (Nunc) at 0.5 $X10^5$ cells per well in 1ml of medium and incubated overnight at 37°C prior to transfection. Plasmid DNA (1µg) was made up to a 10µl volume in HEPES buffer (20mM HEPES, cell culture grade pH7.4, sterile filtered) in a 15ml sterile reaction tube. In a separate sterile reaction tube DOTAP (6µl/transfection) was diluted to a final volume of 20µl/transfection

with HEPES buffer. The DOTAP/HEPES mixture (20µl/transfection) was mixed with the DNA/HEPES sample by a gentle pipetting the sample several times. The sample was incubated at RT for 10-15min, then 1ml of the appropriate medium was added and gently mixed. The culture medium was removed from the appropriate cells and replaced by the culture medium containing the DNA/DOTAP mixture and the cells incubated at 37°C for varying amounts of time (24-48hr) before immunofluorescence was performed. In some cases the DNA/DOTAP/medium mix was replaced after 10hr with fresh medium and incubation at 37°C continued for 14hr after which immunofluorescence was performed.

2B3.3 Indirect immunofluorescence

Transfected cell monolayers (Section 2B3.2) were washed twice with PBS(A) and fixed for 10min at RT with 2% sucrose, 5% formaldehyde in PBS(A). The cells were washed 3 times with PBS(A), then left for 10min at RT with 0.5% NP40, 10% sucrose in PBS(A). The cells were again washed 3 times with PBS(A) supplemented with 1% FCS. Aliquots (20µl) of the appropriate primary antibodies diluted in PBS(A)1% FCS were added to each coverslip for 45-60min. Anti-pp65 MAb was used at 1/300, anti-Vmw110 MAb 11060 was used at 1/2000, anti-PML MAb 5E10 was used at 1/20, anti-PML r8 sera was used at 1/1000, and the anti-HAUSP r201 and r206 sera were used at 1/200. After incubation with the primary antibody the cells were washed 6 times in PBS(A)1% FCS. The coverslips were then incubated with 20µl aliquots of the appropriate secondary antibodies diluted in PBS(A)1% FCS and left at RT for 30min in the dark. Goat anti-mouse FITC-labelled and goat anti-rabbit TRITC-labelled secondary antibodies (Sigma Immunochemicals) were used at 1/100. After the cells were washed 6 times with PBS(A)1% FCS, they were rinsed in distilled water and air dried and mounted on glass slides with 3µl Citifluor, a glycerol/PBS solution (UKC Chemical Laboratories). If necessary DAPI, 4',6'-diamidino-2-phenylindole, (an anti-DNA stain), was present in the Citifluor. Cells were examined using a Nikon MICROPHOT-SA fluorescence microscope with appropriate filters and photographed using Kodak ASA400 black and white film.

2B3.4 ^{35}S-Methionine radiolabelling and extract preparation

Confluent monolayers of WS HeLa cells grown on 140mm diameter tissue culture plates were labelled with ^{35}S-methionine and soluble protein cell extracts prepared for use in GST 'pull-down' assays and immunoprecipitations.

Plates were seeded at 1.5 X10^7 cells per 140mm plate in 30ml complete medium and incubated overnight. The medium was then removed and the cell sheet washed twice with PBS-complete (prewarmed to 37°C), then the cells were incubated with 15ml PBS-complete containing 1,500μCi ^{35}S-Methionine for 2hr at 37°C. Afterwards, the cells were washed twice in ice-cold PBS-complete and harvested by scraping them from the plate using a rubber policeman into 5ml PBS-complete and transfering them to a 15ml Falcon tube. To ensure complete removal of the cell sheet the remaining cells were scraped into 5ml more PBS-complete and transferred to the same Falcon tube. The cells were pelleted by centrifugation at 1,000 rpm for 5min at 4°C and resuspended in 1ml PBS-complete, then repelleted and resuspended in 1ml of ^{35}S-Methionine labelled cell extraction buffer containing 50mM HEPES (pH7.4), 0.2mM NaCl, 1mM β-mercaptoethanol, 0.1% NP40, 1mM PMSF, 0.5μg/ml leupeptin and 40μg/ml bestatin. The cells were then lysed by sonication in a sonibath (Kerry) for 30-60sec and the cell debris pelleted by centrifugation at 1,000rpm for 10min at 4°C. The soluble protein extract was stored at -70°C until required.

2B3.5 Infection of cells with HSV-1 and extract purification

Confluent monolayers of WS HeLas grown on 100mm diameter tissue culture plates were infected with the appropriate HSV-1 strain and soluble protein cell extracts prepared for use in GST 'pull-down' assays.

2B3.5a Absorption of virus by cells

Plates were seeded at 4 X10^6 cells per 100mm plate in 15ml medium and incubated overnight. The medium was removed and replaced with 2ml fresh medium. Virus was added at a multiplicity of 5pfu per cell and the plates gently swirled prior to incubation at 37°C for 1hr. This was interspersed by periods of gentle swirling every 10-15min to ensure complete absorption of the virus. The cells were then overlaid with 8ml of the appropriate medium and left to incubate at 37°C for a further 16hr.

2B3.5b Harvesting of the virus infected cell soluble protein extract

The medium was removed from the plates and the cells washed twice with ice-cold PBS-complete. The cells were then harvested as in Section 2B3.4. The cells were pelleted by centrifugation at 1,000rpm for 5min at 4°C, then the supernatant was discarded and the

pellet resuspended in 220µl homogenisation buffer containing 50mM Tris pH8.5, 0.2M NaCl, 10mM β-mercaptoethanol, 0.1mM zinc acetate, 0.5µg/ml leupeptin and 1µg/ml aprotinin. The cells were lysed using a dounce homogeniser and the cell extract transferred to a fresh eppendorf. The cell debris was pelleted by centrifugation at 10,000rpm for 15min at 4°C. The supernatant containing the virus infected soluble cell extract was stored at -70°C.

2B4 Protein-protein interaction assays

2B4.1 Prediction of regions important for protein-protein interactions

Computer analysis of protein sequences were performed on the Digital Alpha computer system using the University of Wisconsin Genetics Computer Group (GCG Inc., Madison, Wis.). For details refer to Genetics Computer Group (1996) Program Manual for the GCG Package, Version 9.0, 575 Science Drive, Madison, Wisconsin, USA 53711.

2B4.2 Preparation and transformation of competent *E.coli* cells for protein expression

Plasmids which encoded proteins that were to be expressed as part of a bacterial extract were transfected into a fresh batch of the appropriate *E.coli* host competent cells immediately before use. Plasmids under the control of the strong T7 promoter were transfected into the BL21 (DE3) pLysS strain, whilst the pGEX series were transfected into the BL21 strain and the plasmids which were required for use in the standard deubiquitinase assay were transfected into the NovaBlue (DE3) strain.

Bacteria were streaked onto LB agar containing the appropriate antibiotics 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_{450nm} was about 0.3. Cells were then pelleted by centrifugation at 3,000rpm for 5min at 4°C and resuspended in 0.5ml 0.1M $MgCl_2$ (cooled to 4°C). The cells were then repelleted by centrifugation at 3,000rpm for 2min at 4°C and resuspended in 0.5ml 0.1M $CaCl_2$ (cooled to 4°C). These cells were then left at 4°C for 30min before being used for transfection. The required transfections were then carried out using 100µl aliquots of the relevant cells and 10ng of the appropriate plasmid as described in Section 2B1.6.

2B4.3 Expression of proteins under the control of an IPTG-inducible promoter and preparation of bacterial extracts

A single fresh *E.coli* colony transformed with the desired plasmid was inoculated into 2.5ml of LB and shaken overnight at 37°C. An aliquot (1ml) of this culture was added to 100ml 2YT containing the appropriate antibiotics and the culture was grown at 37°C for 3-4hr until the OD_{450nm} was about 0.3. IPTG was added to a concentration of 0.1mM to induce protein expression and incubation continued for a further 3hr. Bacterial pellets were harvested by centrifugation at 3,000rpm for 15min at 4°C and the cell pellet was resuspended in 2ml PBS(A). The bacterial cells were lysed by sonication. This involved exposure to three 10sec bursts of the Branson sonifier 450 soni-probe set at a 50% duty cycle and an output control of 5. After each 10sec burst of the soni-probe the samples were left to cool on ice for 1min. Once the cells had lysed Triton-X100 was added to a 1% concentration and the lysate was incubated on ice for 5min. The cell debris was then pelleted by centrifugation at 9,500rpm in a Sorvall SS34 rotor for 5min at 4°C. The soluble protein extract supernatant was decanted into a fresh tube and stored at -20°C.

2B4.4 Expression and crude purification of T7E52 and T7E58

An overnight culture was set up in 10ml LB from a single fresh *E.coli* colony, strain BL21 (DE3) pLysS, transformed with either pT7E52 or pT7E58 and left shaking overnight at 37°C. The culture was added to 1 litre of 2YT containing Amp at 70µg/ml and grown for 3-4hr at 37°C until the OD_{450nm} was about 0.3. IPTG was then added to a concentration of 0.1mM to induce protein expression and the incubation continued for a further 3hr. The bacterial pellet was harvested by centrifugation at 5,000rpm in a Sorvall GS3 rotor for 5min at 4°C. The cell pellet was resuspended in 10ml cold Extract Buffer (50mM HEPES pH7.5, 100mM NaCl, 0.4% CHAPS, 1mM PMSF, 0.1mM DTT) and stored at -20°C overnight. After thawing, 10µl 20mg/ml DNase1, 25µl 2M $MgCl_2$ and 30µl 10mg/ml lysozyme were added to the mixture and the cells were lysed using a soni-probe as described in Section 2B4.3. This was followed by the addition of 250µl 5M NaCl and 50µl 10% polymin P to the lysate which was then left on ice for 30min. Cell debris and precipitated nucleic acids were pelleted by centrifugation at 9,500rpm for 5min at 4°C. Proteins were precipitated by addition of saturated ammonium sulphate to the supernatant to 40% volume and the mixture stirred on ice for 30min. Precipitated proteins were pelleted by centrifugation at 9,500rpm for 10min at 4°C and the pellet resuspended in 2ml

50mM HEPES (pH7.5), 50mM NaCl, 0.01% CHAPS, 1mM PMSF, 0.1mM DTT. The extract was then filtered using a 0.45µm millipore filter and stored at -20°C.

2B4.5 SDS polyacrylamide gel electrophoresis (SDS-PAGE) of proteins.

Proteins were resolved by electrophoresis through SDS polyacrylamide minigels (Laemmli, 1970) using the Bio-Rad miniprotein II apparatus. The resolving gel mixes were prepared first and poured into glass plate sandwiches in the BioRad gel former apparatus, overlaid with butanol and left to set. Once the gel set, the butanol was washed away with water. The set gel was then overlaid with stacking gel mixture and a comb inserted. Details of how the resolving and stacking gel mixes were prepared are given in Table 2B4.5. Protein samples were mixed with the relevant gel loading buffer and placed in a boiling water bath for 2min prior to loading. Gels were run in tank buffer at 200V until the tracking dye reached the bottom.

Solution	*7.5% resolving gel*	*Stacking gel*
30% acrylamide *(2.5% cross linker)*	2.5ml	0.4ml
RGB	2.5ml	NA
SGB	NA	0.6ml
Water	5ml	1.4ml

Table 2B4.5: Solutions used to make SDS-polyacrylamide gels. Amounts of 30% acrylamide and water were adjusted accordingly for preparation of 6%, 10% or 12.5% resolving gel mixes. To each resolving gel mix 80µl 10% APS and 8µl of TEMED were added and 20µl 10% APS and 3µl TEMED were added to stacking gel mixes, in order to catalyse setting of the gels.

Glycine SDS-PAGE buffers:

i- 3X gel loading buffer: 29% SGB, 6% SDS, 2M β-mercaptoethanol, 29% glycerol, 1mg/ml bromophenol blue
ii- RGB (resolving gel buffer): 1.5M Tris.HCl, 0.4%SDS, pH8.9
iii- SGB (stacking gel buffer): 0.5M Tris.HCl, 0.4% SDS, pH6.7
iv- Tank buffer: 0.05M Tris, 0.05M glycine, 0.1% SDS

2B4.6 Coomassie staining of SDS-PAGE gels:

The gels were stained with Coomassie Blue for five minutes and destained in protein gel destain.

Solutions for coomassie staining:

Coomassie stain:	0.2% Coomassie Brilliant Blue in 50:50:7 ratio of methanol: water:acetic acid
Protein gel destain:	5% methanol, 7% acetic acid in water

2B4.7 Western blot analysis of proteins.

2B4.7a- Electroblotting to a nitrocellulose filter

Proteins resolved on SDS-PAGE minigels were transferred to a nitrocellulose filter by the method of Towbin *et al.*, 1979 in a BioRad 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 BioRad and blotting carried out in Towbin buffer at 0.25A for 3hr.

2B4.7b- Detection of ^{35}S-Methionine-labelled proteins

After completion of transfer of proteins to the nitrocellulose membranes, detection of ^{35}S-Methionine labelled proteins was achieved by air drying the blot for 1hr prior to exposure to Kodak X-OMAT S film.

2B4.7c- Immunodetection of proteins

2B4.7ci- After completion of transfer of proteins to the nitrocellulose membranes, proteins were immunologically labelled using either of the two methods:

Gelatin blocking method

This involved incubating the membranes in 3% gelatin (made in TTBS/TPBS) for 1hr (replacing the gelatin with a fresh batch after 30min) and ensuring complete coverage of the blot by placing it in a shaker. The blots were then washed 3 times for 5min each time at RT in TTBS/TPBS, then incubated with the first antibody (made up to the appropriate dilution in 20ml of 1% gelatin in TTBS/TPBS) and left gently shaking overnight at RT.

After this incubation the blots were washed 6 times, for 5min each time, in TTBS/TPBS at RT, then incubated in the relevant secondary antibody for 60-90min at RT, which was followed by 6 final 5min washes with TTBS/TPBS. The secondary antibodies were diluted in 20ml of 1% gelatin in TTBS/TPBS: Protein A HRP and anti-mouse IgG whole molecule peroxidase conjugates were used at a 1/1,000 dilution.

Marvel blocking method

This involved incubating the blot in 5% marvel (made in TTBS/TPBS) overnight at 4°C (ensuring that the blot was completely covered). The blots were washed 3 times for 5min each time at RT in TTBS/TPBS, then incubated with the first antibody (made up to the appropriate dilution in 20ml of 5% marvel in TTBS/TPBS) and left shaking for 2-4hr at RT. Following this the blots were washed 6 times, for 5min each time, in TTBS/TPBS at RT and then incubated with the relevant secondary antibody for 60-90min at RT, which was followed by 6 final 5min washes with TTBS/TPBS. The secondary antibodies were all diluted in 20ml of 2% marvel in TTBS/TPBS: Protein A HRP and anti-mouse IgG whole molecule peroxidase conjugates were used at a 1/1,000 dilution and goat anti-rabbit IgG whole molecule peroxidase was used at a 1/50,000 dilution.

2B4.7cii- Protein detection by enhanced chemiluminescence (ECL)

Bound antibodies were detected using the Amersham enhanced chemiluminescence (ECL) system. The two reagents provided in the kit 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 variable lengths of time.

2B4.7ciii- Stripping and reprobing membranes

The complete removal of primary and secondary antibodies from membranes was sometimes required in order to reprobe a membrane. This involved submerging the membrane, after immunodetection, in stripping buffer (100mM β-mercapto-ethanol, 2% SDS, 62.5mM Tris.HCl, pH6.7) and incubating it at 55°C for 60min with occasional agitation. The membrane was then washed twice for 10min in about 100ml of TTBS/TPBS at RT. The membrane was then reprobed using one of the blocking protocols, followed by the detection of proteins by ECL as described above.

Solutions for western blotting:

TBS: 0.02M Tris, 0.5M NaCl, pH7.5 with HCl
Towbin blotting buffer: 25mM Tris.HCl, 192mM glycine, 20% methanol, pH8.3
TPBS: PBS(A) plus 0.1% TWEEN-20
TTBS: TBS plus 0.05% TWEEN-20

2B4.8 Standard GST 'pull-down' assay

These standard assay conditions formed the basis by which all the GST 'pull-down' assays, to test the interaction between a GST fusion protein X and non-GST fused protein(s) Y, were carried out. However, the exact details varied significantly. The initial step of purification of fusion proteins by interaction with glutathione agarose beads was based upon the method of Smith & Johnson, 1988. Glutathione agarose beads (Sigma) were prepared for use in the experiments by pre-swelling in 10X volume PBS(A) for 1hr at RT. The beads were then washed 3 times in excess PBS(A) (pelleting the beads by centrifugation at 13,000rpm for 15-20sec between washes). After the final wash the beads were resuspended in a equal volume of PBS(A) and stored as a 50% slurry for up to a month at 4°C.

An aliquot of glutathione agarose beads in a 50% bead slurry (35µl) were added to 300µl bacterial extract (containing a GST fused protein X) and mixed end-over-end for 1hr at 4°C. The beads were pelleted by centrifugation at 13,000rpm for 15-20sec and washed 3 times with 1ml of ice-cold PBS(A), recovering the beads by a brief centrifugation between washes. After the final wash the beads were resuspended in a 50% slurry and stored on ice. Aliquots (5µl) were run on a SDS-PAGE gel (Section 2B4.5) with size standard markers, to help normalise (equalise) the amounts of bound GST fusion proteins used in the 'pull-down' experiment. Based on the results of this gel, appropriate aliquots of the bead slurry for each bound GST fusion protein were taken and mixed with the initial bead slurry so that each sample had a total volume of 30µl, but would contain approximately equal quantities of GST fusion protein. These samples were then mixed end-over-end at 4°C for 1hr with 300µl of a pre-cleared extract (see below) containing the Y protein/s in a buffer optimised for the assay. The beads were recovered by spinning at 13,000rpm for 15-20sec, washed 3X for 5min in 1ml of an optimised wash buffer (this differed for each experiment and has been described where necessary) and finally resuspended in a 50% slurry.

The extracts containing the Y protein/s were routinely pre-cleared by incubation for 1hr at 4 ° C end-over-end with glutathione agarose beads carrying the GST protein expressed by pGEX2TN3 vector plasmid. This step depleted the extract of proteins which non-specifically bound to the beads or GST.

Bound proteins were then eluted from the beads by the addition of 20µl 50mM reduced glutathione and incubation at RT for 20min (interspersed by a gentle agitation of the beads every 5min). The beads were then spun down and the supernatant was transferred to a fresh tube. This elution step was repeated and the eluates mixed with 3X SDS gel loading buffer and boiled for 2min and if necessary stored at -70°C. The samples were again boiled for 2min prior to loading on a SDS-PAGE gel (Section 2B4.5). The presence of particular proteins was then detected by western blotting analysis (Section 2B4.7).

2B4.9 Standard Immunoprecipitation experiment

Proteins were immunoprecipitated using a version of the following standard protocol, to test the interaction between proteins X + Y, which was modified where necessary to suit the purpose of the experiment. An extract containing the X + Y proteins was pre-cleared end-over-end at 4°C for 1hr with Protein-A-Sepharose beads in a 50% slurry (made up in the same buffer used in the later stages of this procedure). Next the beads were pelleted by centrifugation at 13,000rpm for 1min at 4°C in an Ole Dich Instrument Makers 157.MP microfuge. The supernatant was split into two equal amounts and incubated end-over-end at 4°C for 3hr with preimmune or immune serum. This was followed by the addition of 60µl of Protein-A-Sepharose beads (50% slurry) and the reactions were incubated for a further 1hr at 4°C. The Protein-A-Sepharose beads were pelleted by a brief centrifugation at 13,000rpm and washed 3X with 1ml of an optimised wash buffer (which differed depending on the purpose of the experiment and has been described where necessary) to remove any non-specifically bound proteins. The beads were pelleted by a brief centrifugation between washes. After the final wash the specifically bound proteins were eluted by addition of 20µl 1X SDS gel loading buffer, then the samples were boiled for 2min, before being separated by SDS polyacrylamide gel electrophoresis (Section 2B4.5). The presence of particular proteins was then detected by western blotting analysis (Section 2B4.7).

RESULTS CHAPTER 3

3A To investigate the potential deubiquitinase activity of HAUSP

3A1 Introduction

The aim of this project was to understand in more detail the role of the novel protein HAUSP which was found to strongly and specifically interact with the HSV-1 IE protein Vmw110. Prior to the start of this project, analysis of the novel cDNA sequence encoding HAUSP identified two highly conserved motifs belonging to the USP family (Everett *et al.*, 1997). As HAUSP contained no other highly conserved features it was thought that investigating its deubiquitinase activity would be a good strategy to begin to understand its role both within the cell and for viral infection.

There are a variety of methods by which the deubiquitinase activity of a protein can be tested. One of the most widely used methods involves coexpressing in *E.coli* the protein and a gene encoding a Ub-X-β-gal fusion protein, in which ubiquitin is fused to the N-terminus of β-gal via a linker amino acid X (Varshavsky, 1992). The standard *in vivo* deubiquitinase assay used in this investigation was based on this method and is outlined in Figure 3A1.

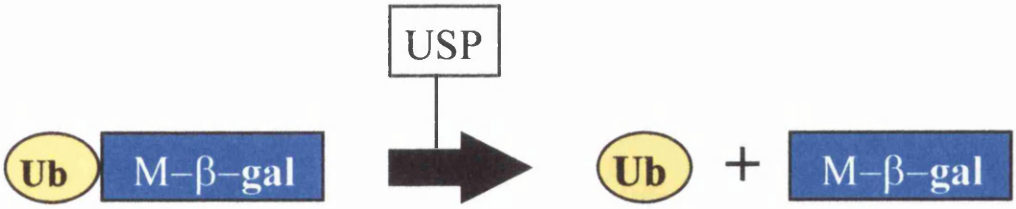
The experiments in sections 3A1-3 describe the use of the above assay to determine the deubiquitinase activity of HAUSP and to identify the active site residues essential for this activity.

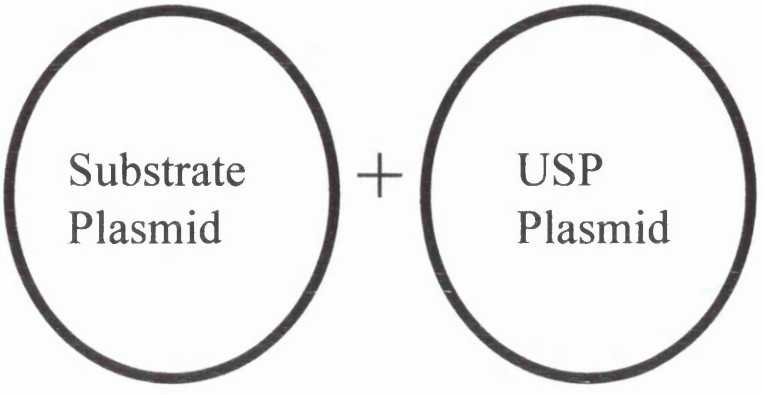
3A2 Cleavage of model substrates by HAUSP using a standard *in vivo* deubiquitinase assay

3A2.1 HAUSP is enzymatically active on the model substrate Ub-M-β-gal expressed in bacteria

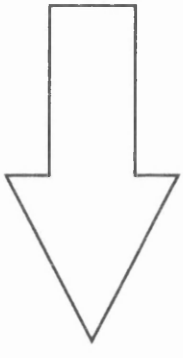
The results of using the standard *in vivo* deubiquitinase assay, in which Ub-M-β-gal was used as a model substrate, are shown in Figure 3A2.1. As can be seen both the positive control (UBP2) and HAUSP cleaved the 121kD Ub-M-β-gal model substrate to the lower molecular weight 113kD M-β-gal product. However, constitutive expression of UBP2 was sufficient to completely cleave the low levels of substrate present, whereas even after

Figure 3A1: An outline of the standard *in vivo* deubiquitinase assay.
The pAC-M-β-gal plasmid (which is in a pACYC-184 Cm^r replicon) expressing the Ub-M-β-gal fusion protein substrate was coexpressed with a USP encoding compatible replicon. The plasmids encoding the USPs were either pRB105 expressing the yeast UBP2 USP enzyme from an IPTG-inducible tac promoter in a pKK-based plasmid (pBR322 Amp^r replicon), or a T7-driven HAUSP plasmid expressing HAUSP from an IPTG-inducible T7 promoter in a pET-based plasmid (pBR322 Amp^r/Kan^r replicon). These plasmids were coexpressed in the NovaBlue *E.coli* strain for a number of reasons. Firstly, *E.coli* lacks a ubiquitin system, secondly, the NovaBlue strain expresses a truncated endogenous β-gal which can be distinguished from the model β-gal fusion protein substrate. Thirdly this strain also encodes a $laqI^q$ repressor which reinforces the repression of IPTG-inducible promoters until IPTG becomes available. Finally NovaBlue bacteria encode a phage T7 RNA polymerase which is required for induction of expression from the T7-driven plasmids. Deubiquitinase activity could then be detected by running the IPTG induced and uninduced bacterial extracts on a SDS-PAGE gel and detecting β-gal products by immunoblotting techniques. In this way cleavage of the model Ub-M-β-gal substrate to the M-β-gal product, could be detected by the appearance of a reduced molecular weight M-β-gal product on the western blot.

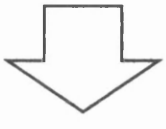




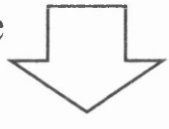
Plasmids Compatible:
a- Different replicons
b- Different drug resistance markers
c- IPTG inducible



Coexpressed in Nova Blue cells
•Lack Ub system
•Express truncated endogenous β-gal
•Express $lacI^q$
•Phage T7 RNA polymerase encoded in genome



No deubiquitinase activity



deubiquitinase activity

121kD

113kD

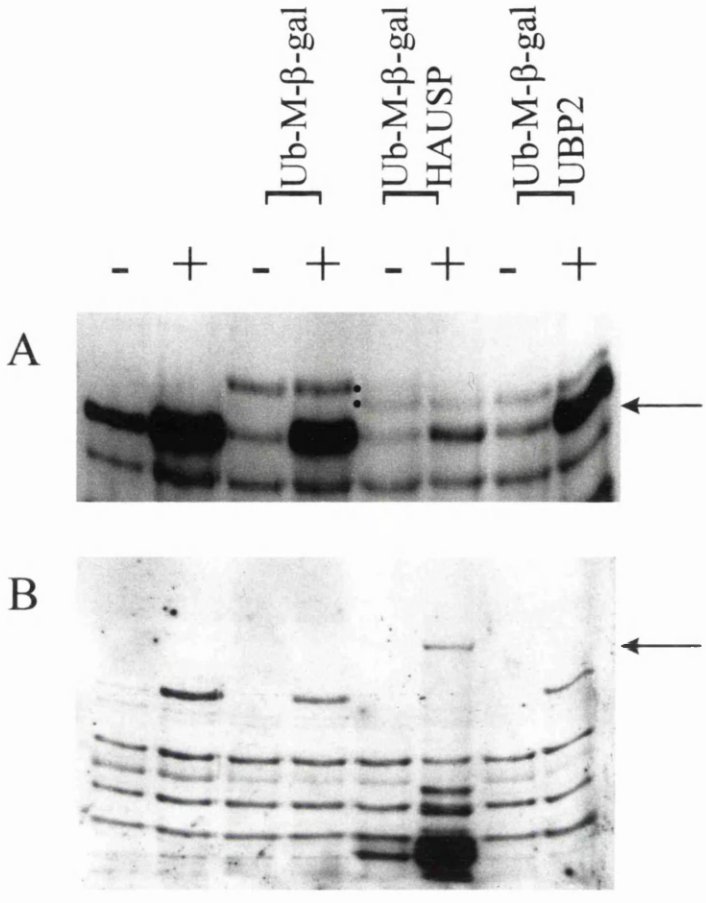


Figure 3A2.1: Deubiquitination by HAUSP of a standard ubiquitin fusion protein expressed in bacteria.

A standard *in vivo* deubiquitinase assay was carried out as described in Section 2B2 using bacteria harbouring plasmids expressing the Ub-M-β-gal model substrate (pAC-M-β-gal) and either HAUSP (pT7135) or UBP2 (pRB105).

A- The western blot of the bacterial cultures probed with anti-β-gal antiserum r12741. The labels on top of the tracks indicate the proteins which were expressed in the cultures and whether their expression had been induced by the addition of IPTG or left uninduced (as indicated by + and - respectively). The upper dot between lanes 4 and 5 indicates the uncleaved substrate and the lower dot indicates the correctly cleaved M-β-gal product (as defined by the UBP2-positive control in lanes 7 and 8). The endogenous truncated β-gal expressed by the NovaBlue bacteria is indicated by the arrow on the right of the gel.

B- The western blot was stripped and reprobed for HAUSP using the r201 anti-HAUSP antipeptide antibody in a 1 in 1,000 dilution. The arrow on the right of the gel indicates the position of HAUSP, indicating that it was correctly expressed.

induction, HAUSP expressing cultures cleaved the substrate incompletely. This may have been a reflection of the lower growth rates observed for bacterial cultures in which HAUSP expression had been induced compared with those cultures in which HAUSP was absent.

Basal levels of HAUSP expression, by cultures transfected with a HAUSP encoding plasmid and grown in the absence of IPTG, appear to be sufficient to cleave a substantial quantity of the substrate (Figure 3A2.1 part A lane 5). Furthermore, as this expression of HAUSP was not detectable by western blotting techniques (Figure 3A2.1 part B lane 5) it suggests that HAUSP is a potent USP cleaving substrate efficiently even at low levels.

3A2.2 HAUSP cleaves the model substrate Ub-R-β-gal at the junction between the ubiquitin and the fusion protein.

The second stage in this investigation was to test that cleavage of the model substrate by HAUSP takes place at the junction between the C-terminus of the ubiquitin and the β-gal moiety of the fusion protein. In order to test this an assay was performed based on the standard *in vivo* deubiquitinase assay, with the exception that the pAC-R-β-gal plasmid was used which encoded the Ub-R-β-gal fusion protein, in place of the pAC-M-β-gal plasmid. In this case deubiquitination of the model substrate would result in the production of R-β-gal which is an unstable protein and is rapidly degraded in *E.coli* (Tobias *et al.*, 1991). The results of this assay are shown in Figure 3A2.2 part A. As can be seen, both UBP2 and HAUSP cleave the full length model Ub-R-β-gal substrate, however lower molecular weight β-gal products did not appear, indicating that cleavage is resulting in the production of an unstable product, presumably R-β-gal. Residual levels of substrate were again observed in samples in which HAUSP expression had been induced. The western blot of the deubiquitinase assay samples was probed for HAUSP encoding sequences (shown in Figure 3A2.2 part B) to prove that HAUSP was being expressed as expected. As can be seen a relatively high level of background HAUSP expression could be observed even in the absence of induction by IPTG. This is probably the result of using the BL21 strain of *E.coli* for this assay, as opposed to the Nova Blue strain which were normally used, as BL21 (DE3) bacterial cells lack the $lacI^q$ repressor which provides tighter control over basal expression of IPTG-inducible promoters than the genomic wild type repressor.

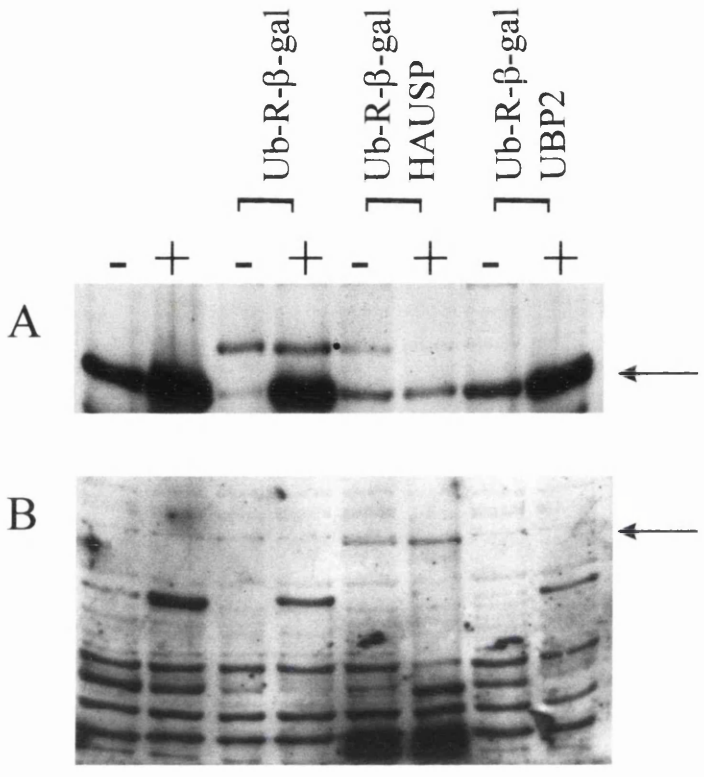


Figure 3A2.2: Cleavage of Ub-R-β-gal by HAUSP occurs at the junction between Ub and the C-terminal fusion protein.

A standard *in vivo* deubiquitinase assay was carried out as described in Section 2B2 using bacteria harbouring plasmids expressing the Ub-R-β-gal model substrate (pAC-R-β-gal) and either HAUSP (pT7135) or UBP2 (pRB105), with the exception that the BL21 strain of *E.coli* was used as opposed to the Nova Blue strain.

A- Western blot of the bacterial cultures probed with anti-β-gal antiserum r12741. The labels on top of the tracks indicate proteins which were expressed in the cultures and whether their expression had been induced by the addition of IPTG or left uninduced (as indicated by + and - respectively). The dot between lanes 4 and 5 indicates the uncleaved substrate. The endogenous truncated β-gal expressed by the NovaBlue bacteria is indicated by the arrow on the right of the gel.

B- The western blot was stripped and reprobed for HAUSP using the r201 anti-HAUSP antipeptide antibody in a 1 in 1,000 dilution. The arrow on the right of the gel indicates the position of HAUSP.

3A3 Identification of residues essential for the enzyme activity of HAUSP

3A3.1 Prediction of residues required for the enzyme activity of HAUSP

The strategy used to define residues within the active site domains essential for the enzyme activity of HAUSP, was to first identify potential target residues and then to perform site-directed mutagenesis, in order to generate mutants in which these residues have been altered.

The cysteine 223, histidine 456 and histidine 464 residues targeted were identified by comparison of the HAUSP highly conserved cysteine and histidine motifs with those of other USPs as shown in Figure 3A3.1. The targeted residues are those indicated in bold face.

3A3.2 Construction of HAUSP active site mutants

Construction of the HAUSP active site mutants was achieved by performing site-directed mutagenesis using the C223S, H456L and H464L oligonucleotides (Section 2B1.10 and Table 2B1.10). The successful incorporation of the mutations was checked by restriction enzyme analysis using restriction sites introduced in each mutagenic primer which did not affect the coding potential. Then for the C223S, H456L and H464L pET24a135 clones, a small region surrounding the inserted mutation was subcloned into the original wild type pET24a135 plasmid DNA. This was done in order to reduce the likelihood of any additional mutations which may have arisen as part of the site-directed mutagenesis procedure, interfering with the results. A figure illustrating the positions of restriction sites within the open reading frame of HAUSP which were relevant to the construction of plasmids encoding HAUSP, has been given in Figure 3A3.2 and an additional loose leaf copy has been placed in a pocket at the back of this thesis.

The C223S pET24a135 subclone was synthesised in a ligation reaction with two fragments isolated from the relevant digestion mixes: these were the *Bsr*G1(193)-*Bsr*GI(241) C223S pET24a135 insert fragment and the *Bsr*GI(241)-*Bsr*GI(193) wild type pET24a135 vector fragment (see Figure 3A3.2).

HAUSP Cysteine domain:

```
215aa-GLKNQGATCYMNSLLQTLF
      GL N GnT**C**OMNSOLQcLO
```

HAUSP Histidine domain:

```
448aa-YILHAVLVHSGDNHGGHYVVYLNPKGDGKWCKFDD
      Y L  V  **H** G    G**H**Y  O k      WO ODD

484aa-VVSRCTKEEAIEHNYGGHDDDLSVRHCTNAYMLVY
                                      AYOLfY
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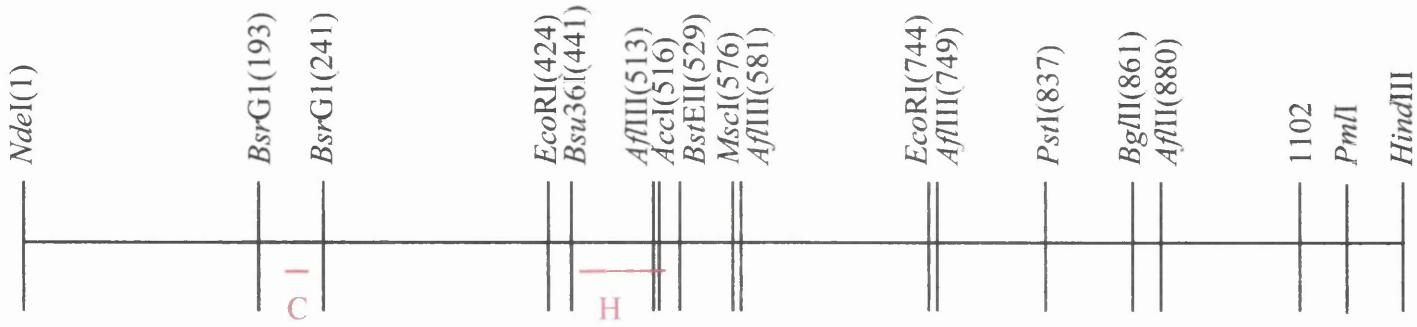
Figure 3A3.1: HAUSP active site sequences aligned against cysteine and histidine USP consensus motifs. These consensus sequences were derived from alignments of 20 other members of the USP family accession numbers: Sc9952x6, Q09879, P38187, P39967, p34547, A49132, Z54218, Sc9959x5, P40453, P36026, P32571, P39944, P35123, U20657, P35125, P39538, P40818, P25037, Q01476, Z47811x3.
The highly conserved residues within the cysteine and histidine motifs of the USP family are indicated under the HAUSP sequence, with residues that are not present in HAUSP written in lower case, the letter O depicting conserved hydrophobic residues and those written in bold indicating predicted active site residues. Figure modified from Everett *et al.*, 1997 with permission.



Scale:

—— =50 HAUSP residues

Figure 3A3.2: Restriction map of the sequence encoding HAUSP. The positions of restriction sites within the HAUSP open reading frame which are relevant to the construction of plasmids made for the purposes of this thesis have been indicated and their codon position marked. A scale has been given to indicate the length of line representing 50 codons. The red lines indicate the approximate positions of the cysteine (C) and histidine (H) active site domains.
N.B. Where in the text a restriction site has been followed by a number in parentheses, the number is an indication of the codon position within the HAUSP open reading frame at which the fragment had been digested.

Both the H456L and H464L pET24a135 subclones were synthesised in similar ligation reactions with two fragments isolated from the relevant restriction digestion mixes: these were the *Bsu*36I(441)-*Bst*EII(529) insert fragment from either the H456L or H464L pET24a135 clones and the *Bst*EII(529)-*Bsu*36I(441) wild type pET24a135 vector fragment (see Figure 3A3.2).

Sequencing (Section 2B1.9) was then performed, using the sequencing primers (Table 2B1.9) 1664 for the C223S pET24a135 mutant and 1782 for the H456L and H464L pET24a135 mutants, to ensure the correct incorporation of the mutations.

3A3.3 Investigation of the deubiquitinase activity of HAUSP active site mutants

The ability of these HAUSP active site mutants to cleave model ubiquitin fusion proteins was tested by including them in the standard *in vivo* deubiquitinase assay, the results of which are displayed in Figure 3A3.3. As can be seen substitution of the key Cys 223 residue with a serine and either of the two conserved histidine 456 or 464 residues with leucine resulted in abolition of the deubiquitinase activity of HAUSP. The fact that the lack of deubiquitinase activity cannot be explained by reduction in the levels of full length HAUSP expression was established by reprobing the western blot of the bacterial extracts with anti-HAUSP serum (Figure 3A3.3 part B). This blot shows that full length HAUSP is expressed to comparable levels for both the wild type and mutant proteins.

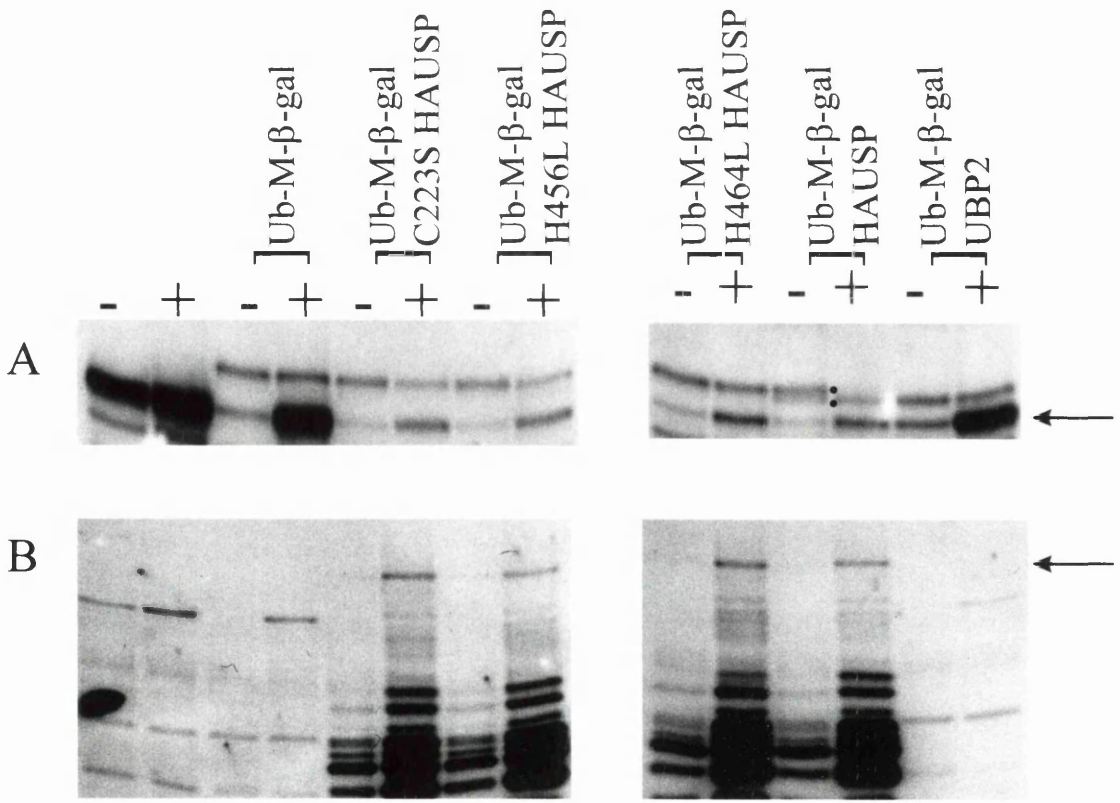


Figure 3A3.3: HAUSP active site mutants C223S, H456L and H464L are unable to cleave a model ubiquitin fusion protein expressed in bacteria.

A standard *in vivo* deubiquitinase assay was carried out as described in Section 2B2, using bacteria harbouring plasmids expressing the Ub-M-β-gal model substrate (pAC-M-β-gal) and either C223S HAUSP, H456L HAUSP, H464L HAUSP, or wild type HAUSP (pET24a135), or UBP2 (pRB105).

A-The western blot of the bacterial cultures probed with anti-β-gal antiserum r12741. The labels on top of the tracks indicate which proteins were expressed in the cultures and whether their expression had been induced by the addition of IPTG or left uninduced (as indicated by + and - respectively). The upper dot between lanes 11 and 12 indicates the uncleaved substrate and the lower dot indicates the correctly cleaved M-β-gal product (as defined by the UBP2-positive control in lanes 13 and 14). The endogenous truncated β-gal expressed by the NovaBlue bacteria is indicated by the arrow on the right of the gel.

B- The western blot was stripped and reprobed for HAUSP using the r201 anti-HAUSP antipeptide antibody diluted 1 in 1,000. The arrow on the right of the gel indicates the position of HAUSP.

3A4 Development of an *in vitro* cleavage assay

3A4.1 Introduction

In order to confirm the deubiquitinase activity of HAUSP, attempts were made to develop an *in vitro* cleavage assay. The experimental strategy to develop such an assay involved first purifying HAUSP and then testing its ability to cleave a purified model substrate of USPs. The model substrate chosen for use in the assay was Ub-GST, as it could be purified from bacterial extracts using glutathione agarose beads.

3A4.2 Confirmation of the ability of HAUSP to cleave Ub-GST using an *in vivo* based assay

Before testing Ub-GST in the *in vitro* cleavage assay it was decided to first ensure that Ub-GST was recognised by HAUSP as a substrate for cleavage. This was tested using a modified version of the standard *in vivo* deubiquitinase assay which has been described in detail in the legend of Figure 3A4.2. This involved using a plasmid expressing Ub-GST in place of one which expresses Ub-M-β-gal. It is clear from the tracks derived from samples in which HAUSP or UBP2 protein expression was induced, that a protein of lower molecular weight, similar to that expected for GST alone, has been produced. This suggests that both these proteins recognise Ub-GST as a substrate for cleavage. However, HAUSP expression in this assay only resulted in approximately 50% cleavage of the Ub-GST. This is in contrast to the sample in which UBP2 had been expressed in which 100% cleavage was observed. The root cause of this observation is not clear, but may be due to the differences in the substrate specific activity of these two proteins or as a result of lower expression levels of HAUSP in comparison to UBP2.

3A4.3 Purification of Ub-GST substrate

The initial step in developing an *in vitro* cleavage assay for deubiquitinase activity was to obtain purified substrate. This involved transforming the pRB307 plasmid encoding Ub-GST into the *E.coli* NovaBlue strain. Cultures were grown, protein expression induced by the addition of IPTG and soluble protein extracts prepared as described in Sections 2B4.2 and 2B4.3. An aliquot of 900µl of the soluble protein extract was then taken and mixed with 150µl of glutathione agarose beads and left to incubate for 1hr end-over-end at 4°C. The beads were then washed 4 times with 1ml PBS(A), pelleting them by a brief centrifugation in between every wash. Beads-bound proteins were then eluted by addition

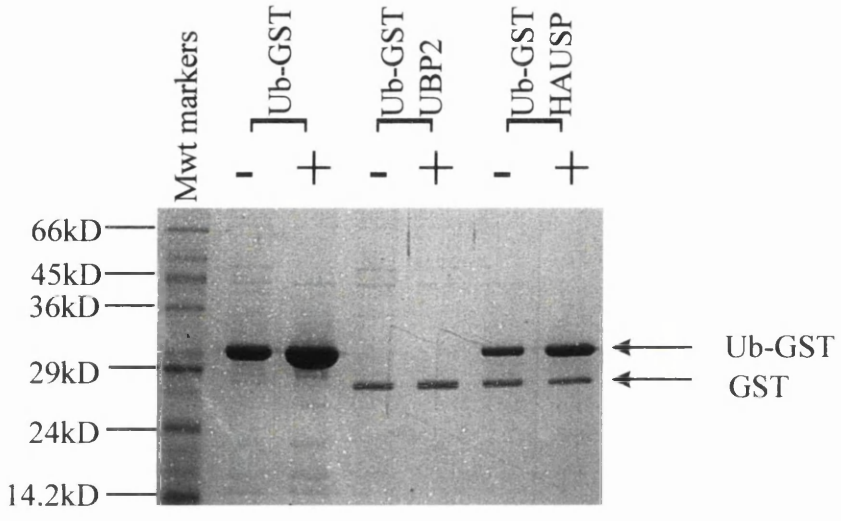


Figure 3A4.2: *In vivo* assay confirming the cleavage of Ub-GST by HAUSP.
A modified *in vivo* assay based on the standard *in vivo* deubiquitinase assay described in Section 2B2 was performed. The first modification involved using the plasmid pRB307 which encodes the Ub-GST substrate in place of the Ub-M-β-gal expressing plasmid. This plasmid was coexpressed with a compatible plasmid encoding a USP either UBP2 (pACYCUBP2), or HAUSP (pACYCHAUSP) in NovaBlue cells and the cell extracts harvested as in the standard assay. The cell extracts were then sonicated using a sonibath and the cell debris pelleted by centrifuging the extract at 9,500rpm for 10min at 4°C. The soluble bacterial extracts were then decanted into fresh eppendorf tubes and mixed with 40µl GST beads and left to incubate end-over-end at 4°C for 1hr. The beads were then washed with 1ml PBS(A) three times, pelleting the beads by a brief centrifugation in between each wash. GST fused proteins bound to the beads were then eluted by the addition of 20µl of 1X SDS loading buffer and the boiling of the samples for 2min. The beads were then pelleted by a brief centrifugation (20sec at 13,000rpm) and the supernatant decanted into a fresh eppendorf. If necessary the eluted samples were then boiled again for 2min prior to loading on a 12.5% SDS-PAGE gel. An aliquot (2µl) of molecular weight markers was also run on the gel, the sizes of which have been indicated on the left of the gel. The labels on top of the tracks indicate which proteins were expressed in the cultures and whether their expression had been induced by the addition of IPTG or left uninduced (as indicated by + and - respectively). The upper arrow indicates the position of the uncleaved substrate and the lower arrow indicates the correctly cleaved product.

of 60µl elution buffer (50mM reduced glutathione pH7.4, 1mM DTT, 0.2M NaCl and 0.05% NP40), followed by a 30min incubation at RT. The reducing agent DTT was present in the elution buffer as its presence was thought to optimise USP activity by ensuring the reduced status of the thiol group of the active site cysteine residue. The beads were then pelleted and eluted proteins decanted and stored at -70°C. A 2µl aliquot of this eluted sample was then run on a 10% SDS-PAGE gel alongside a standard molecular weight marker to ensure that the protein had been purified successfully (data not shown).

3A4.4 Investigation of the ability of HAUSP to cleave Ub-GST using an *in vitro* based assay

Testing the ability of HAUSP to cleave Ub-GST using an *in vitro* based assay involved first the purification of HAUSP and then the use of this protein in a cleavage reaction in which the Ub-GST purified substrate was present (the experimental procedures for which are described in detail in the legend of Figure 3A4.4a). HAUSP was purified using cell extracts by immunoprecipitation onto Protein-A-Sepharose beads, using anti-HAUSP r201 serum. Cleavage reactions were then set up, which involved adding purified Ub-GST substrate to the beads obtained from the immunoprecipitation experiment, altering the conditions to optimise USP activity (as described in the legend of Figure 3A4.4a) and leaving them to incubate for 2hr at 37°C. The protein constituents of the supernatant samples (containing the purified GST proteins) and bead samples (from the immunoprecipitation experiment), were analysed by coomassie staining and western blotting techniques respectively. The results of this preliminary experiment can be seen in Figure 3A4.4a. It is clear from the gel in Panel A that in the sample provided with the immunoprecipitated HAUSP (as verified by the immunoblot in Figure 3A4.4a part B), only minimal cleavage of the Ub-GST to a lower molecular weight GST product was observed. Furthermore, a lower molecular weight product was also seen to a lesser extent in samples in which HAUSP was not present. This may have been produced as a result of background cleavage from other proteases which were precipitated from the cell extract by the constituents of the preimmune rabbit r201 serum, or secondly it may have been a natural degradation product of the Ub-GST substrate.

As the *in vitro* cleavage activity of HAUSP, if any, was of very low efficiency, an attempt was made at optimising the levels of cleavage. This consisted of increasing the number of hours of incubation and shaking the samples continuously to ensure the beads were fully

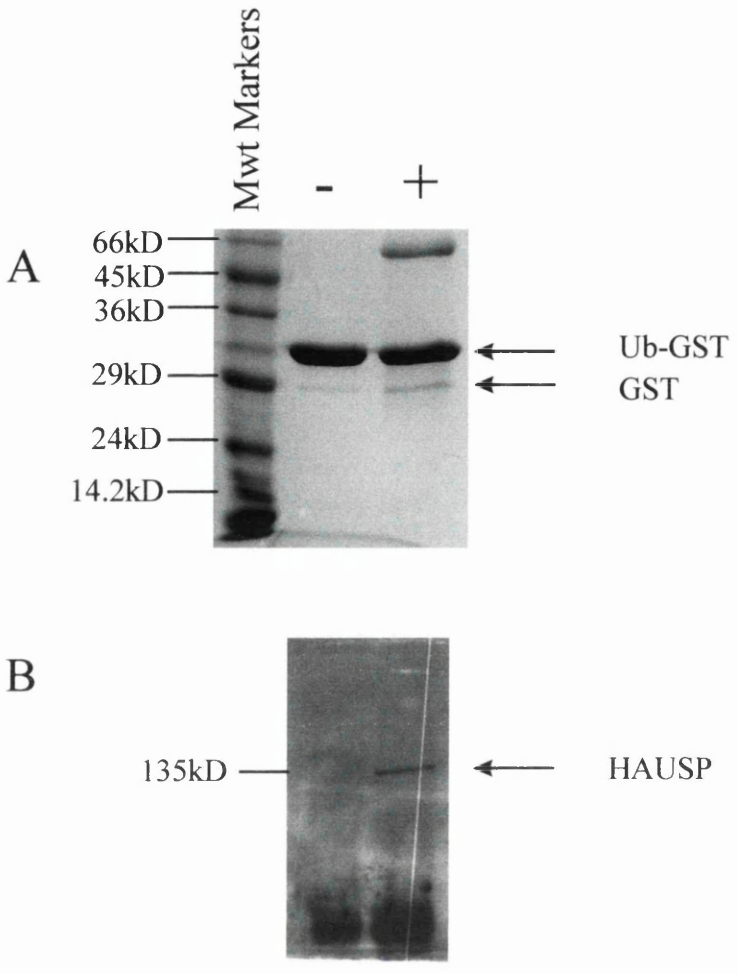


Figure 3A4.4a: Preliminary attempt to develop an *in vitro* deubiquitinase assay for HAUSP.
The first step in this experiment was the purification of HAUSP. As such, two WS HeLa cell extracts were made from 100mm plates, as described in Section 2B3.5b, with the exception that the cell pellets were resuspended in 500µl of USP assay buffer (50mM Tris pH7.4, 1mM DTT, 0.2M NaCl). An immunoprecipitation was then carried out, as described in Section 2B4.9, adding either 20µl preimmune r201 serum or 20µl immune r201 serum to the pair of Protein-A-Sepharose pre-cleared cell extracts. Another exception to the method described in Section 2B4.9 was that USP assay buffer was used to wash the beads from any non-specific interactions. In addition to this, immunoprecipitated proteins were not eluted into 1X SDS gel loading buffer from the beads, rather the beads were used as a source of HAUSP for the *in vitro* cleavage assay. Cleavage reactions were then set up in fresh eppendorf tubes. This involved firstly adding 1.5µl purified Ub-GST to both the mock and test immunoprecipitated beads and making the final volume up to 20µl with USP assay buffer. The beads were then left at 37°C for 2hr, mixing the supernatant and beads samples every 10min. The supernatant and beads samples were then separated by a brief centrifugation and placed separately into fresh eppendorf tubes. An aliquot of 10µl of 3X SDS loading buffer was added to each decanted supernatant sample and 20µl aliquots of 1X SDS loading buffer were used to elute proteins from the beads samples. The samples were then boiled for 2min prior to loading on SDS-PAGE gels.
Panel A: Shows the soluble products of an *in vitro* cleavage reaction between purified Ub-GST and either immunoprecipitated HAUSP bound to Protein-A-Sepharose beads (+), or Protein-A-Sepharose beads mock immunoprecipitated using a pre-immune serum (-), run onto a 12.5% SDS-PAGE gel and visualised by coomassie staining. An aliquot (2µl) of molecular marker weights was also run on this gel, the sizes of which are indicated on the left of the gel. The arrows on the right of the gel correspond to the positions of Ub-GST and a lower molecular weight product most likely GST.
Panel B: Shows the products eluted from the Protein-A-Sepharose beads samples derived from the *in vitro* cleavage reaction, run on a 6% SDS-PAGE gel and transferred to a nitrocellulose filter and then probed for HAUSP using the 16613 anti-HAUSP monoclonal antibody. The arrow on the right of the gel indicates the position of the band corresponding to HAUSP.

immersed in the cleavage reaction. The results of this experiment are in Figure 3A4.4b. As can be seen, neither the extra hours of incubation nor continual shaking appeared to have significantly altered the efficiency of *in vitro* cleavage by HAUSP.

3A4.5 Limitations in the development of an *in vitro* cleavage assay

The reasons for the low level of *in vitro* cleavage by HAUSP are not clear. Whether the problem was intrinsic to HAUSP or to the design of the assay could not be confirmed. As I was not able to establish whether the assay worked in the presence of other USPs due to a lack in the availability of antibodies against such proteins.

However, other groups including that of Dr. John Mayer (Nottingham) and Dr. Rohan Baker (Canberra, Australia) have also attempted to study the *in vitro* cleavage activity of HAUSP. In these cases they applied *in vitro* cleavage assays which had previously been used successfully to confirm the activity of other USPs. They too observed *in vitro* cleavage by HAUSP, but again the levels of cleavage appeared to be very low. As such this supports the concept that the low level of *in vitro* cleavage observed for HAUSP in the assay developed here is an intrinsic problem related to HAUSP rather than a problem of developing a working assay.

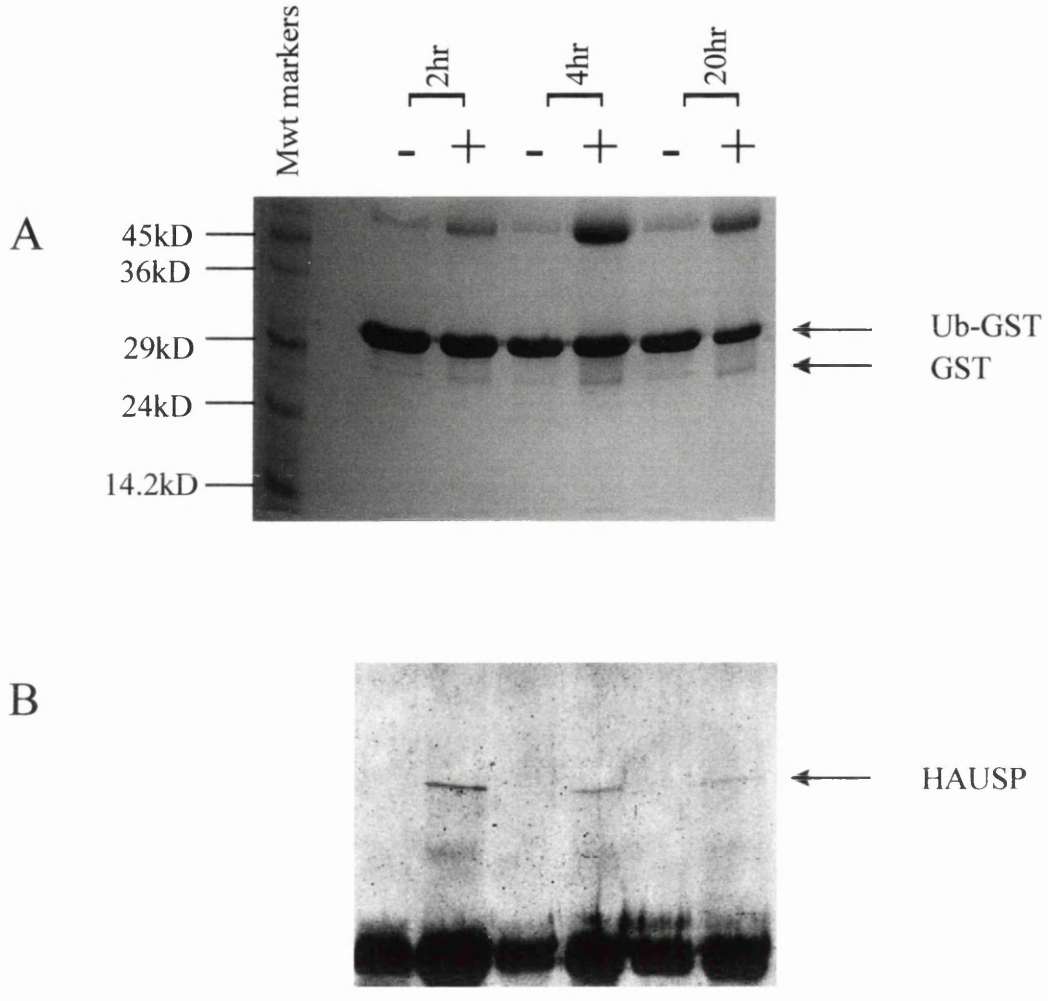


Figure 3A4.4b: An *in vitro* cleavage assay testing the effects of varying the incubation conditions on the ability of HAUSP to cleave Ub-GST.

For this assay, cleavage reactions were set up in 6 wells of a 96 well microtitre plate (Nunc). The first step was to make three sets of immunoprecipitated beads, prepared in the absence or presence of active r201 serum, using the same method as described in the previous figure. These beads were then added to the wells of the microtitre plate (using a cut off yellow gilson tip). The next step was to add 1.5µl purified Ub-GST to each well and make the final volume up to 20µl with USP assay buffer. The three sets of beads were then left shaking at 37°C, for 2hr, 4hr or 20hr respectively. At the end of each incubation period the supernatant and beads samples were removed from the relevant wells and placed into separate eppendorf tubes. Proteins from the samples were then analysed as described in the legend of Figure 3A4.4a.

Panel A shows the 12.5% SDS-PAGE gel with the supernatant samples and Panel B shows the immunoblot of the 6% SDS-PAGE gel with the beads samples, both of which have been labelled in a similar fashion as for the gels described in the legend of Figure 3A4.4a. Additionally, the number of hours the cleavage reaction was incubated for has been indicated above the gel.

3A5 Summary of the investigation into the potential deubiquitinase activity of HAUSP

- Established that HAUSP has deubiquitinase activity using a bacterial based *in vivo* assay.
- Demonstrated that HAUSP cleavage of the model substrate occurs at the junction between the ubiquitin and the fusion protein.
- Confirmed that the conserved active site residues C223, H456 and H464 are essential for deubiquitinase activity.
- Development of an *in vitro* cleavage assay for HAUSP proved difficult.

3B To test the effects of transiently expressing HAUSP in eukaryotic cells

3B1 Introduction

In order to investigate the role of HAUSP within the cell it was decided to try to transiently express HAUSP in eukaryotic cells. The strategy used involved transfecting eukaryotic cells with a plasmid expressing HAUSP under the control of the strong CMV promoter and testing these cells for overexpression of HAUSP using indirect immunofluorescence (Sections 2B3.2 and 2B3.3). The preliminary data from the initial experiments has not been shown, but typical images are presented in the later parts of this section.

3B2 Construction of the pCMV135 plasmid which encodes HAUSP for expression in eukaryotic cells

Plasmid pCMV135 was designed to transiently express HAUSP in mammalian cells under the control of the strong simian CMV promoter. The vector used was pJ7Ω, which includes the simian CMV IE94 promoter flanked 3' by a polylinker, an intron and a transcriptional termination signal which is linked to a pBR322 derived backbone. Construction of the pCMV135 plasmid was achieved through ligation of the *Xba*I-*Pml*I fragment of the pT7135 plasmid (which encodes the entire HAUSP coding region), into the *Xba*I-*Sma*I restriction sites of the pJ7Ω polylinker. The junction between the vector and HAUSP encoding regions was checked by sequencing (Section 2B1.9) using the 2141 sequencing primer (Table 2B1.9).

3B3 Investigation of the effects of transient transfection of pCMV135 in eukaryotic cells

The pCMV135 plasmid was tested for its ability to express HAUSP in eukaryotic cells by firstly transfecting WS HeLa cells or Hep2 cells with pCMV135 using the DOTAP method of transfection (Section 2B3.2). Indirect immunofluorescence was then performed on cells at 24hr and 48hr post transfection (Section 2B3.3), using the anti-N-terminal HAUSP anti-peptide r206 as the primary antibody. The Vmw110-harbouring plasmid p111 was used as a positive control for transfection in these experiments using the anti-Vmw110 MAb 11060 as the primary antibody. Although the transfection efficiency using plasmid p111 was satisfactory it was concluded that no significant differences could be observed between cells mock transfected and those transfected with pCMV135 and probed with r206. This

suggested that pCMV135 was not expressing HAUSP. This could have been due to the targeting of excess HAUSP for degradation.

3B4 Construction of pp65CMV135

It was decided to construct a plasmid which would incorporate a pp65 epitope tag on the N-terminus of HAUSP. This was done to distinguish between endogenous and exogenous expression of HAUSP by use of an anti-pp65 epitope MAb. Also, it was hoped that incorporation of a pp65 tag at the N-terminus might lead to an increase in the stability of exogenously expressed HAUSP. Details of the oligonucleotide sequences designed for the construction of the pp65 tag are illustrated in Figure 3B4 part A.

The pp65CMV135 plasmid was constructed through a ligation reaction with three fragments which encoded the pp65 epitope tag, the HAUSP coding region and the pJ7Ω vector backbone respectively. These fragments were isolated from the following digestion mixes: the *Xba*I-*Nde*I(1) digestion of the 5' phosphorylated pp65 epitope tag (the preparation of which is outlined in Figure 3B4 part B), the *Kpn*I-*Xba*I digestion of the pJ7Ω vector and *Nde*I(1)-*Kpn*I digestion of the pCMV135 plasmid respectively. The junction between the pp65 tag and HAUSP encoding regions was checked by sequencing (Section 2B1.9) using the 2141 sequencing primer (Table 2B1.9).

3B5 Expression of pp65-tagged HAUSP in eukaryotic cells

The pp65CMV135 plasmid was tested for its ability to express HAUSP in eukaryotic cells using a similar method as used for pCMV135 (Section 3B3). This involved firstly transfecting WS HeLa cells or Hep2 cells with pp65CMV135 using the DOTAP method of transfection. Indirect immunofluorescence was then performed on cells at either 24hr or 48hr post transfection using both the anti-HAUSP N-terminal r206 serum and the anti-pp65 MAb in the primary antibody mix. The p111 plasmid was again used as a positive transfection control. The general conclusion from repeated experiments was that pp65-tagged HAUSP expression was detectable in cells probed with the anti-pp65 antibody 24hr after transfection and to a lesser degree 48hr after transfection. However the number of cells positively transfected with the pp65CMV135 plasmid was much lower (approximately a ten fold reduction), compared to those transfected with p111. Furthermore, no significant differences were seen when using Hep2 or WS HeLa cells.

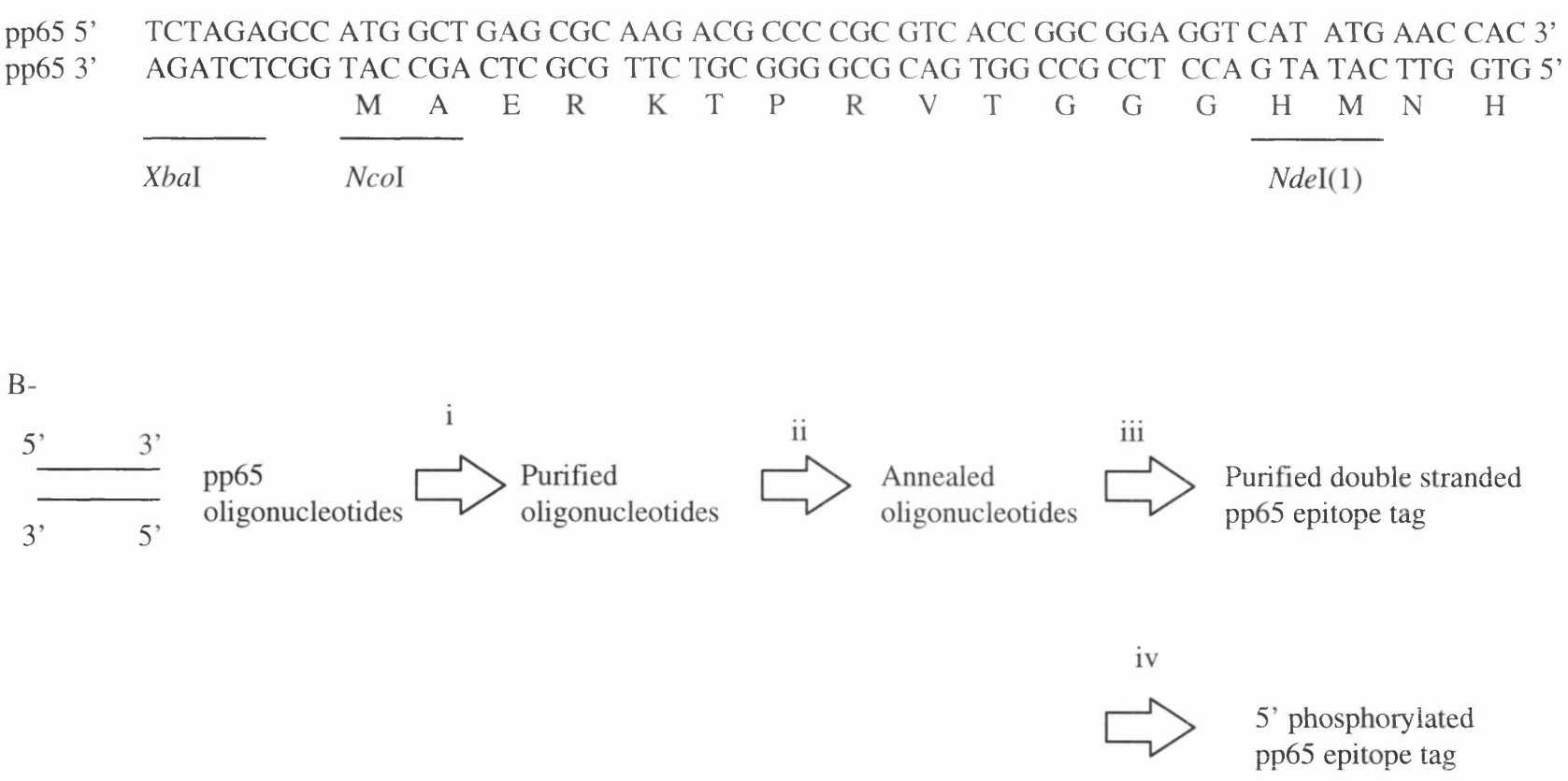


Figure 3B4: Design and preparation of the pp65 epitope tag.
A- The sequence of the pp65 epitope tag. The predicted amino acid sequence has been indicated below, along with the encoded restriction sites (underlined).
B- The preparation of the double stranded pp65 epitope tag for use in the ligation reaction required several stages. i-The first stage was purification of the pp65 epitope encoding oligonucleotides (Section 2B1.3). ii-These oligonucleotides were then annealed by using 0.5µg of each and adjusting the total volume to 50µl in a final concentration of 0.1M NaCl and then heating this sample to 90°C to break any secondary structure and finally cooling it to 30°C. iii-The double stranded oligonucleotide was then purified by running it on a 8% non-denaturing acrylamide gel (Section 2B1.4c) and excising the corresponding band from the gel following visualisation of the DNA by U.V. transillumination. The band was then crushed and the DNA eluted in 400µl 1X TE overnight at 37°C in a shaking incubator. The liquid phase containing the DNA was separated from the gel fragments by filtering through siliconised glass wool and then ethanol precipitated and resuspended in 14µl dH_2O. iv-The double stranded oligonucleotide was then 5' phosphorylated by a kinase reaction done in 1mM ATP, 1X Kinasing buffer with 10 units of T4 polynucleotide kinase and the total volume adjusted to 20µl and left at 37°C for 30min.

However as Hep2 cells have more distinguishable ND10 domains it was decided to continue the work using this cell line.

3B6 Investigation of why so few pp65-tagged HAUSP expressing cells were detectable

It is not clear why so few pp65-tagged HAUSP expressing cells were detectable. It is speculated that this could be a result of the cells stringent regulation of the level of cellular HAUSP. Hence any excess HAUSP produced may have been targeted for degradation or the cells in which HAUSP was produced in excess may themselves be targeted for apoptosis.

Furthermore, although only a low percentage of cells showed detectable expression of pp65-tagged HAUSP, within those cells excess amounts of the exogenously expressed HAUSP were always detected. An explanation for this may be that it is only when cells are at a particular stage in the cell cycle that a cellular factor is produced which enables detection of excess levels of HAUSP.

3B6.1 Investigation of whether the pp65-tagged HAUSP was being degraded by the proteasome

To investigate the possibility that pp65-tagged HAUSP was being targeted for degradation by the proteasome, a modified version of the standard indirect immunofluorescence experiment was performed. This involved carrying out transfections in Hep2 cells using either no plasmid or the pp65CMV135 plasmid and then incubating the cells for 24hr posttransfection (Section 2B3.2). Proteasome inhibitors were then added to the transfected cells, in fresh medium. To investigate which proteasome inhibitor was most effective and at which concentration: lactone lactacystein, β-lactone-lactacystein and MG132 were tested at varying concentrations. Lactone lactacystein was used at: 0μm, 1μm, 5μm and 10μm; β-lactone lactacystein at 10μm and MG132 at 5μM. To investigate the incubation period at which the proteasome inhibitors were most effective transfected cells containing a particular concentration of a proteasome inhibitor were incubated for: 2hr, 4hr and 8hr periods. At the end of the incubation period, cells were fixed as normal and left in approximately 200μl PBS(A) overnight. This was followed by continuation of the indirect immunofluorescence procedure, using both the anti-pp65 MAb and anti-HAUSP anti-peptide antibody r201 in the primary antibody mix (Section 2B3.3).

Comparison between transfected cells which were either exposed, or not, to the proteasome inhibitors for a particular duration revealed no significant differences in the number of pp65-tagged HAUSP expressing cells. The distribution or level of the tagged HAUSP between these cells was also not significantly altered. This suggests that pp65-tagged HAUSP is not a target for the proteasome.

However, cells in which the transfection mix had been replaced by fresh medium and incubated for 8hr post transfection had an approximately two fold increase in the number of pp65-tagged HAUSP expressing cells (irrespective of whether or not proteasome inhibitor was present), compared to cells that were fixed straight after the 24hr transfection period. This suggests that replacement of transfection mix with fresh medium may increase the number of detectable pp65-tagged HAUSP expressing cells. In order to test this, it was decided to transfect Hep2 cells with pp65CMV135, using the DOTAP transfection method, and leave the transfection mix on the cells for either 10hr, 14hr or 24hr and then replace it with fresh medium. Indirect immunofluorescence was then performed 24hr after the start of transfection using both the anti-pp65 MAb and r201 serum in the primary antibody mix. Examination of the coverslips revealed that replacement of the transfection mix with fresh medium 10hr after transfection gave the highest number of detectable pp65-tagged HAUSP expressing cells. All transfections done subsequently were based on this protocol.

3B6.2 Investigation of the effect of expression of pp65-tagged HAUSP on the state of the cell

As explained previously, the low levels of cells expressing detectable pp65-tagged HAUSP may have been the result of targeting cells overexpressing HAUSP for apoptosis. An observation which may support this theory was that a minor proportion of the pp65-tagged HAUSP expressing cells appeared sickly and often blebing of the plasma membranes was seen, similar to that observed for apoptotic cells (Figure 3B6.2). Thus it was decided to further investigate whether these cells had other features of apoptotic cells such as alterations in their nuclear structure (Martin *et al.*, 1994). Hep2 cells were transfected with pp65-tagged HAUSP harbouring plasmid and indirect immunofluorescence performed using anti-pp65 MAb as the primary antibody. The fluorochrome DAPI which stains DNA was also used. A fair representation of the results of this experiment are illustrated in Figure 3B6.2. Panel A shows the most prominent phenotype observed for a pp65-tagged HAUSP positively transfected cell in which the cell membrane appears normal. Panel B

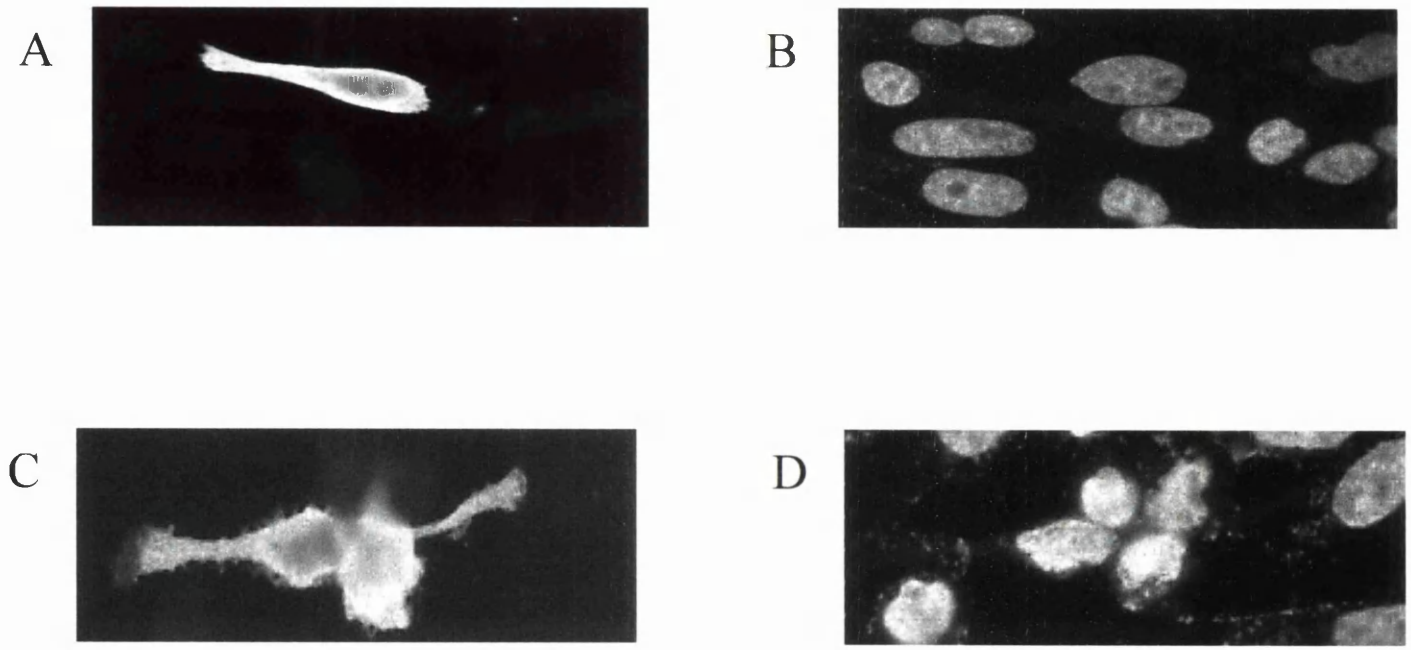


Figure 3B6.2: Immunofluorescence experiment illustrating the appearance and location of DNA in cells expressing pp65-tagged HAUSP.
Hep2 cells were transfected with pp65CMV135 and incubated for 10hr, after which the DNA/DOTAP/medium mix was replaced by fresh medium and incubated for a further 14hr (Section 2B3.2). Indirect immunofluorescence was then carried out on these cells, using the anti-pp65 MAb antibody and FITC labelled goat anti-mouse IgG secondary antibody (Section 2B3.3). Coverslips were then mounted on microscope slides using a citifluor solution in which the fluorochrome DAPI was present. A representation of the phenotypes observed after examination of several thousand cells can be seen in panels A-D. Panels A/B and panels C/D show the same field of cells. In the left hand panels (A/C) cells probed for pp65-tagged HAUSP expression have been illustrated and in the right hand panels (B/D) cells probed for DNA have been illustrated.

represents the same field of cells and shows the distribution of the DNA. Thus it is clear that, in this case, tagged HAUSP expression does not significantly alter DNA location. However panels C and D illustrate two cells positively transfected with tagged HAUSP that have the membranous blebing effect previously described. It is interesting to see that the DNA appearance within these cells appears somewhat altered. Changes in or degradation of nuclear architecture are one of the signs of apoptosis, but it would be necessary to investigate these phenotypes in more detail in order to come to any conclusion on the fate of overexpressing exogenous HAUSP.

3B6.3 Investigation into the effect of enzymatic inactivation of HAUSP for transient expression in eukaryotic cells

Another possible reason for the low level of pp65-tagged HAUSP expressing cells is due to HAUSP enzyme activity being toxic to the cells. Thus to investigate what effect lack of deubiquitinase activity would have on HAUSP transient expression in eukaryotic cells, an enzymatically inactive version of pp65CMV135 was made. The C223S pp65CMV135 mutant was made by subcloning a small fragment of the HAUSP coding region in which the C223S mutation had been incorporated into the relevant sites of the wild type pp65CMV135 plasmid. In particular, a ligation reaction was set up between the *Bsr*GI(193)-*Bsr*GI(241) C223S pET24a135 insert fragment and the *Bsr*G1(241)-*Bsr*G1(193) wild type pp65CMV135 vector fragment (see Figure 3A3.2 for positions of these sites within the HAUSP coding sequence).

The C223S pp65CMV135 mutant was then transfected alongside wild type pp65CMV135 in Hep2 cells and indirect immunofluorescence was carried out using both the anti-pp65 MAb and r201 serum in the primary antibody mix. No differences were observed between the level or distribution of tagged HAUSP expression in cells positively transfected with wild type or mutant pp65-tagged HAUSP. However, an approximately three fold increase in the number of positive cells expressing tagged HAUSP was seen for the enzyme inactive mutant. Thus, this work suggests that HAUSP enzyme activity is toxic to the cells and as such excess production of active HAUSP may cause cell death.

3B7 Investigation of the distribution of transiently expressed tagged HAUSP within Hep2 cells

Once pp65-tagged HAUSP expression in eukaryotic cells was optimised to an acceptable level the next stage was to design indirect immunofluorescence experiments to observe the

distribution of tagged HAUSP within the cells, the results of which can be seen in Figure 3B7. From the several thousand cells examined, the dominant phenotype observed was that shown in panel A where pp65-tagged HAUSP is mainly located in the cytoplasm. The next most common phenotype is displayed in panel B where the pp65-tagged HAUSP is both located in the nucleus and cytoplasm, while panel C shows the least prominent, but still significant phenotype in which pp65-tagged HAUSP expression is mainly in the nucleus. In contrast to this endogenous HAUSP is mainly located in the nucleus in a diffusely punctate form as part of the ND10 domains (Everett *et al.*, 1997). It has been speculated that this punctate localisation is a result of protein-protein interactions between HAUSP and a component of the ND10 domains. The observed difference between the distribution of endogenous and exogenous HAUSP may be a result of the exogenous HAUSP expression exceeding the level of the ND10 protein with which it interacts, resulting in excess HAUSP being relocated to the cytoplasm.

3B8 Characterisation of exogenously expressed HAUSP

To better characterise the pp65-tagged HAUSP being expressed, pp65CMV135 was transfected into Hep2 cells and indirect immunofluorescence carried out using both the anti-pp65 MAb and either: the N- or C-terminal anti-HAUSP serum in the primary antibody mix. The results of these experiments are displayed in Figure 3B8a and 3B8b. As can be seen from Figure 3B8a, cells which appeared to be positively transfected with the pp65-tagged HAUSP (detected using the anti-pp65 MAb), gave a similar staining pattern when probed using the anti-N-terminus HAUSP anti-peptide antibody r206. This suggests that the N-terminus of pp65-tagged HAUSP is expressed correctly.

More interestingly, the cells which appeared to express pp65-tagged HAUSP (detected using the anti-pp65 antibody), did not give a similar staining pattern when probed using the anti-C-terminus HAUSP anti-peptide antibody r201. Indeed, a number of different phenotypes were seen (Figure 3B8b Panels A-F). The most predominant phenotype, as before, was of cells which express pp65-tagged HAUSP mainly in the cytoplasm (Panel A). Surprisingly, in these cells the C-terminus of HAUSP was only detected in the nucleus at a level comparable to that of the surrounding mock transfected cells (Panel B). This suggests that pp65-tagged HAUSP, but not endogenous HAUSP, is being truncated at the C-terminal. This could be the result of cleavage by a cellular protease, but why only the tagged HAUSP should be targeted is unclear. A second phenotype observed was for cells

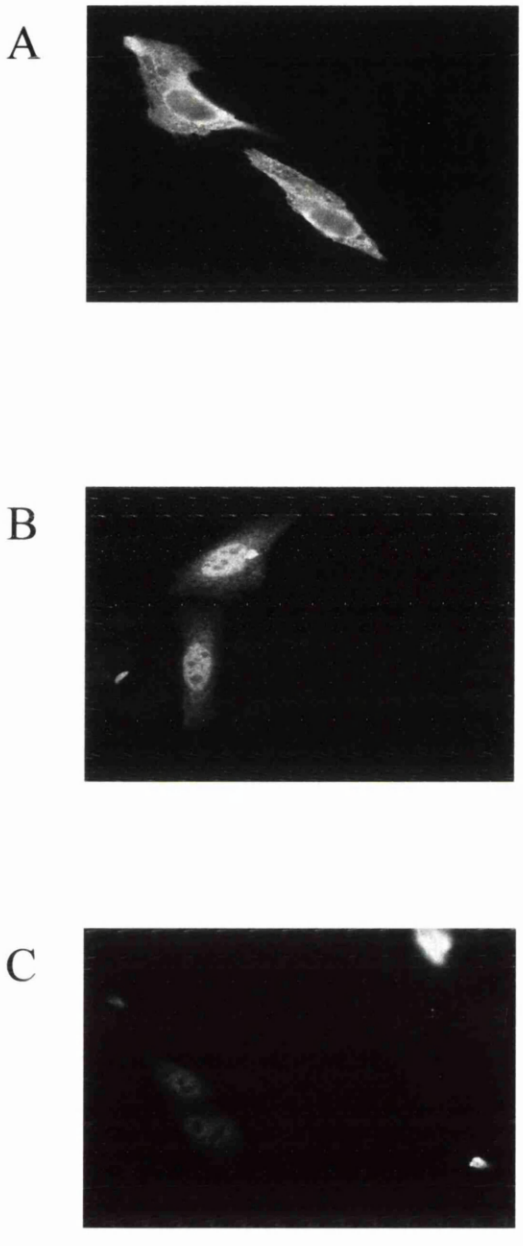


Figure 3B7: Indirect immunofluorescence experiment to investigate the cellular location of transiently expressed pp65-tagged HAUSP.
Hep2 cells were transfected with pp65CMV135 and incubated for 10hr, after which the DNA/DOTAP/medium mix was replaced by fresh medium and incubated for a further 14hr (Section 2B3.2). Indirect immunofluorescence was then carried out on these cells using the MAb anti-pp65 antibody and FITC labelled goat anti-mouse IgG secondary antibody (as described in Section 2B3.3). The fields of cells shown have been selected, after examination of several thousand cells, to illustrate the range of phenotypes observed. The dominant phenotype was that shown in panel A where pp65-tagged HAUSP is mainly located in the cytoplasm. Panel B displays the next most common phenotype where the pp65-tagged HAUSP is located in both the nucleus and cytoplasm, while panel C shows the least prominent, but still significant, phenotype in which pp65-tagged HAUSP expression is observed mainly in the nucleus.

A

B

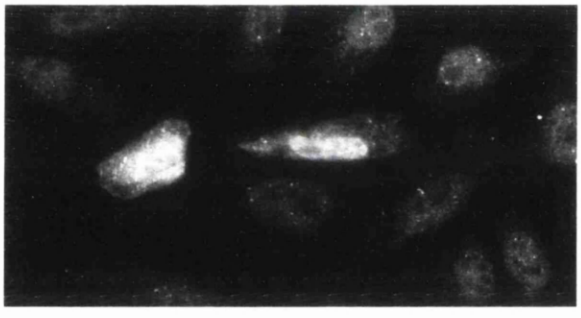


Figure 3B8a: Immunofluorescence experiment illustrating the quality of the N-terminus of the pp65-tagged HAUSP protein.
Hep2 cells were transfected with pp65CMV135 and incubated for 10hr, after which the DNA/DOTAP/medium mix was replaced by fresh medium and incubated for a further 14hr (Section 2B3.2). Indirect immunofluorescence was then carried out on these cells using both the anti-pp65 MAb and the anti N-terminal HAUSP serum r206 in the primary antibody mix (as described in Section 2B3.3).
Panels A and B show the main phenotype observed after examination of several thousand cells. Binding against pp65 was detected using the FITC labelled goat anti-mouse IgG (illustrated in panel A). Binding against HAUSP was detected using TRITC labelled goat anti-rabbit IgG (illustrated in panel B).

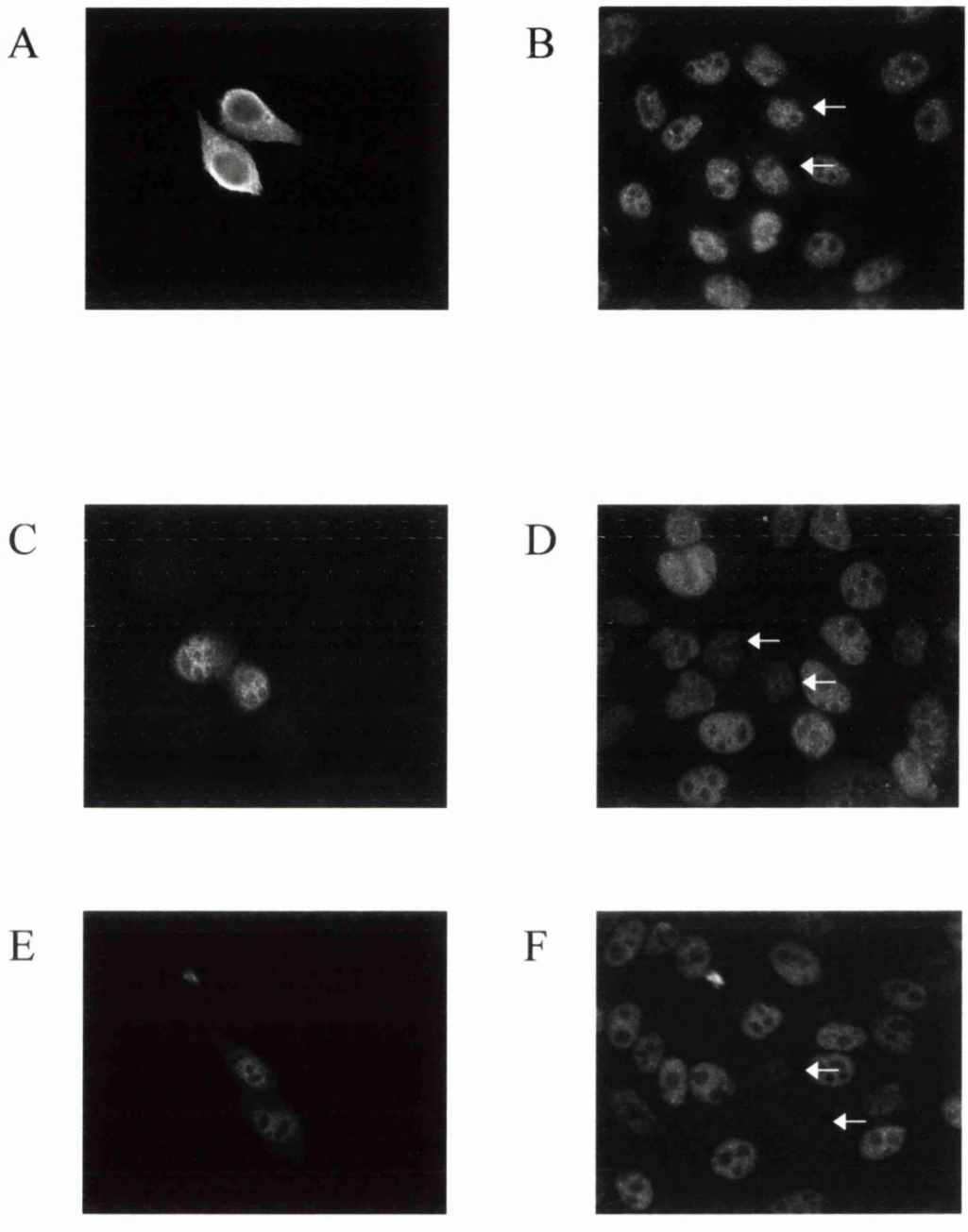


Figure 3B8b: Immunofluorescence experiment illustrating the quality of the C-terminus of the pp65-tagged HAUSP protein and the effect of exogenous HAUSP expression on levels of endogenous HAUSP.
Hep2 cells were transfected with pp65CMV135 and incubated for 10hr, after which the DNA/DOTAP/medium mix was replaced by fresh medium and incubated for a further 14hr (Section 2B3.2). Indirect immunofluorescence was then carried out on these cells, using both the anti-pp65 MAb and the anti C-terminal HAUSP serum r201 in the primary antibody mix (as described in Section 2B3.3).
After examination of several thousand cells three main phenotypes were observed. These three phenotypes can are displayed in panels A/B, C/D and E/F. Binding against pp65 was detected using the FITC labelled goat anti-mouse IgG (illustrated in left hand panels A,C and E). Binding against HAUSP was detected using TRITC labelled goat anti-rabbit IgG (illustrated in right hand panels B, D and F). The white arrows illustrated in the right hand panels indicate the position of the cells shown in the left hand panels.

in which the pp65-tagged HAUSP was expressed both in the cytoplasm and nucleus (Panel C). In this case the level of HAUSP C-termini detected in the nucleus was lower than that observed for the surrounding cells (Panel D). This suggests that when the C-terminal truncated tagged HAUSP enters the nucleus, it may displace the endogenous full length HAUSP to the cytoplasm or target it for cleavage or degradation. This is further supported by a third phenotype observed where the cells express pp65-tagged HAUSP mainly in the nucleus (Panel E). In these cases no C-termini of HAUSP were detected in the nucleus (Panel F). This strengthens the theory that a protein exists in the ND10 domains which interacts with HAUSP and which limits its nuclear localisation. Furthermore, it implies that this interaction does not require the C-terminal of HAUSP. Alternatively, localisation of exogenously expressed HAUSP in the nucleus results in partial degradation of endogenous HAUSP.

3B9 Investigation of the effect of expression of pp65-tagged HAUSP on distribution of the ND10 constituent PML

As it has been observed that HAUSP colocalises with PML in a subset of ND10 domains (Everett *et al.*, 1997). It was decided to investigate the effect of expression of tagged HAUSP on the distribution and level of endogenous PML. This was done in order to explore the idea that PIC-1, or ubiquitin-tagged PML may be substrates of HAUSP. As such, it was thought pp65-tagged HAUSP expression may cause a change in the stability of endogenous PML, which would result in a change in its levels observed within the cell. Thus cells were transfected with the plasmid encoding pp65-tagged HAUSP and indirect immunofluorescence carried out using the anti-pp65 MAb and anti-PML rabbit serum r8 in the primary antibody mix. A fair representation of the results have been provided in Figure 3B9. No significant alteration can be seen in either the distribution or level of endogenous PML expressed in cells positively transfected with pp65-tagged HAUSP in comparison to the surrounding untransfected cells. This result suggests that alteration of the intracellular levels of HAUSP by transfection does not effect PML or ND10 domains.

3B10 Investigation into the role of different domains of HAUSP for transient expression in eukaryotic cells

It was postulated that by expressing only specific regions of HAUSP we would be able to formulate a clearer idea of the role each region played in the expression of HAUSP in the cell. Therefore, N- and C-terminal mutants of HAUSP were constructed.

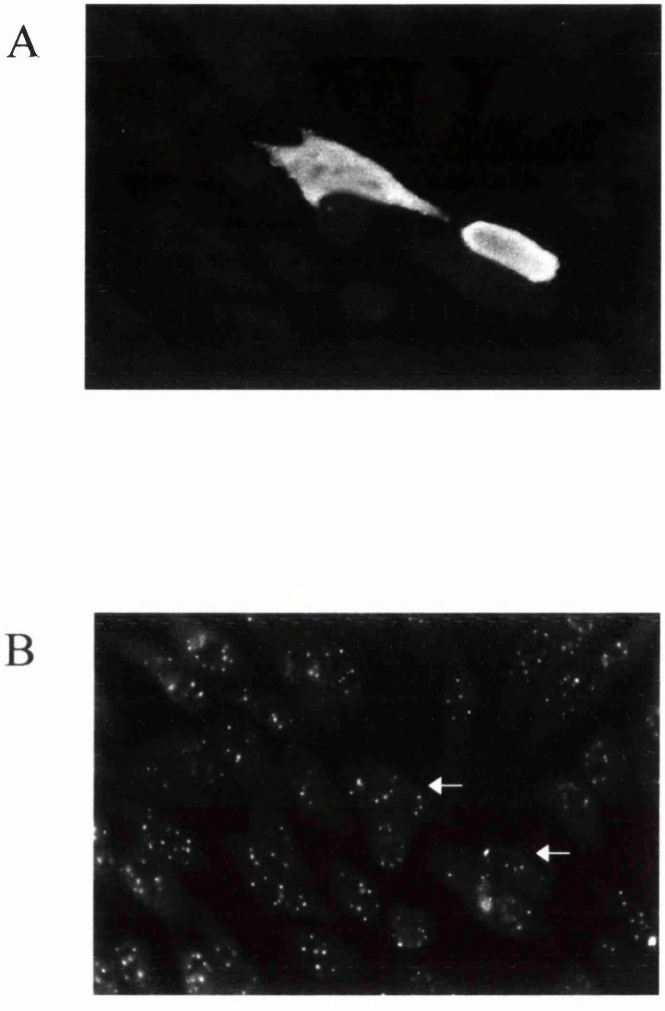


Figure 3B9: Immunofluorescence experiment illustrating that the level of endogenous PML is not altered by expression of pp65-tagged HAUSP.
Hep2 cells were transfected with pp65CMV135 and incubated for 10hr, after which the DNA/DOTAP/medium mix was replaced by fresh medium and incubated for a further 14hr (Section 2B3.2). Indirect immunofluorescence was then carried out on these cells, using both the anti-pp65 MAb and anti-PML serum r8 in the primary antibody mix (as described in Section 2B3.3).
Panels A and B show the same field of cells. FITC labelled goat anti-mouse IgG was used to detect the pp65 binding (panel A) and TRITC labelled goat anti-rabbit IgG was used to detect the PML binding (panel B). The white arrows in panel B illustrate the position of the cells shown in panel A.

The 18 residue N-terminal pp65CMV135 deletion mutant was synthesised in a ligation reaction with three fragments: the *Xba*I-*Nde*I(1) pp65CMV135 vector fragment (in which the *Nde*I site was blunt ended), the *Ava*I(18)-*Bst*EII(529) pp65CMV135 HAUSP fragment (in which the *Ava*I site was blunt ended) and the *Bst*EII(529)-*Xba*I pp65CMV135 fragment (see Figure 3B10). The junction between the pp65 and HAUSP encoding regions was checked by sequencing (Section 2B1.9) using the sequencing primer 1868 (Table 2B1.9).

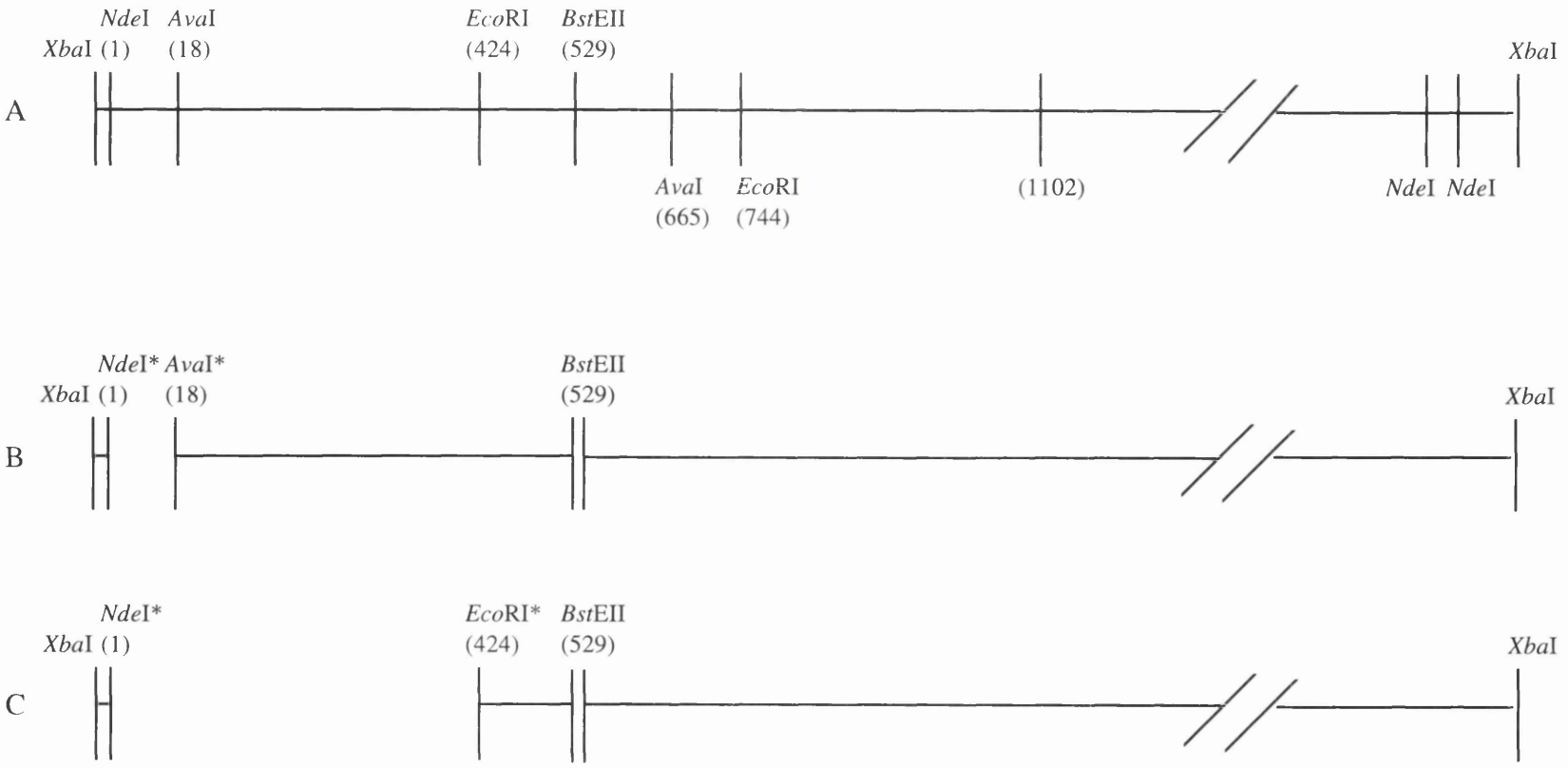
The 424 residue N-terminal pp65CMV135 deletion mutant was synthesised in a ligation reaction with three fragments: the *Xba*I-*Nde*I(1) pp65CMV135 vector fragment (in which the *Nde*I site was blunt ended), *Eco*RI(424)-*Bst*EII(529) pp65CMV135 HAUSP fragment (in which the *Eco*RI site was blunt ended) and the *Bst*EII(529)-*Xba*I pp65CMV135 fragment (see Figure 3B10). The junction between the pp65 and HAUSP encoding regions was checked by sequencing (Section 2B1.9) using the sequencing primer 424NTRUNC (Table 2B1.9).

The 441 residue C-terminal truncated pp65CMV135 mutant was synthesised by ligation of a stop linker (Table 2B1.9) into the pp65CMV135 plasmid which had been linearised within the sequence encoding residue 441 of HAUSP. This linearised pp65CMV135 plasmid was isolated after digestion of pp65CMV135 with *Bsu*36I(441) followed by blunt ending and dephosphorylation of the fragment (Section 2B1.2).

The 576 residue C-terminal truncated pp65CMV135 mutant was synthesised by ligation of a stop linker (Table 2B1.9) into the pp65CMV135 plasmid which had been linearised within the sequence encoding residue 576 of HAUSP. This linearised pp65CMV135 plasmid was isolated after digestion of pp65CMV135 with *Msc*I(576) followed by blunt ending and dephosphorylation of the fragment (Section 2B1.2). The correct incorporation of the termination signal at codon 576 was checked by sequencing (Section 2B1.9) using the sequencing primer H+8 (Table 2B1.9).

The 880 residue C-terminal truncated pp65CMV135 mutant was synthesised by ligation of a stop linker (Table 2B1.9) into the pp65CMV135 plasmid which had been linearised within the sequence encoding residue 880 of HAUSP. This linearised pp65CMV135 plasmid was isolated after digestion of pp65CMV135 with *Afl*II(880), followed by blunt ending and dephosphorylation of the fragment (Section 2B1.2).

Figure 3B10: Construction of the N-terminal pp65CMV135 deletion mutants.
Part A: Shows a linearised plasmid map of pp65CMV135 in which all the restriction sites have been indicated for the enzymes used in the construction of the N-terminal pp65CMV135 deletion mutants. The restriction sites above the line are the ones which were directly used for the cloning procedure. Positions 1-1102 represent the HAUSP coding sequence which has been drawn to scale, unlike the vector sequences marked by the // sign.
Part B: Shows the three pp65CMV135 fragments used in the ligation reaction for the construction of the 18 residue N-terminal pp65CMV135 deletion mutant. Restriction sites which were blunt ended have been indicated by a * sign.
Part C: Shows the three pp65CMV135 fragments used in the ligation reaction for the construction of the 424 residue N-terminal pp65CMV135 deletion mutant. Restriction sites which were blunt ended have been indicated by a * sign.


A
NdeI AvaI
XbaI (1) (18)
EcoRI BstEII
(424) (529)
XbaI
AvaI EcoRI
(665) (744)
(1102)
NdeI NdeI
B
NdeI* AvaI*
XbaI (1) (18)
BstEII
(529)
XbaI
C
NdeI*
XbaI (1)
EcoRI* BstEII
(424) (529)
XbaI

The wild type and mutant pp65CMV135 plasmids were transfected into Hep2 cells and indirect immunofluorescence carried out using both the anti-pp65 MAb and either r201 serum (in the case of the N-terminal deletion mutants), or r206 serum (in the case of the wild type and C-terminal truncated mutants), in the primary antibody mix. Examination of these transfected cells showed no significant differences in subcellular distribution or level of HAUSP expression between cells expressing the 18 residue N-terminal deleted, 441 residue C-terminal truncated or 880 residue C-terminal truncated mutants of pp65-tagged HAUSP with that of cells expressing wild type pp65-tagged HAUSP. As such no solid conclusions could be made as to the roles of these missing regions for HAUSP function within the cell.

Interestingly, expression was not detectable for the 424 residue N-terminal deleted or 576 residue C-terminal truncated mutants of tagged HAUSP. The reason for this lack of expression is unclear. However, the possibility existed that loss of the respective residues from these mutants lead to their improper folding or deletion of a stability element which resulted in targeting them for degradation. In order to test whether these mutant proteins were being degraded by the proteasome, Hep2 cells were first transfected with these mutants and left for 24hr (Section 2B3.2). Next the DNA/DOTAP/medium transfection mix was replaced with fresh medium containing MG132 to a final concentration of 5μM and the cells incubated for a further 8hr. Indirect immunofluorescence was then carried out using both the anti-pp65 MAb and either: r201 serum (for the N-terminal deletion mutant) or r206 serum (for the C-terminal truncated HAUSP mutant), in the primary antibody mix. However, no pp65-tagged HAUSP expression was detectable.

3B11 Summary of the investigation into the effects of transiently expressing HAUSP in eukaryotic cells

- Exogenous expression of pp65-tagged HAUSP was detectable. As exogenous expression of untagged HAUSP was not detectable it was suggested that the N-terminal pp65 tag is required for stable expression.

- Expression of pp65-tagged HAUSP was detectable in a lower number of cells compared with the expression of another exogenously expressed protein. This implied that intracellular levels of HAUSP are closely monitored.

- Investigations into possible causes for the low number of detectable pp65-tagged HAUSP expressing cells suggested:

1. Exogenously expressed pp65-tagged HAUSP is not degraded by the proteasome.
2. Exogenous expression of HAUSP may be triggering cells for apoptosis.
3. The deubiquitinase activity of exogenously expressed HAUSP may be toxic for cells.

- Although pp65-tagged HAUSP expression was observed in the nucleus, its expression in the cytoplasm was the dominant phenotype observed. This is in contrast to the endogenous HAUSP which is expressed in punctate domains in the nucleus.

- Characterisation of the pp65-tagged HAUSP suggested that it was truncated at its C-terminal end.

- Exogenous expression of pp65-tagged HAUSP does not effect the level or distribution of the ND10 constituent PML.

- Expression of various N-terminal deletion and C-terminal truncation mutants of pp65-tagged HAUSP did not alter the distribution pattern observed for the protein.

3C Identification of cellular proteins that interact with HAUSP

3C1 Introduction

A key to understanding the function of a protein in a cell often lies in understanding its interactions with other proteins. As such it was decided to initiate a search for the cellular proteins which interact with HAUSP.

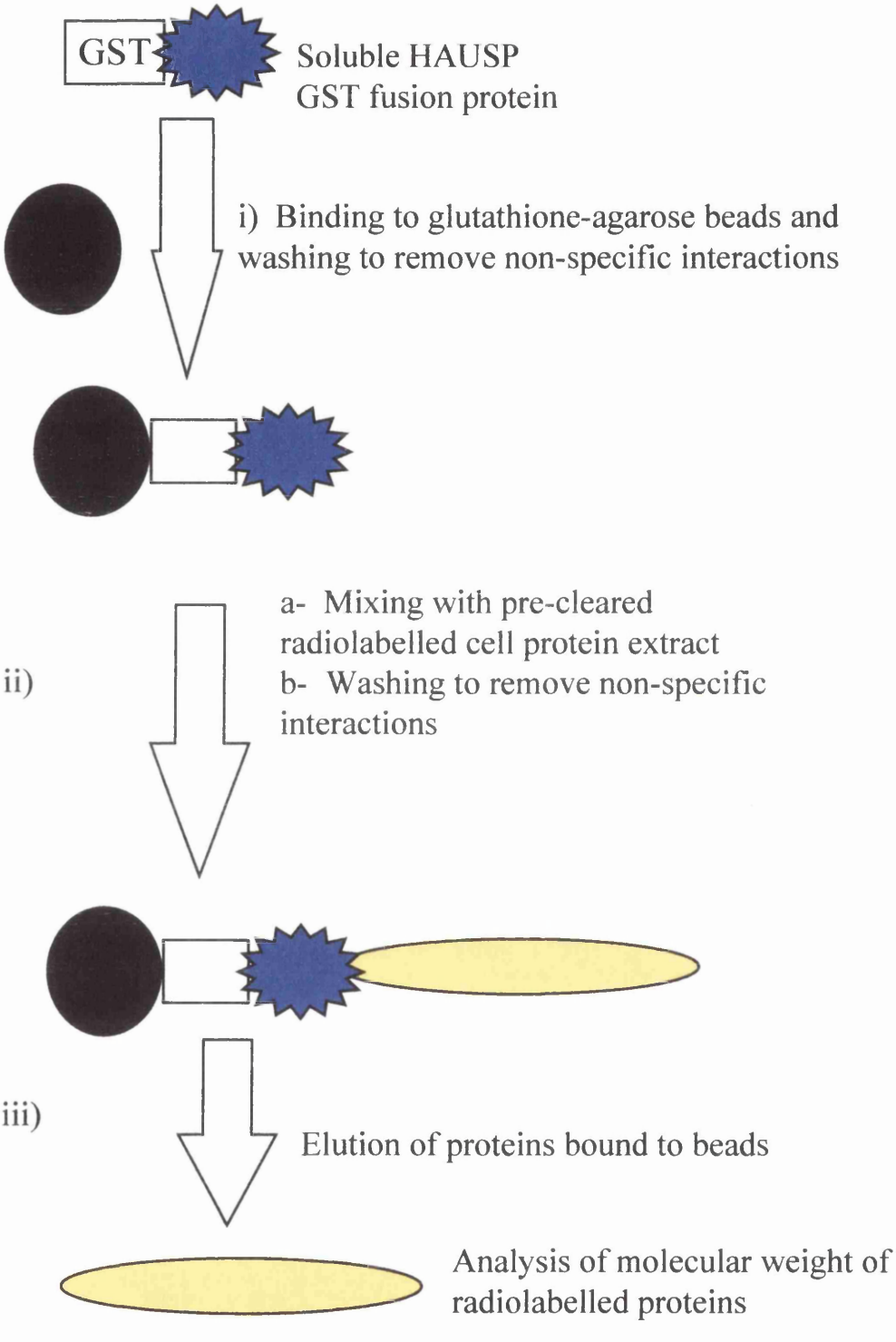
From the wide variety of methods that exist to detect specific protein-protein interactions, it was decided to use one based on the GST 'pull-down' technique. This technique involves the synthesis of fusion proteins between a protein, or protein segment, of interest and GST. The expressed GST fusion proteins are then purified from bacterial lysates by adsorption to glutathione agarose beads (Smith & Johnson, 1988). These beads, on which the GST fusion proteins are immobilised, are then used as 'bait'. In this manner, proteins which naturally interact with the GST fusion proteins can then be purified from a sample, first by the addition of the sample to the beads and then subsequent washing of the beads which involves their centrifugation.

The fundamental reasoning for using this technique is that it had been used successfully as the method by which HAUSP itself was identified as the protein that interacts with Vmw110 (Meredith *et al.*, 1994). In addition to this, results arising from this work will be used to support the work of another member of the group who is using the yeast two hybrid assay to identify cellular proteins that interact with HAUSP.

An outline of the 'pull-down' designed to identify cellular proteins that interact with HAUSP is given in Figure 3C1. The design of the initial 'pull-down' assay implemented was based on the conditions used originally to identify HAUSP as being a cellular protein that interacts with Vmw110 (Meredith *et al.*, 1994). Thus, by using conditions under which it is known that Vmw110 and HAUSP would interact, it was hoped that any cellular proteins identified as interacting with HAUSP would be of biological significance.

This section describes experiments based on this assay which led to the identification of ~40kD, 100kD and 105kD cellular proteins that strongly interact with specific regions of HAUSP. Furthermore, it describes the use of immunoprecipitation to support this data. It also describes the use of this assay to define more precisely the residues of HAUSP required for these interactions. Finally the GST 'pull-down' assay was also used to

Figure 3C1: Outline of the GST 'pull-down' assay implemented to identify cellular proteins that interact with HAUSP.
Bacterial extracts containing GST fusion proteins were mixed with glutathione agarose beads and the beads-fusion complexes were then washed to remove non-specific interactions.
Aliquots of the beads were run on a 10% SDS-PAGE gel and coomassie stained. This was done in order to calculate the amounts of beads-fusion complexes to be used, such that the fusion proteins would be present in equalised quantities (normalised) for subsequent 'pull-down' experiments.
Normalised amounts of beads-fusion complexes were mixed with pre-cleared radiolabelled soluble cell extract and washed to remove non-specific interactions.
Proteins bound to the beads were then eluted by competition with reduced glutathione. The eluted proteins were separated on an SDS-PAGE gel and transferred to a nitrocellulose filter. The molecular weight of any eluted radiolabelled proteins was observed by exposing the nitrocellulose blot to a film.


GST
Soluble HAUSP
GST fusion protein
i) Binding to glutathione-agarose beads and
washing to remove non-specific interactions
ii)
a- Mixing with pre-cleared
radiolabelled cell protein extract
b- Washing to remove non-specific
interactions
iii)
Elution of proteins bound to beads
Analysis of molecular weight of
radiolabelled proteins

investigate the susceptibility of the ~40kD, 100kD and 105kD proteins to proteasomal degradation.

3C2 Targeting of regions within HAUSP predicted to be important for protein-protein interactions

The first stage in this process was to synthesise HAUSP GST fusion proteins encoding regions of HAUSP potentially important for protein-protein interactions. Such regions were first predicted using computer protein structure prediction packages such as PepPlot, Plot Structure and Helical Wheel which predicted the secondary structure of HAUSP. The most significant findings from these analyses were those indicating that HAUSP had regions which were strongly predicted to form α-helices. As can be seen from Figure 3C2 the N-terminal 19 residues and segments between residues 515-572, 920-960 and 1008-1043 are predicated to have a high α-helical content. GenBank database searches using the NCBI non-redundant database were also performed. The database comparisons revealed no other highly conserved features outside the USP active site domains. However the results of this work did show that the regions specified to have high α-helical content also showed low level similarity to the helical bundles of the involucrin family of proteins which form an extended flexible rod and are thought to allow multiple intermolecular interactions (Yaffe *et al.*, 1992). Thus it was postulated that these helical regions in HAUSP were involved with protein-protein interactions. The regions of HAUSP to be used as bait in the GST 'pull-down' assay were chosen based on these results.

3C3 Plasmids expressing GST fusion proteins

The pGEX2TN3 plasmid (Figure 3C3) was used for expression of GST sequences and was used as a control in the 'pull-down' assay.

The pGEX4222 plasmid was used for expression of a HAUSP GST fusion protein encoding the HAUSP residues 26-1102.

The plasmids pGEX135N/B, pGEX135B/B, pGEX135A/P, pGEX135A/A', pGEX135A'/A'', pGEX135A''/P, pGEX135M/P, pGEX135A/H and pGEX135P/H were all derived from the pGEX2T vector (Figure 3C3). These plasmids were used for the expression of HAUSP GST fusion proteins encoding the HAUSP residues: 1-193, 241-441, 516-837, 513-581, 581-749, 749-837, 576-837, 516-1102 and 837-1102 respectively. An outline of how they were all constructed is given in Table 3C3.

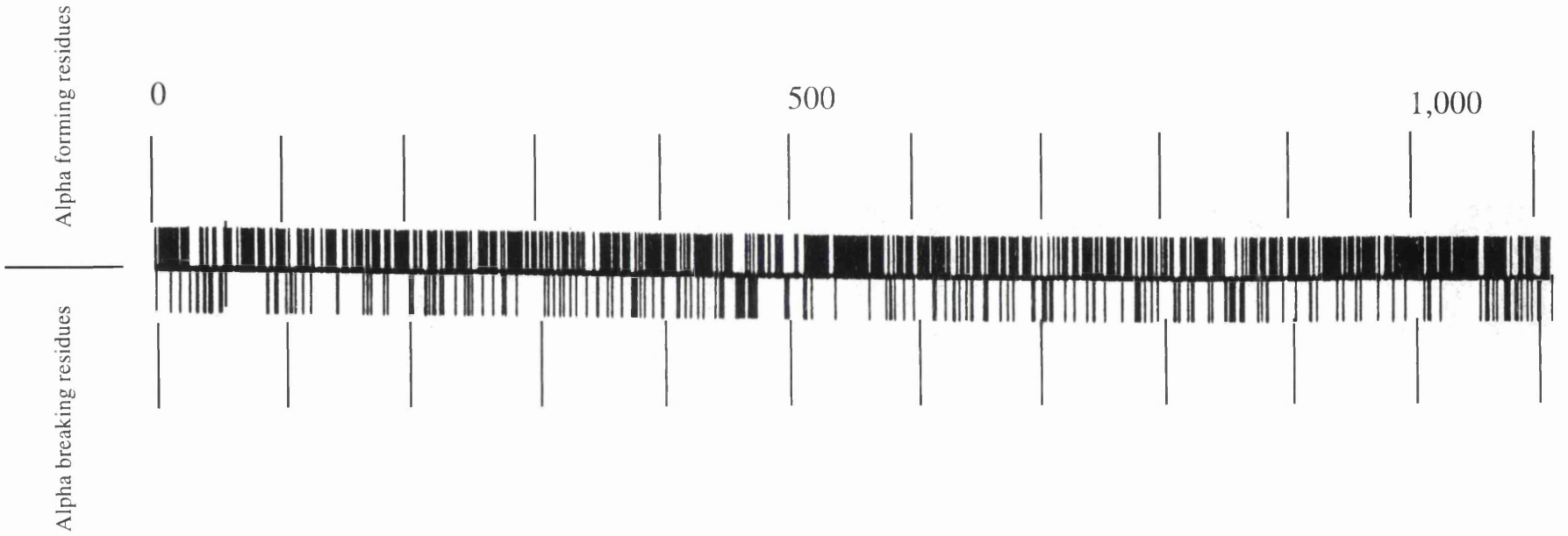
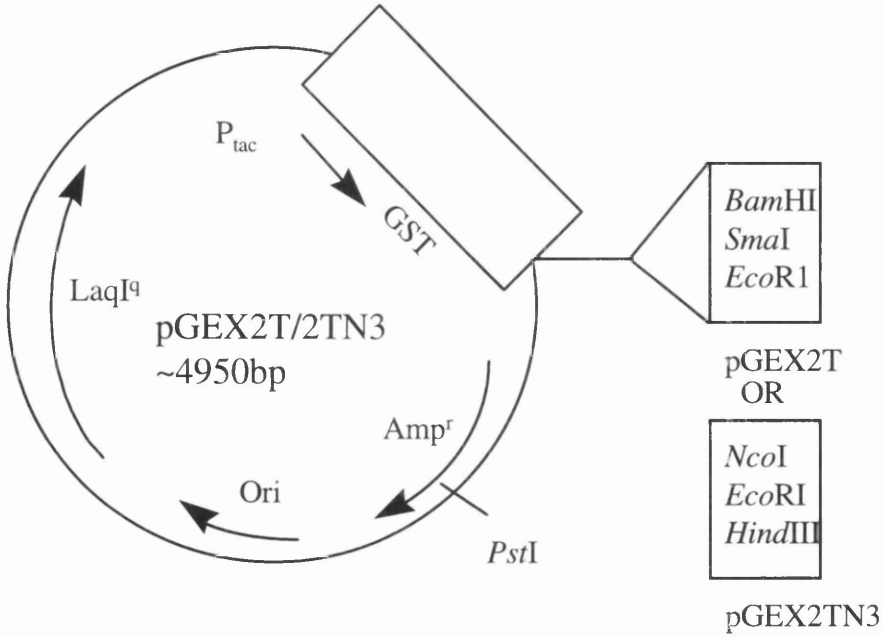


Figure 3C2: Prediction of the high alpha helical structure of HAUSP. The secondary structure of the translated HAUSP sequence was analysed using the GCG computer software package Pepplot. The diagram above shows the most significant results of this work in which the HAUSP residues most likely to form alpha helicies are marked above the central line and those that are predicted to break such a structure are marked below the line.

Figure 3C3: Structure of pGEX2T and pGEX2TN3.
Restriction sites relevant to the construction or further use of the plasmids are shown. The sequences encoding the C-terminal of GST have also been provided for both plasmids, underneath of which the positions of restriction sites have been indicated.



pGEX2T

P R G S P G I H R D STOP

CCGCGTGGATCCCCGGGAATTCATCGTGACTGACTGACTG

*Bam*HI *Eco*RI

*Sma*I

pGEX2TN3

P R G S T M A R I R K L N S S STOP

CCGCGTGGATCGACCATGGCTAGAATTCGGAAGCTTAATTCATCGTGACTGA

*Nco*I *Eco*RI *Hind*III

Clone	Vector fragment	HAUSP fragment	HAUSP residues encoded	5' junction sequence
pGEX135N/B	pGEX2T/*Sma*I#	*Nde*I*-*Bsr*GI*	1-193	ATC/CCC/ TAT/GAA
pGEX135B/B	pGEX2T/*Sma*I#	*Bsr*GI*-*Bsu*36I*	241-441	ATC/CCC/ GTA/CAT
pGEX135A/P	pGEX2T/*Sma*I#	*Acc*I*-*Pst*I*	516-837	ATC/CCC/ CTA/CAT
pGEX135A/H	pGEX2T/*Sma*I-*Pst*I pGEX2TN3/*Pst*I-*Hind*III	*Acc*I*-*Hind*III	516-1102	ATC/CCC/ CTA/CAT
pGEX135P/H	pGEX2T/*Sma*I-*Pst*I pGEX2TN3/*Pst*I-*Hind*III	*Pst*I*-*Hind*III	837-1102	ATC/CCC/ GTT/TTT
pGEX135A/A'	pGEX2T/*Sma*I#	*Afl*III*-*Afl*III*	513-581	ATC/CCC/ CAT/GTT
pGEX135A/A''	pGEX2T/*Sma*I#	*Afl*III*-*Afl*III*	581-749	ATC/CCC/ CGT/GTC
pGEX135A''/P	pGEX2T/*Sma*I#	*Afl*III*-*Pst*I*	749-837	ATC/CCC/ CAT/GTA
pGEX135M/P	pGEX2T/*Sma*I#	*Msc*I-*Pst*I*	576-837	ATC/CCC/ CCA/CCA

Table 3C3: The construction of various plasmids encoding HAUSP GST fusion proteins.
Each HAUSP GST fusion protein encoding plasmid was constructed in a ligation reaction with fragments isolated from the relevant digestion mixes of the pGEX2T/2TN3 vector(s) and HAUSP encoding plasmids. A # sign indicates that this vector fragment has been dephosphorylated. A * sign indicates the restriction site next to which it has been marked has been blunt ended. Six bases either side of the 5' junction between sequences encoding GST and HAUSP have been listed for each clone.

The pGEX135B/E plasmid was used for the expression of a GST fusion protein encoding HAUSP residues 516-744. It was made in a ligation reaction with two fragments isolated from the relevant digestion mixes, the *Bam*HI-*Eco*RI pGEX2T vector fragment and the *Bam*HI-*Eco*RI(744) pGEX135A/P HAUSP fragment.

The pGEX4222/B plasmid was used for the expression of a GST fusion protein encoding HAUSP residues 26-193. It was made by first digesting the pGEX4222 plasmid with *Bsr*GI and *Hind*III and blunt ending the resulting fragments. The large *Hind*III-*Bsr*GI(193) fragment was then isolated and self-ligated.

The junction between the GST and HAUSP encoding regions of the pGEX plasmids constructed were all checked by sequencing (Section 2B1.9), using the sequencing primer GEX2T (Table 2B1.9).

3C4 Expression of GST fusion proteins

The expression of GST fusion proteins was done in several stages. Firstly, the pGEX plasmids were transformed into the *E.coli* BL21 strain (Section 2B4.2). The next step was to grow cultures in which protein expression was induced from the P_{tac} promoter by addition of IPTG and from these cultures protein extracts were prepared as described in Section 2B4.3.

Expression of the fusion proteins was detected by mixing bacterial extracts containing GST fusion proteins with glutathione agarose beads and washing them with PBS(A) to remove any non-specific interactions. Aliquots of these beads were analysed on SDS-PAGE gels and the gels coomassie stained. As expression of the proteins varied, a second gel was run on which the quantity of beads used was adjusted so that each fusion protein was present in equalised (normalised) amounts. The coomassie stained gels on which normalised levels of these GST fusion proteins have been run can be seen in Figures 3C4a and 3C4b.

The molecular weights were estimated by plotting a graph of the logarithmic molecular weight of the standard proteins against distance migrated on the acrylamide gel. These results were then compared to the predicted molecular weight calculated using the GCG 'Translate' computer program to ensure that the fusion proteins were of full length.

Despite attempts to normalise the amounts of beads-bound proteins run on the gels, it is still evident that some proteins either had high degradation levels, were bound to the beads

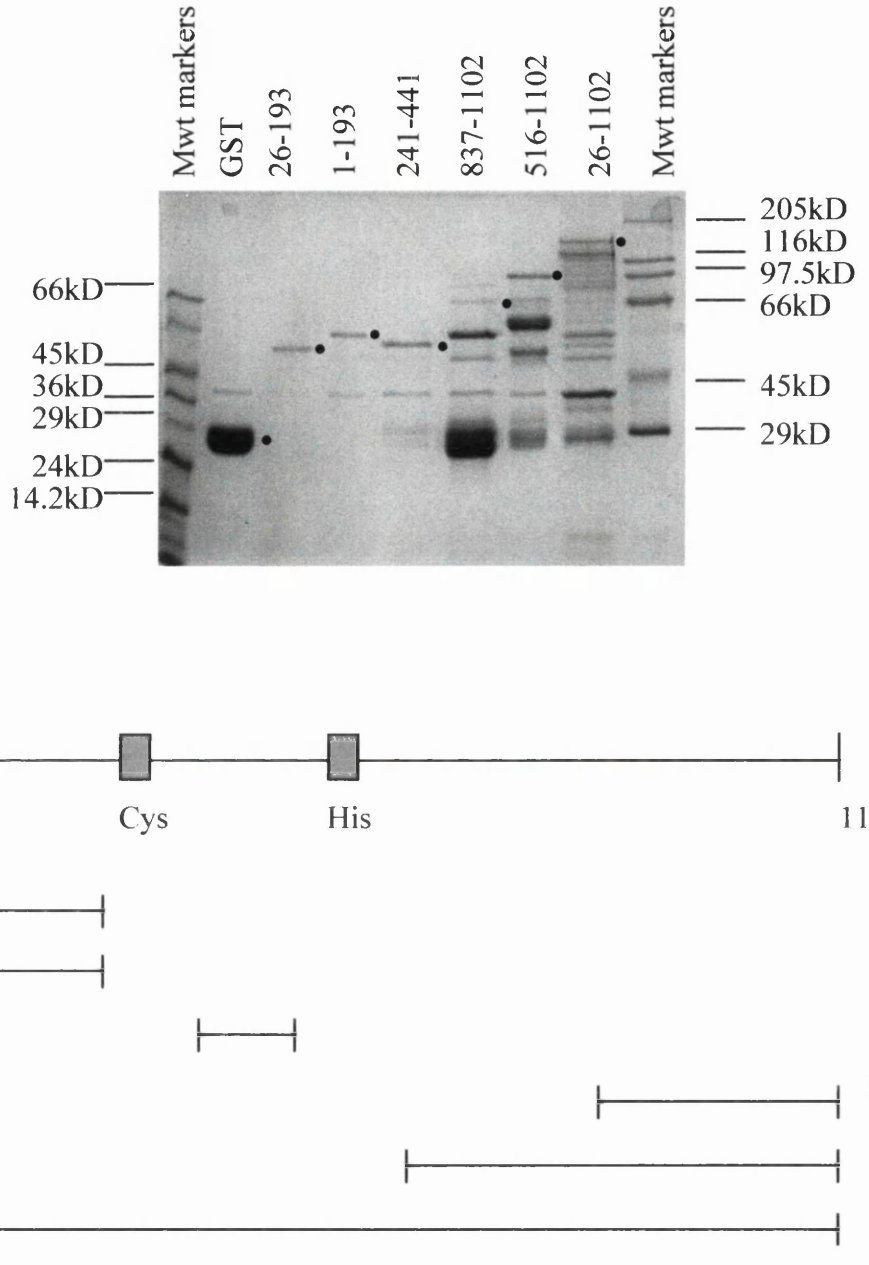


Figure 3C4a: Coomassie stained 10% polyacrylamide gel showing samples of fusion proteins bound to beads. Aliquots of bacterial extracts (300µl) were incubated with 30µl of a 50% slurry of glutathione agarose beads for 1hr at 4°C end-over-end. The beads were washed three times in 1ml cold PBS(A) and the beads-bound proteins eluted into 20µl reduced glutathione. The samples were then boiled for 2min after addition of 10µl 3X SDS loading buffer and 10µl aliquots loaded on a 10% SDS-PAGE gel, alongside molecular weight markers and the gel coomassie stained (Section 2B4.6). Based on these results another 10% polyacrylamide gel was run in which normalised amounts of the beads-bound fusion proteins were loaded. However, it is still evident that degradation of some fusion proteins was very high in comparison to others. The diagram underneath the gel illustrates HAUSP fragments expressed as fusion proteins within the context of the entire sequence of HAUSP.

The 'GST' track shows the eluants of the beads-bound to the GST protein. The numbered tracks represent the residues of HAUSP encoded by the purified GST HAUSP fusion protein that was loaded on that track. Bands representing the full length beads-bound fusion protein are marked with a • next to the corresponding band. The sizes of the molecular weight marker proteins are indicated at the side of the gel.

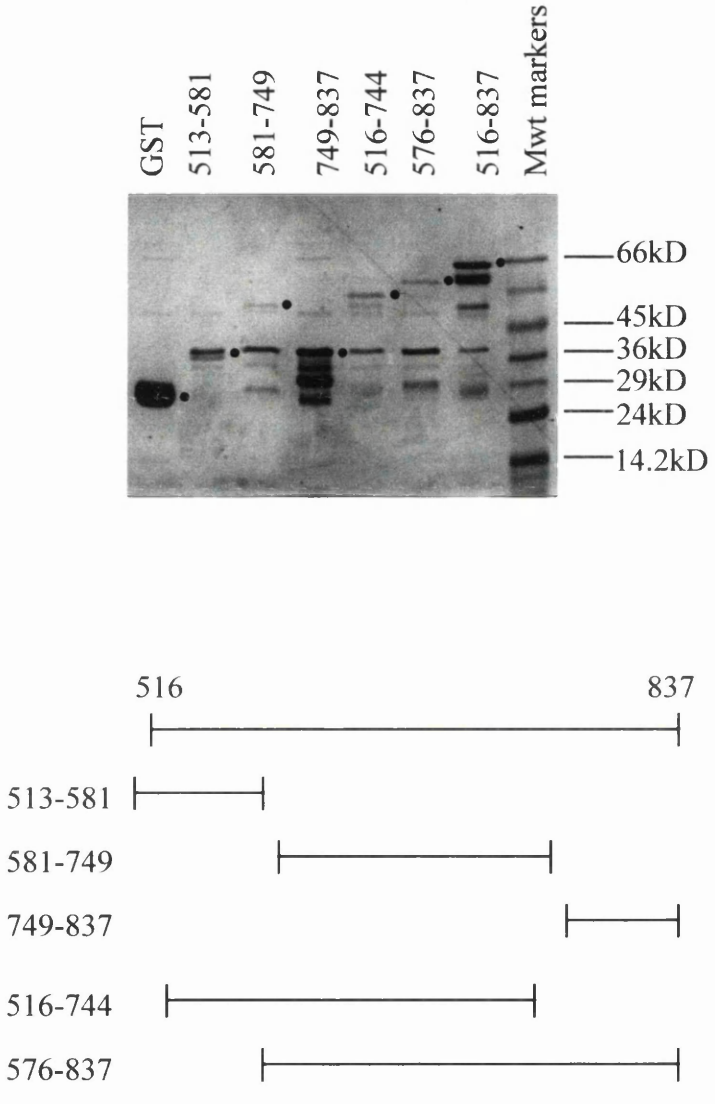


Figure 3C4b: Coomassie stained 12.5% polyacrylamide gel showing samples of fusion proteins bound to beads.

The above gel was prepared using the procedure outlined in the legend of Figure 3C4a, with the exception that a 12.5% SDS-PAGE gel was run in place of a 10% SDS-PAGE gel. A similar annotation pattern has been used as described in the legend of Figure 3C4a.

with less affinity or were expressed at reduced levels. For example, it is clear that the GST fusion proteins encoding the C-terminal regions of HAUSP are more susceptible to degradation. In general, the subclones of the 513-837 region appear to be expressed at reduced levels compared to the other fusion proteins. The GST fusion protein encoding the HAUSP residues 26-1102 was also produced at low levels. Thus, to minimise these problems, fresh extracts were generally used for the GST 'pull-down' assays. Secondly the amounts of the fusion proteins used in each assay were normalised, by running beads-fusion complexes on a gel and then adjusting the amounts of these complexes used for the subsequent 'pull-down' assay to provide equalised levels of the fusion proteins.

3C5 Initial experiment to identify cellular proteins that interact with segments of HAUSP

The initial experiment to identify cellular proteins that interact with segments of HAUSP involved carrying out a 'pull-down' assay using glutathione agarose beads bound to HAUSP GST fusion proteins encoding HAUSP residues 1-193 and 516-1102. These beads were mixed with radiolabelled WS HeLa cell extracts and then washed with a 0.2M NaCl wash buffer to remove non-specific interactions. Details of the experiment are explained in the legend of Figure 3C5.

The results of the autoradiographs of 7.5% and 12.5% SDS-PAGE gels on which the eluted samples of the 'pull-down' assay were run are shown in Figure 3C5. Bands that specifically interacted with HAUSP GST fusion proteins were clearly observable due to only a minimum of proteins being pulled down by the control GST beads. In particular, observation of the lower concentration (7.5%) acrylamide gel shows that the 1-193 HAUSP GST fusion protein appears to interact with 40kD, 100kD and 105kD cellular proteins and the 516-1102 HAUSP GST fusion protein with 40kD and 80kD cellular proteins (Figure 3C5 part A). The sizes of these proteins were estimated by the relative mobility of the corresponding bands compared to the rainbow markers.

This lead to speculation that may be both the N- and C-terminal HAUSP GST fusion proteins were interacting with the same 40kD cellular protein. However, when these samples were run on the higher percentage (12.5%) acrylamide gel, on which the molecular weight of proteins around 40kD can be better resolved, it is clear that these proteins are different (Figure 3C5 part B). In fact the 1-193kD HAUSP fusion protein appears to interact with a 45kD cellular protein, whereas the 516-1102 HAUSP GST fusion protein

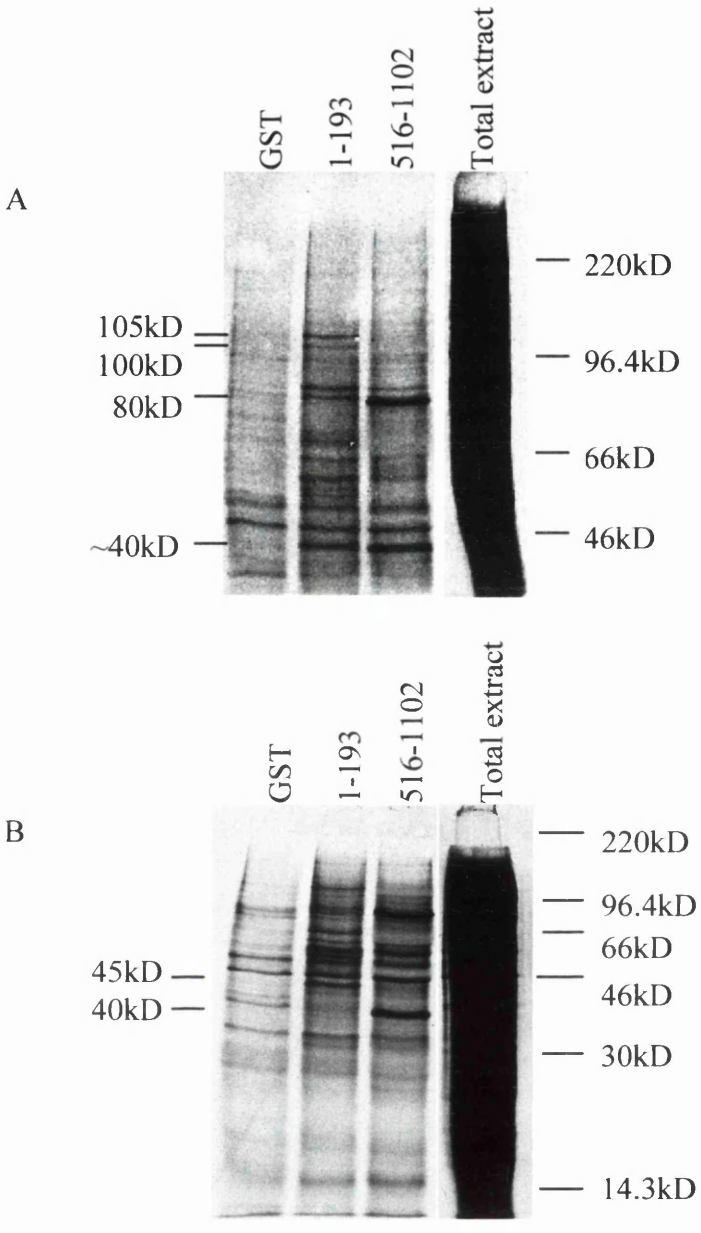


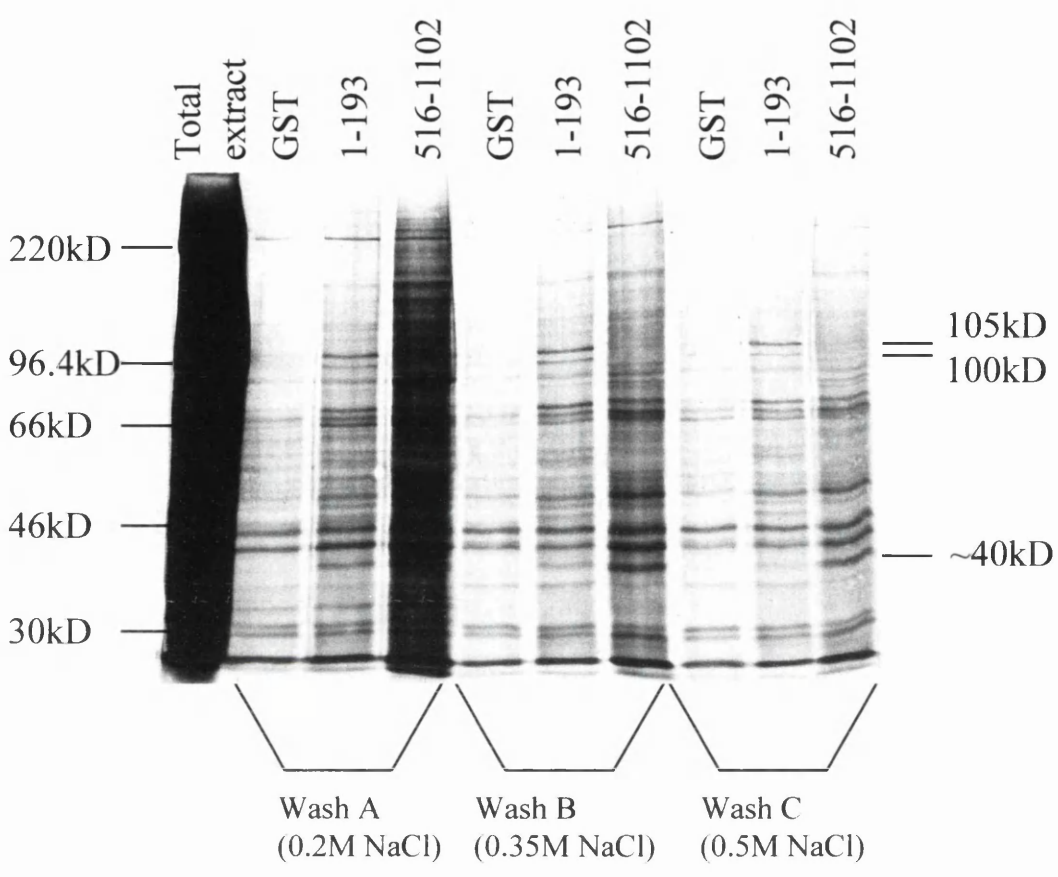
Figure 3C5: Preliminary GST 'pull-down' assay to identify cellular proteins that interact with HAUSP.
A GST 'pull-down' assay was carried out using the standard assay conditions described in Section 2B4.8, with the following modifications. Normalised amounts of glutathione agarose beads-bound to GST fusion proteins (encoding: GST alone, 1-193 HAUSP GST, and 516-1102 HAUSP GST), were prepared. These beads were then mixed with 300µl quantities of pre-cleared ^{35}S-radiolabelled WS HeLa cell extracts (the production of which is described in Section 2B3.4) and samples incubated for 1hr at 4°C end-over-end. The samples were then washed three times with 1ml of wash buffer containing: 0.2M NaCl, 0.05M Hepes pH7.4, 0.1% NP40, 1mM β-mercaptoethanol, 0.5µg/ml leupeptin, 1mM PMSF and 40µg/ml bestatin. After elution of the bound proteins by reduced glutathione, 3X SDS loading buffer was added and samples boiled for 2min. Samples were then loaded on 7.5% and 12.5% SDS-PAGE gels. Proteins were then transferred to a nitrocellulose filter and once dried these blots were exposed to film (as described in Section 2B4.7).
Panel A shows the results from the 7.5% SDS-PAGE gel and panel B shows the results from the 12.5% SDS-PAGE gel. A 2µl sample of the ^{35}S-radiolabelled WS HeLa cell extract was run in the last lane. The tracks labelled 'GST', '1-193' and '516-1102' show radiolabelled proteins eluted from the beads-fusion complexes of the GST fusion proteins encoding: GST alone, 1-193 HAUSP GST and 516-1102 HAUSP GST respectively. The positions of the 40kD, 45kD, 80kD, 100kD and 105kD proteins are indicated on the left of the gels. The mobilities of the 40kD and 45kD proteins were not distinguishable on the 7.5% gel. The sizes of the rainbow molecular weight markers have been indicated on the right of the gels.

interacts with a protein of about 40kD molecular weight. This is a reproducible result. However, all the subsequent experiments described in this section involved running samples on either 7.5% or 10% acrylamide gels. On these gels the difference in the mobility between these ~40kD cellular proteins, that interact with either the N- or C-terminal HAUSP GST fusion protein, cannot be distinguished. Therefore both these proteins are hereafter referred to as ~40kD cellular proteins.

3C6 Optimisation of the GST 'pull-down' assay

We wished to investigate the relative stabilities of protein-protein interactions observed between HAUSP GST fusion proteins and components of the cellular extract described in Section 3C5. A modified GST 'pull-down' assay was therefore designed, based on the assumption that increases in salt concentrations disrupt weak protein-protein interactions. This 'pull-down' assay involved using the GST fusion proteins encoding: GST alone, 1-193 HAUSP GST and 516-1102 HAUSP GST, bound to glutathione agarose beads. Each beads-bound fusion protein sample was then divided into three aliquots and mixed with radiolabelled WS HeLa cell extract (made up in a buffer containing 0.2M NaCl). After a 1hr incubation at 4 °C, the three aliquots of each fusion protein sample were washed three times, using 1ml of buffer A, B or C (which differed only in their final salt concentrations: buffer A-0.2M NaCl, B-0.35M NaCl, C-0.5M NaCl). The 'pull-down' assay was then continued as described previously (see the legend of Figure 3C6 for the experimental details of this assay).

The results of this 'pull-down' are shown in Figure 3C6. For samples washed in the lowest salt concentration, 0.2M NaCl, a large number of proteins remained bound to all the GST fusion proteins. However close observation of the gel reveals three proteins of approximately 40kD, 100kD and 105kD that interact with the N-terminal HAUSP GST fusion protein and an approximately 40kD protein that interacts with the C-terminal HAUSP GST fusion protein. The bands corresponding to these proteins were better defined after using the 0.35M NaCl wash buffer B. The most clear results were observed for samples made using the 0.5M NaCl wash buffer C. Indeed, in these samples the only different bands that are apparent between the control track and HAUSP GST fusion protein tracks, are the 100kD and 105kD proteins which interact with the N-terminal HAUSP GST fusion and the ~40kD cellular protein that interacts with the C-terminal HAUSP GST fusion protein. This means that, apart from the ~40kD protein that interacts with the N-



Wash buffers :- xM NaCl, 50mM HEPES pH7.4, 0.1% NP40
1mM βME, 1mM PMSF, 0.5μg/ml leupeptin, 40μg/ml bestatin

Figure 3C6: GST 'pull-down' assay investigating the relative stabilities of interactions between HAUSP and cellular proteins.

A GST 'pull-down' assay was carried out using the standard assay conditions described in Section 2B4.8, with the following modifications. Normalised amounts of glutathione agarose beads bound to GST fusion proteins (encoding: GST alone, 1-193 HAUSP GST, and 516-1102 HAUSP GST), were prepared and each sample divided into three aliquots. These beads were then mixed with 300μl quantities of pre-cleared ^{35}S-radiolabelled WS HeLa cell extracts (the production of which is described in Section 2B3.4) and samples incubated for 1hr at 4°C end-over-end. The next stage was to wash the beads with 1ml wash buffer. A different wash buffer A, B or C (described in diagram) was used for each of the three aliquots of the GST fusion beads-bound complexes. After elution of the bound proteins by reduced glutathione, 3X SDS loading buffer was added and samples boiled for 2min prior to loading on a 10% SDS-PAGE gel. Proteins were then transferred to a nitrocellulose filter and once dried the blot was exposed to film (as described in Section 2B4.7).

A 2μl sample of the ^{35}S-radiolabelled WS HeLa cell extract was run in the first lane. The tracks labelled 'GST', '1-193' and '516-1102' show radiolabelled proteins eluted from the beads-fusion complexes of the GST fusion proteins encoding: GST alone, 1-193 HAUSP GST and 516-1102 HAUSP GST respectively. The type of wash buffer used has been indicated below the relevant samples. The positions of the ~40kD, 100kD and 105kD proteins are indicated on the right of the gel. The sizes of the molecular marker weight proteins are indicated on the left of the gel.

terminal HAUSP GST fusion protein, the other interactions appear to be both strong and specific. Therefore, future efforts were focused on better defining these interactions.

It is also worth noting that no band corresponding to 80kD was seen to interact with the 516-1102 HAUSP GST fusion protein in this experiment. This is in contrast to all the other GST 'pull-down' assays executed using this fusion protein. However, it is thought this could be an artefact of running the SDS-PAGE gel at a different percentage (10%), from the percentage at which it was most regularly run (7.5%) in which this interaction is observed. As such the 80kD protein may have been pulled down in this experiment too, but its appearance could have been masked by a background protein which had a similar gel mobility.

3C7 Comparison of the interactions observed between HAUSP and cellular proteins using either an immunoprecipitation or GST 'pull-down' based assay

An immunoprecipitation experiment was designed (as described in the legend for Figure 3C7), to identify interactions that occur between wild type HAUSP and cellular proteins. This was done in order to compare these interactions with those already identified to occur with segments of HAUSP using the GST 'pull-down' assay. The results of this immunoprecipitation experiment can be seen in Figure 3C7. Comparison of proteins precipitated in the mock IP and test IP tracks, demonstrates that a band most likely representing HAUSP (of approximately 135kD), is the major additional band that appears in the test IP track. There are a number of other bands that coprecipitate, including two which have mobilities similar to the 105kD and ~40kD bands, identified through the GST 'pull-down' assay using the GST fusion proteins encoding HAUSP residues 1-193 and 516-1102 respectively (Figure 3C6). A band running at a slightly lower gel mobility than 105kD can also be observed in the test IP sample but not the mock IP sample. It is uncertain whether this band runs with the same mobility as the 100kD protein band observed in the GST 'pull-down' assay using the GST fusion protein encoding HAUSP residues 1-193. The complexity of the proteins which appear to coimmunoprecipitate with HAUSP precludes definative analysis, but the results of the immunoprecipitation appear to provide support to those found using the GST 'pull-down' assay. However, the question of whether substrates of HAUSP would interact for a long enough duration to be coimmunoprecipitated (as such interactions are usually extremely transitory), should be taken into consideration when interpreting data from this experiment.

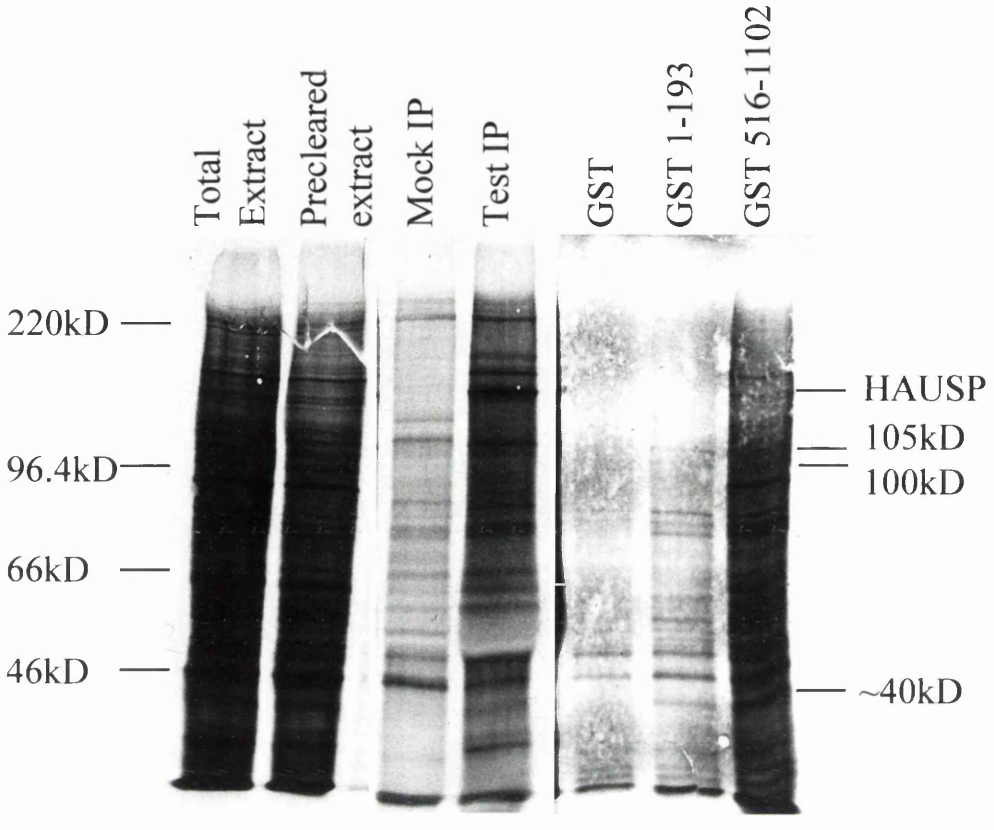


Figure 3C7: Comparison of the interactions observed between HAUSP and cellular proteins using either an immunoprecipitation or GST 'pull-down' based assay.

An immunoprecipitation was carried out based on the standard assay conditions described in Section 2B4.9, with the following modifications. A ^{35}S-radiolabelled WS HeLa cell extract was made as described in Section 2B3.4 and pre-cleared with Protein-A-Sepharose beads. Aliquots (100µl) of pre-cleared ^{35}S-radiolabelled WS HeLa cell extract were mixed with either: an aliquot (20µl) of the pre-immune anti-HAUSP r201 serum, or an aliquot (20µl) of the active anti-HAUSP r201 serum. The total volume of each sample was made up to 500µl containing: 0.2M NaCl, 0.05M HEPES pH7.4, 0.1% NP40, 1mM β-mercaptoethanol, 0.5µg/ml leupeptin, 1mM PMSF and 40µg/ml bestatin. These samples were left to incubate for 3hrs at 4°C end-over-end. An aliquot (60µl) of a 50% slurry of Protein-A-Sepharose beads was added to the samples and left for 1hr at 4°C. The samples were then washed three times with a buffer containing: 0.2M NaCl, 0.05M HEPES pH7.4, 0.1% NP40, 1mM β-mercaptoethanol, 0.5µg/ml leupeptin, 1mM PMSF and 40µg/ml bestatin. Immuneprecipitated proteins were eluted in an aliquot (20µl) of 1X SDS loading buffer. Samples were boiled for 2min prior to loading on a 10% SDS-PAGE gel. Proteins were then transferred to a nitrocellulose filter by western blotting and once dried the blot was exposed to film (as described in Section 2B4.7).

Samples (2µl) of the total and pre-cleared ^{35}S-radiolabelled WS HeLa cell extracts were run in the first two lanes. The Mock IP track shows radiolabelled proteins that were immunoprecipitated when pre-immune r201 serum was used. The Test IP track shows radiolabelled proteins that were immuneprecipitated when active r201 serum was used. Aliquots (10µl) of samples from the GST 'pull-down' assay described in Figure 3C6 using the 0.5M NaCl wash buffer C were loaded in the 'GST', 'GST1-193', 'GST516-1102' tracks. These tracks show radiolabelled proteins eluted from the beads-fusion complexes of the GST fusion proteins encoding: GST alone, 1-193 HAUSP GST, and 516-1102 HAUSP GST respectively. The positions of the ~40kD, 100kD and 105kD proteins are indicated. The sizes of the molecular marker weight proteins are indicated on the left of the gel.

3C8 An investigation to determine which HAUSP residues are required to bind cellular proteins

The next stage, after establishing the existence of strong and specific interactions between segments of HAUSP and cellular proteins, was to better define the region of HAUSP required for these interactions. In order to do this GST 'pull-down' assays were carried out as outlined in the legend of Figure 3C8, using an array of GST fusion proteins. The results can be seen in Figure 3C8.

It is evident from the results shown in panel A that among the ~40kD, 100kD and 105kD proteins pulled down by the 1-193 HAUSP GST fusion protein, only the ~40kD and 105kD proteins are pulled down by the N-terminal truncated 26-193 HAUSP GST fusion protein. This suggests residues 1-26 of HAUSP interact with the 100kD protein and residues 26-193 interact with the ~40kD and 105kD proteins.

Furthermore, it appears that only the 516-837 and not the 837-1102 HAUSP GST fusion protein pulled down the ~40kD band (Figure 3C8 part A), suggesting that residues 516-837 are sufficient for this interaction. Interestingly none of the subclones of the 513-837 region fused to GST, encoding HAUSP residues: 513-581, 581-749, 749-837, 516-744 and 576-837, pulled down the ~40kD protein (illustrated in Figure 3C7 part B). This suggests that the integrity of the entire 516-837 region is required to keep the interacting domain in a suitable conformation, even if it is potentially a more local interaction.

Additionally, this experiment also demonstrated that the 80kD protein, which interacts with HAUSP residues 516-1102, was not pulled down by either of the GST fusion proteins encoding HAUSP residues 516-837 or 837-1102. This may suggest that sequences encoded in the entire 516-1102 C-terminal region of HAUSP need to be present in order to form a structure to which the 80kD protein can bind. Alternatively these results may suggest that a minimum of residues which span the region around the 837 residue of HAUSP are required for this interaction.

3C9 Investigation of the susceptibility of the ~40kD, 100kD and 105kD proteins to proteasomal degradation

To investigate whether the ~40kD, 100kD and 105kD proteins were substrates for the ubiquitin proteasome degradation pathway a modified GST 'pull-down' assay was attempted, described in the legend of Figure 3C9. In this 'pull-down' assay three

Figure 3C8: GST 'pull-down' assays investigating HAUSP residues involved in binding the ~40kD protein. These 'pull-down' experiments were carried out using the conditions described in the legend of Figure 3C5, with the exception that a 10% SDS-PAGE gel was run instead of the 12.5% gel.
Panel A shows the results of the autoradiograph derived from the 7.5% SDS-PAGE gel and panel B shows the results of the autoradiograph derived from the 10% SDS-PAGE gel. The total extract track shows a 2μl aliquot of the ^{35}S-radiolabelled WS HeLa cell extract. The tracks labelled 'GST', '26-193', '1-193', '516-744', '576-837', '513-581', '581-749', '749-837', '516-837', '837-1102', '516-1102' show radiolabelled proteins bound to beads of the GST fusion proteins encoding: GST alone, 26-193 HAUSP GST, 1-193 HAUSP GST, 516-744 HAUSP GST, 576-837 HAUSP GST, 513-581 HAUSP GST, 581-749 HAUSP GST, 749-837 HAUSP GST, 516-837 HAUSP GST, 837-1102 HAUSP GST, 516-1102 HAUSP GST, respectively. The positions of the ~40kD, 80kD, 100kD and 105kD proteins are indicated. The sizes of the molecular marker weight proteins are indicated on the left of the gels.
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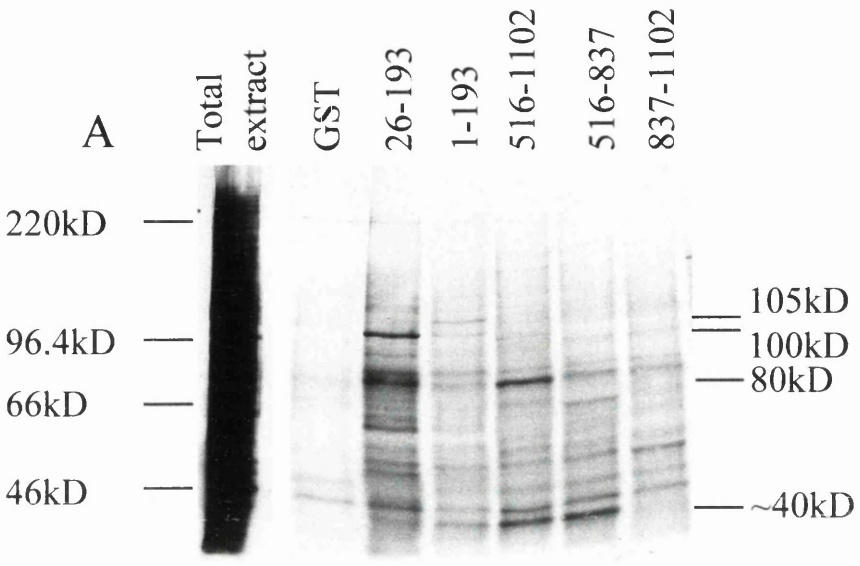
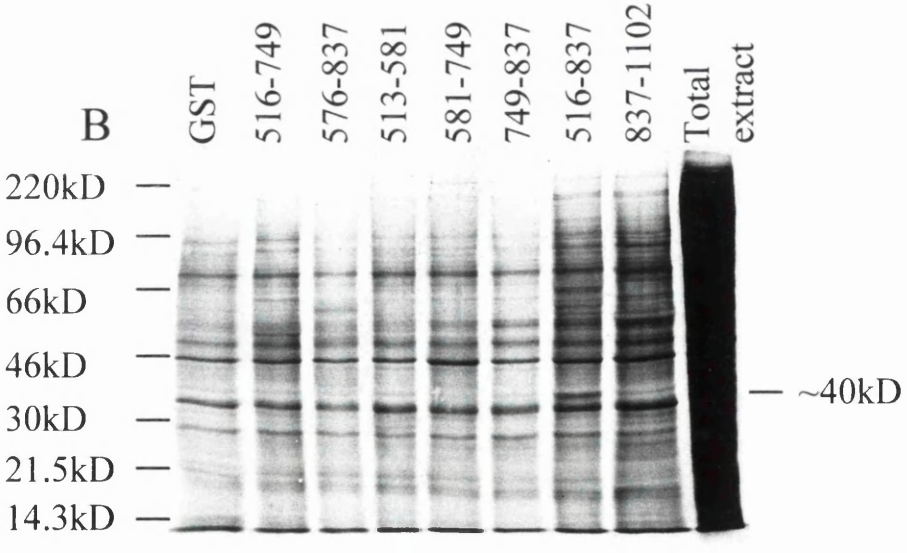

A
Total extract
GST
26-193
1-193
516-1102
516-837
837-1102
220kD
96.4kD
66kD
46kD
105kD
100kD
80kD
~40kD


B
GST
516-749
576-837
513-581
581-749
749-837
516-837
837-1102
Total extract
220kD
96.4kD
66kD
46kD
30kD
21.5kD
14.3kD
~40kD

radiolabelled WS HeLa cell extracts were made (denoted extracts A-C), A being normal extract, B being made 12hr after labelling and C being made 12hr after labelling but in the presence of proteasome inhibitor. The protocol consisted of following the standard 2hr labelling procedure with ^{35}S-methionine (Section 2B3.4) and then, for extract A, WS HeLa cells were harvested as usual. For extract B the label mix was replaced with normal growth medium and then the cells harvested 12hr later and finally, for extract C the label mix was again replaced by normal growth medium, but this time containing 2.5μM MG132 proteasome inhibitor and the cells harvested 12hr later. A 'pull-down' assay was then carried out with the GST alone, 1-193 HAUSP GST fusion protein and 516-1102 HAUSP GST fusion protein using these three extracts. The results are illustrated in Figure 3C9.

As can be seen the 100kD and 105kD proteins appear at equal levels when using all three extracts indicating that these proteins are not substrates for proteasomal degradation. However the band corresponding to the ~40kD protein which interacts with the C-terminal half of HAUSP appears clearly in the normal extract A but, compared to the background bands diminishes when extract B was used in which the cell extract has been chased for 12hr. Interestingly this band was not decreased in the sample made using extract C, which was also chased for 12hr, but in the presence of a proteasome inhibitor. This indicates that the ~40kD protein is broken down during the 12hr chase. Furthermore, as this degradation appears to be prevented in the presence of proteasome inhibitor, it suggests the ~40kD protein is specifically targeted for degradation by the proteasome. As ubiquitinylation of the ~40kD protein is most probably required to signal it for proteasomal degradation, the possibility exists that HAUSP cleaves off these ubiquitins and therefore regulates its degradation.

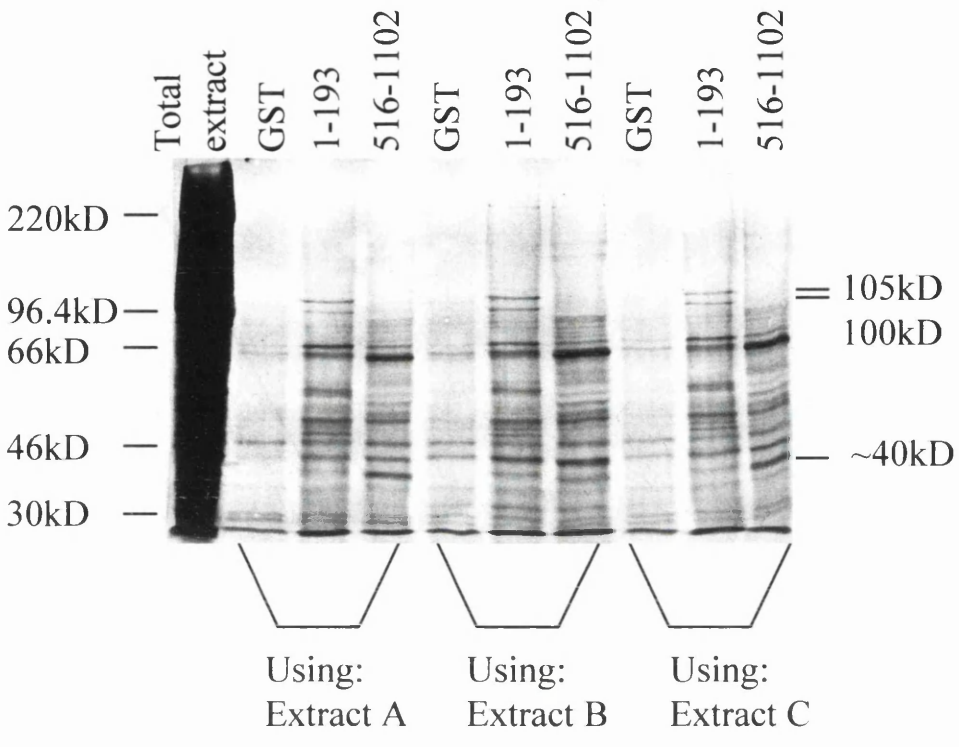


Figure 3C9: Investigation of the susceptibility of the ~40kD, 100kD and 105kD proteins to proteasomal degradation.

A GST 'pull-down' assay was carried out using the standard assay conditions described in Section 2B4.8, with the following modifications. Three different WS HeLa cell extracts were prepared in which the cells were labelled with ^{35}S-Methionine (Section 2B3.4) and were either; harvested immediately for extract preparation (extract A); or the label medium replaced with normal growth medium and the cells harvested after 12hr (extract B); or the label medium replaced with normal growth medium (containing the proteasome inhibitor MG132 at a 2.5μM concentration) and the cells harvested after 12hr (extract C). Normalised amounts of glutathione agarose beads-bound to GST fusion proteins (encoding: GST alone, 1-193 HAUSP GST, and 516-1102 HAUSP GST), were prepared and each fusion protein sample divided into three aliquots. Each of these three aliquots was then mixed with one of the three different extracts prepared A, B, or C and samples incubated for 1hr at 4°C end-over-end. The volumes of extracts A, B and C used for the 'pull-down' assay were calibrated such that equal levels of radioactivity were present. The samples were then washed three times with 0.5M NaCl wash buffer C (described in Figure 3C6). After elution of the bound proteins by reduced glutathione, 3X SDS loading buffer was added and samples boiled for 2min. Samples were then loaded on a 10% SDS-PAGE gel. Proteins were then transferred to a nitrocellulose filter and once dried the blot was exposed to film (as described in Section 2B4.7).

A 2μl sample of the ^{35}S-radiolabelled WS HeLa cell extract was run in the first lane. The tracks labelled 'GST', '1-193' and '516-1102' show radiolabelled proteins eluted from the beads-fusion complexes of the GST fusion proteins encoding: GST alone, 1-193 HAUSP GST and 516-1102 HAUSP GST respectively. The type of extract used has been indicated below the gel. The positions of the ~40kD, 100kD and 105kD proteins are indicated. The sizes of the molecular marker weight proteins are indicated on the left of the gel.

3C10 Summary of studies to identify cellular proteins that interact with HAUSP

- Based on secondary structure predictions and GenBank database searches, regions of HAUSP with potential importance for protein-protein interactions were identified.

- Interaction of HAUSP with a ~40kD cellular protein:

1. Using the GST 'pull-down' technique a ~40kD cellular protein was identified which interacts with residues 516-837 of HAUSP.
2. This interaction appears to withstand increases in salt concentration, suggesting it is a stable interaction.
3. The biological significance of this interaction was supported by the results of an immunoprecipitation assay, in which a protein of similar molecular weight coimmunoprecipitated with wild type HAUSP.

- Interaction of HAUSP with a 80kD cellular protein:

1. The GST 'pull-down' technique also identified a 80kD cellular protein which interacts with residues 516-1102 of HAUSP.

- Interaction of HAUSP with a 100kD cellular protein:

1. The GST 'pull-down' technique also identified a 100kD cellular protein which interacts with residues 1-193 of HAUSP, of which the first 26 residues are essential.
2. This interaction appears to withstand increases in salt concentration suggesting it is a stable interaction.

- Interaction of HAUSP with a 105kD cellular protein:

1. A 105kD cellular protein was also identified using the GST 'pull-down' technique which interacts with residues 26-193 of HAUSP.
2. This interaction appears to withstand increases in salt concentration, suggesting it is a stable interaction.
3. This finding was supported by the results of an immunoprecipitation assay in which a protein of similar molecular weight coimmunoprecipitated with wild type HAUSP.

- Interaction of HAUSP with another ~40kD cellular protein:

1. A second cellular protein with the molecular weight of ~40kD was identified to interact with residues 26-193 of HAUSP, using the GST 'pull-down' technique.
2. This interaction did not withstand increases in salt concentration, suggesting it is not a stable interaction.

- Investigation into the susceptibility of these proteins to degradation by proteasome showed that the ~40kD cellular protein that interacts with HAUSP residues 516-1102 was unstable in the cell and degraded in a proteasome-dependant manner.

3D To define the region of HAUSP that interacts with Vmw110

3D1 Introduction

The aim of the work done in this section was to define the region of HAUSP that interacts with Vmw110. Based on results from these studies, mutants of HAUSP were produced in which residues essential for interaction with Vmw110 were removed. It was hoped that these mutants could then be used in HAUSP functional assays to test for the importance of these regions.

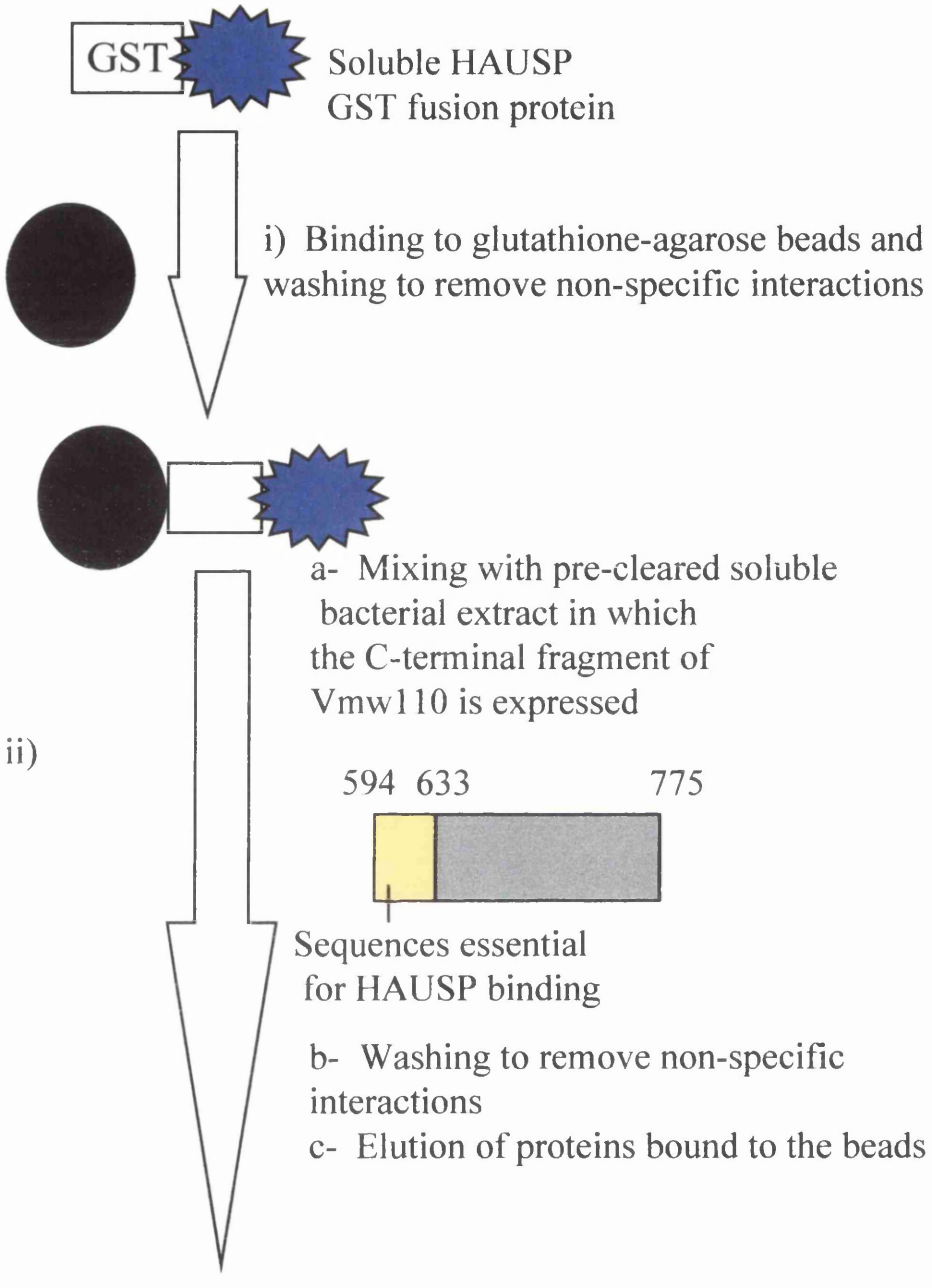
The initial strategy used was based on the GST 'pull-down' system, an outline of the specific method designed is given in Figure 3D1. It involved using the HAUSP GST fusion proteins already described in Section 3C3, the first stage being to immobilise them on glutathione agarose beads. The next stage was to mix the beads-bound proteins with a pre-cleared soluble bacterial extract in which a Vmw110 fragment, encoding residues essential for interaction with HAUSP, was expressed, the synthesis of which is described in Section 2B4.4. The final steps were the washing of the beads and elution of the proteins from the beads. The samples were then analysed for the presence of Vmw110 by probing a western blot of the eluants for Vmw110.

3D2 Initial investigation of the HAUSP residues involved in binding to Vmw110

Initial experiments involved using this 'pull-down' technique (details of which can be seen in the legend of Figure 3D2) and all the HAUSP GST fusion proteins previously described in Section 3C3. The results are shown in Figure 3D2.

From the blot in Figure 3D2 part A, it is clear that only HAUSP GST fusion proteins 516-1102 and 26-1102 'pull-down' the bacterially expressed 594-775 Vmw110 C-terminal fragment and not the 241-441 or 837-1102 HAUSP GST fusion proteins. This suggested that only the region 516-837 of HAUSP is necessary for the interaction with the C-terminal of Vmw110. This was supported by the observation that the GST fusion protein encoding HAUSP residues 516-837 was sufficient for the interaction with the Vmw110 fragment (Figure 3D2 part B).

Figure 3D1: Outline of the GST 'pull-down' assay, in which HAUSP GST fusion proteins were used as 'bait'.
i) Bacterial extracts containing GST fusion proteins encoding selected segments of HAUSP were mixed with glutathione agarose beads and the beads-fusion complexes were washed to remove non-specific interactions.
ii) Normalised amounts of beads-fusion complexes were mixed with pre-cleared soluble bacterial extracts in which the C-terminal of Vmw110 was expressed. The samples were washed to remove non-specific interactions. Proteins bound to the beads were eluted by competition with reduced glutathione.
iii) The eluted proteins were separated on an SDS-PAGE gel and transferred to a nitrocellulose filter. The blots were analysed by immunodetection of Vmw110 encoding sequences using the 10503 monoclonal antibody.



iii) Analysis of eluted proteins for presence of Vmw110 encoding sequences

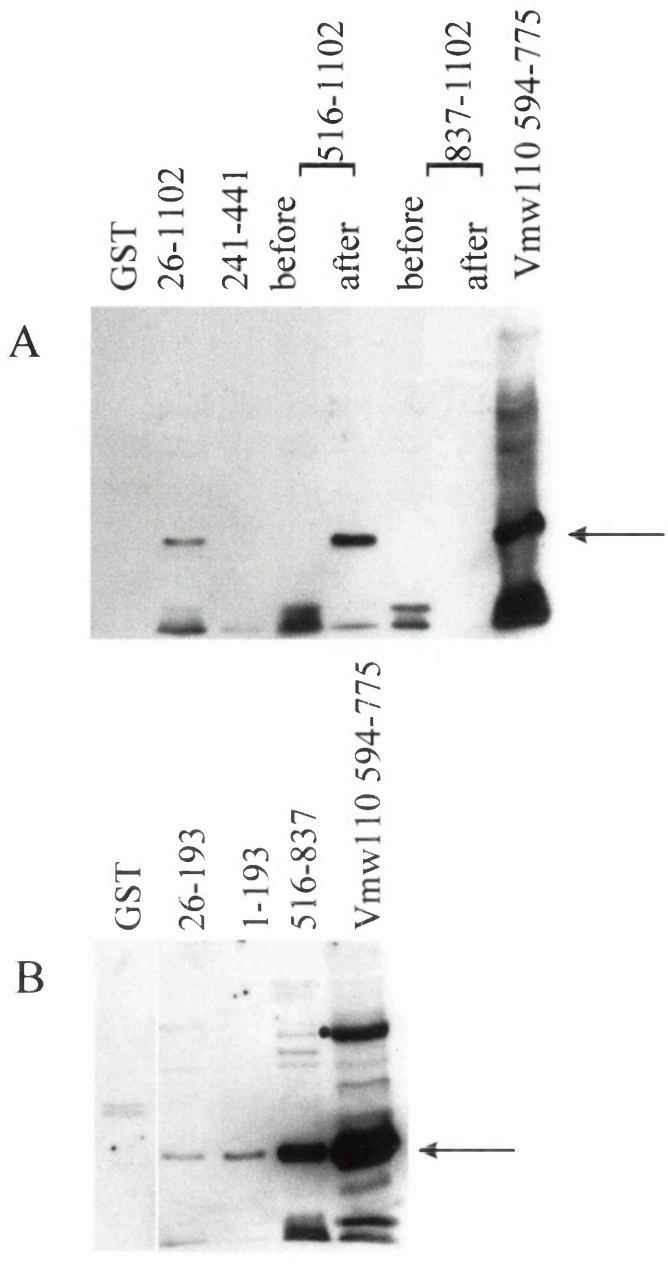


Figure 3D2 Panels A and B: GST 'pull-down' assays investigating HAUSP residues involved in binding to Vmw110.

Panels A-D show 'pull-down' experiments based on the standard assay conditions described in Section 2B4.8. Aliquots of beads bound to control GST protein and GST fusion proteins encoding selected sequences of HAUSP, were adjusted in order to provide normalised levels of all the proteins. These were then added to 300µl quantities of pre-cleared extracts. The pre-cleared extract consisted of 266µl bacterial extraction buffer (50mM HEPES pH7.5, 50mM NaCl, 0.1% NP40), 27µl 5M NaCl and 7µl T7E52 bacterial extract. A protocol for the synthesis of T7E52 is detailed in Section 2B4.4. These samples were then incubated for 1hr at 4°C end-over-end. Beads were then washed three times with 1ml of cold 0.5M NaCl wash buffer (consisting of 50mM Tris, 0.5M NaCl, 1mM EDTA, 0.5% NP40, 1mM PMSF, 1µg/µl leupeptin, 15µg/µl Bestatin). After elution of the bound proteins by reduced glutathione, 3X SDS loading buffer was added. The samples were then boiled for 2min prior to loading on a 12.5% SDS-PAGE gel and then transferred to a nitrocellulose filter. The blots were then analysed by immunodetection (as described in Section 2B4.7c) for the presence of Vmw110 encoding sequences using the 10503 MAb in a 1 in 10,000 dilution.

The 'GST' track shows proteins eluted from beads bound to the GST protein. The numbered tracks show proteins eluted from beads bound to purified GST HAUSP fusion proteins, the numbers representing the residues of HAUSP encoded by the protein. The labels 'before' and 'after' represent samples from the 'pull-down' assay obtained 'before' binding to the pre-cleared extract and those which were eluted 'after' binding to pre-cleared extract. The 'Vmw110 594-775' track shows a 5µl sample of T7E52 bacterial extract. The black arrows to the right of the gels denote the position of the bands representing the Vmw110 fragment encoding residues 594-775.

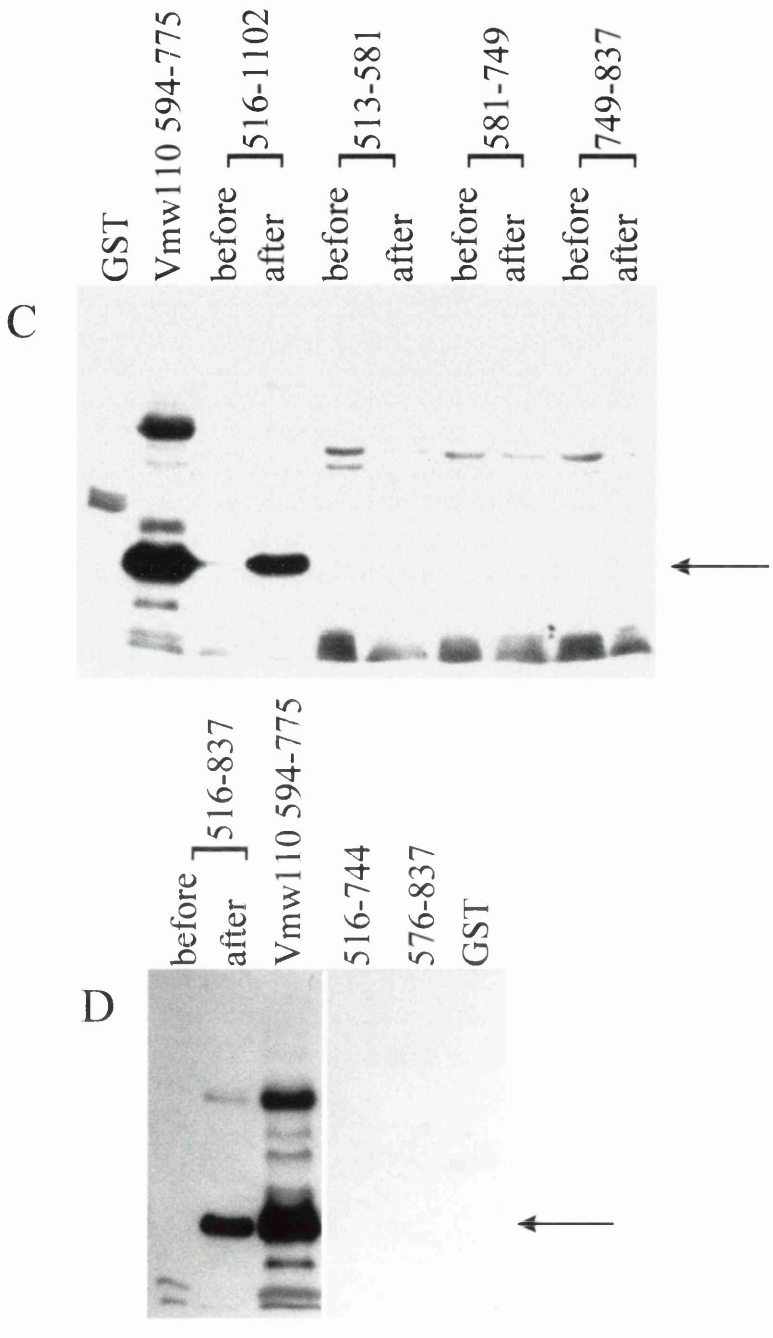


Figure 3D2 Panels C and D: GST 'pull-down' assays investigating HAUSP residues involved in binding to Vmw110.

The results shown in part B revealed that the N-terminal 26-193 and 1-193 HAUSP GST fusion proteins also interact with the C-terminal of Vmw110, but to a lesser degree than the 516-837 HAUSP GST fusion protein. This difference in affinity was very reproducible.

Interestingly, in part A the 26-1102 HAUSP GST fusion protein appeared to pull down the C-terminal of Vmw110 to a lesser degree than the 516-1102 HAUSP GST fusion protein. However, this is probably due to the fact that the 26-1102 HAUSP GST fusion protein is very unstable and so degraded during the 'pull-down' procedure, despite attempts to normalise the amounts of HAUSP GST fusion protein bound to the beads.

The results shown in part C and part D are of 'pull-down' assays in which HAUSP GST fusion proteins encoding subcloned regions of HAUSP residues 513-837 were used in an attempt to identify the minimal region required for interaction with the C-terminal of Vmw110. However, none of these fusion proteins interacted with the Vmw110 fragment, suggesting that the entire region is required for interaction. One possible explanation could be that all these residues need to be present in order that the protein forms a structure in which residues essential for binding to Vmw110 become exposed.

3D3 An investigation into the interaction of HAUSP fusion proteins with various forms of Vmw110 to test their specificity

3D3.1 An investigation into whether the HAUSP GST fusion proteins interacted with the bacterially expressed region of Vmw110 essential for binding to cellular HAUSP

After the regions of HAUSP required for interaction with the 594-775 C-terminal fragment of Vmw110 were identified, the next stage was to ensure that these interactions required the 594-633 residues of Vmw110, demonstrated to be essential for binding to cellular HAUSP (Meredith *et al.*, 1995). Therefore an experiment was designed which tested the ability of the N-terminal 1-193 and C-terminal 516-837 HAUSP GST fusion proteins, to interact with either the 594-775 or the 633-775 C-terminal Vmw110 encoding fragments (details of which are described in the legend to Figure 3D3.1). The results of these experiments can be seen in Figure 3D3.1. Both HAUSP GST fusion proteins only pulled down the 594-775 and not the 633-775 C-terminal fragment of Vmw110 (Figure 3D3.1 parts A and B). This implies that both regions of HAUSP interact with residues within 594-633 of Vmw110 in a 'pull-down' assay and supports the biological specificity of the observed interactions.

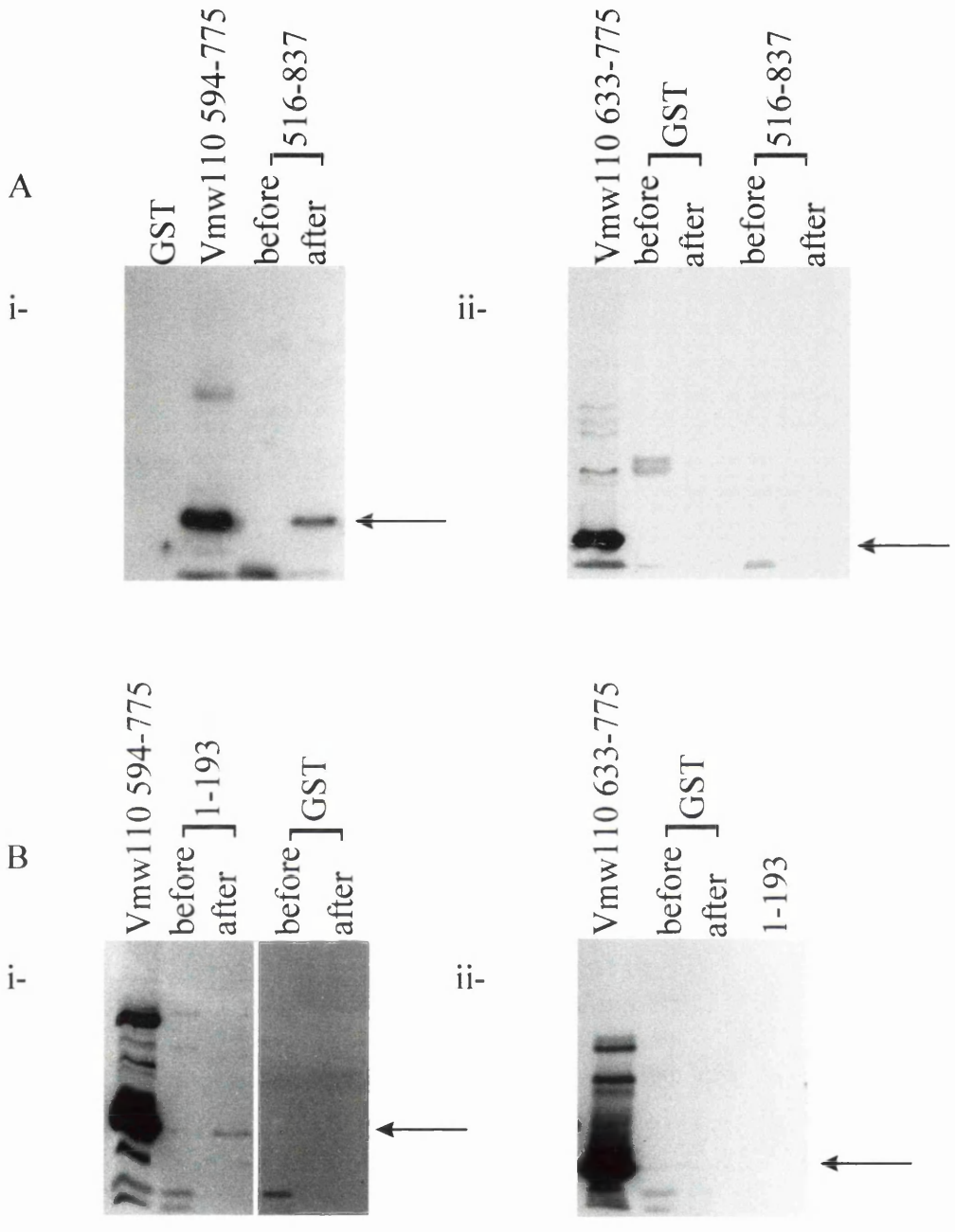


Figure 3D3.1: GST 'pull-down' assays to investigate whether the HAUSP GST fusion proteins interact with the region of Vmw110 essential for binding to cellular HAUSP.

Panels Ai and Bi show 'pull-down' experiments based on the assay conditions described in Figure legend 3D2. The 'pull-down' experiments shown in panels Aii and Bii were done simultaneously based on the same assay conditions, with the exception that the 300µl pre-cleared extract used as the source of Vmw110 for binding to each beads-bound GST fusion protein consisted of 269µl bacterial extraction buffer (50mM HEPES pH7.5, 50mM NaCl, 0.1% NP40), 27µl 5M NaCl, and 4µl T7E58 bacterial extract. The protocol for the synthesis of T7E52 and T7E58 is detailed in Section 2B4.4.

The GST track shows proteins eluted from beads bound to the GST protein. The numbered tracks show proteins eluted from beads bound to purified GST HAUSP fusion proteins, the numbers representing the residues of HAUSP encoded by the protein. The 'before' and 'after' has been indicated to represent samples from the 'pull-down' assay obtained 'before' binding to the pre-cleared extract and those which were eluted 'after' binding to pre-cleared extract. The Vmw110 594-775 track shows a 5µl sample of the T7E52 bacterial extract and the Vmw110 633-775 track shows a 5µl sample of the T7E58 bacterial extract. The black arrows indicated on the right of the gels denote the position of the bands representing either the Vmw110 fragment encoding residues 594-775 or the one encoding residues 633-775.

3D3.2 An investigation of the interaction of HAUSP fusion proteins with Vmw110 expressed during virus infection

To further investigate the biological relevance of these interactions we tested whether the HAUSP GST fusion proteins identified as interacting with bacterially expressed fragments of Vmw110, also interacted with full length Vmw110 expressed during virus infection. An experiment was designed to investigate this, in which the relevant HAUSP GST fusion proteins were used in a 'pull-down' assay with wild type HSV-1 infected cell extract as the source of Vmw110. The results shown in Figure 3D3.2a reveal that the 26-1102, 516-1102 and 1-193 HAUSP GST fusion proteins interact with wild type Vmw110, albeit at different affinities. It should be noted that the 26-1102 HAUSP GST fusion protein became degraded during the course of experiment despite the use of a freshly prepared extract and as such was present in a smaller proportion relative to the other GST fusion proteins. This may explain why the band pulled down by the 26-1102 HAUSP GST fusion protein, which corresponded to wild type Vmw110, was of such low intensity. However, what was really surprising was that the 1-193 HAUSP GST fusion protein appeared to pull down wild type Vmw110 with a greater affinity than the 516-1102 HAUSP GST protein, which is the reverse of what is seen when using the bacterially expressed C-terminal Vmw110 fragment (Section 3D2). Furthermore, this result was reproducible and was not an artefact of less 516-1102 HAUSP GST fusion protein being present compared with the 1-193 HAUSP GST fusion protein, as the amounts of these protein present prior to use in the 'pull-down' were always normalised.

The next stage was to establish whether or not the interactions between the HAUSP GST fusion proteins and Vmw110 expressed during virus infection were specific. In order to test this a slightly modified version of the experiment described above was designed in which the source of Vmw110 was from a cell extract which had been infected with the D12 mutant of HSV-1. This strain of HSV-1 has the 594-633 residues of Vmw110 removed, which are essential for interaction with cellular HAUSP (Meredith *et al.*, 1995). The results shown in Figure 3D3.2b demonstrate that neither the 26-1102 nor the C-terminal HAUSP GST fusion proteins interact with D12 Vmw110. Thus, although the interaction of 516-1102 HAUSP GST fusion protein with full length HSV-1 Vmw110 was of a relatively lower affinity than seen with the N-terminal HAUSP GST fusion protein, this interaction requires residues within Vmw110 that have been demonstrated to be essential for interaction with cellular HAUSP. Hence, this supports the idea that this interaction is

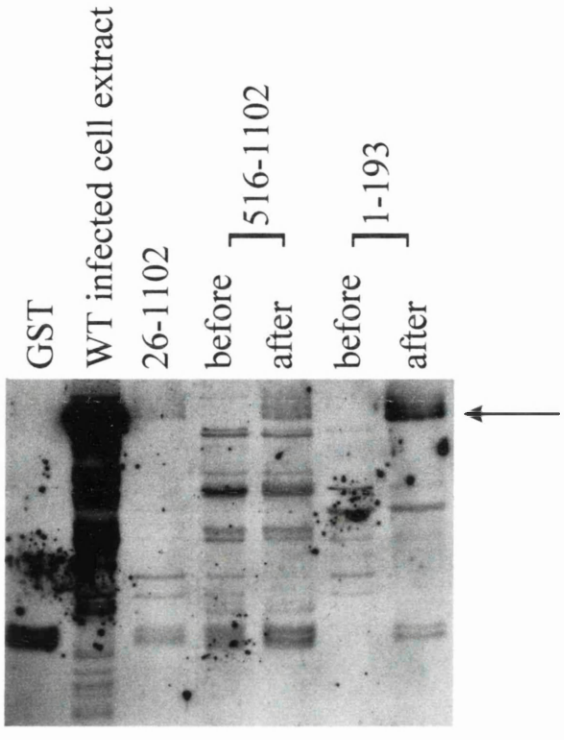


Figure 3D3.2a: GST 'pull-down' assay to investigate if HAUSP GST fusion proteins can interact with wild type Vmw110 from 17 Syn$^+$ HSV-1 infected cell extracts.

A GST 'pull-down' assay was carried out using the assay conditions described in the legend of Figure 3D2, with the exception that the pre-cleared extract used was derived from wild type 17 Syn$^+$ HSV-1 infected cell extract. The protocol for synthesising this cell extract is described in Section 2B3.5.

A similar gel annotation as described in the legend of Figure 3D2 has been used. The track labelled 'WT infected cell extract' represents a 5µl aliquot of the wild type HSV-1 infected cell extract. The black arrow denotes the position of the bands representing wild type Vmw110.

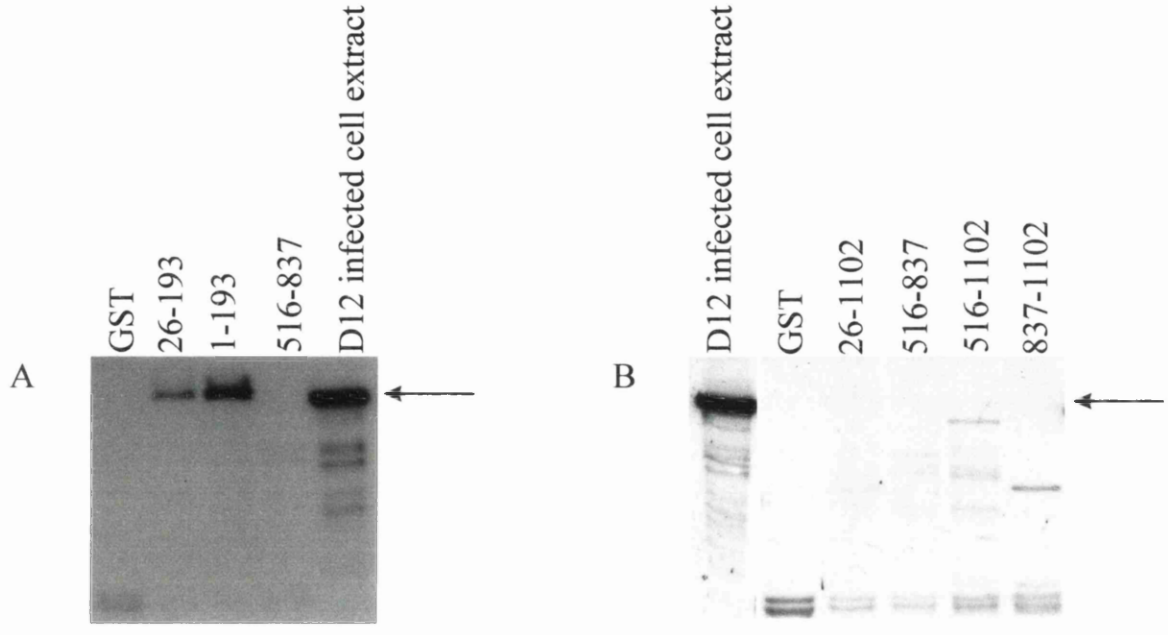


Figure 3D3.2b: GST 'pull-down' assays to investigate if HAUSP GST fusion proteins can interact with D12 Vmw110 from D12 HSV-1 infected cell extracts.

Panels A-B show 'pull-down' assays based on the protocol given in the legend of Figure 3D3.2a, with the exception that the pre-cleared extract is derived from D12 HSV-1 infected cell extract. A similar gel annotation as described in the legend of Figure 3D2 has been used. The track labelled 'D12 infected cell extract' represents a 5µl aliquot of the D12 HSV-1 infected cell extract. The black arrows denote the positions of the bands representing D12 Vmw110.

of biological significance. However this is not the case for the 26-193 and 1-193 HAUSP GST fusion proteins which appeared to strongly interact with D12 Vmw110. This suggests that the interactions seen with the N-terminal HAUSP GST fusion proteins and Vmw110 from HSV-1 infected cell extracts are non-specific. This is in contradiction to the results shown in Section 3D3.1 of the 'pull-down' assay investigating which residues of Vmw110 expressed in bacteria were specific for interaction with the HAUSP fusion proteins. These previous results demonstrated that the N-terminal 26-193 and 1-193 HAUSP GST fusion proteins did require residues 594-633 of Vmw110 for interaction.

3D4 An investigation into the interaction of full length Vmw110 with deletion mutants of HAUSP

Studies using fragments of exogenously expressed HAUSP had suggested that residues 516-837 of HAUSP are required for interaction with Vmw110 (Section 3D2 and 3D3). The significance of these residues for interaction with Vmw110 in the context of full length HAUSP was investigated. In order to test this, mutants of HAUSP were constructed in which residues in the critical region had been removed. The ability of these mutants to interact with Vmw110 was then tested.

3D4.1 Construction of x513-581, x529-576 and x744-861 HAUSP deletion mutants

As residues 516-837 of HAUSP are required for interaction with Vmw110, it was decided to construct two HAUSP mutants in which either residues 513-581 or 744-861 had been deleted. Interestingly, sequence analysis revealed that the region spanning between residues 515-529 of HAUSP, directly downstream of the histidine active site domain, were conserved between members of the USP family. This implied that these residues are important for the normal functioning of HAUSP in the cell. Therefore, another HAUSP deletion mutant was made removing only residues between 529-576 to reduce the likelihood of deleting residues from HAUSP that disrupt a separate function other than its interaction with Vmw110.

The plasmid x513-581pET24a135 was constructed in a ligation reaction of three fragments isolated from relevant digestion mixes: these were the *Xba*I-*Afl*III(513) pET24a135 insert fragment, the *Afl*III(581)-*Eco*RI(744) pET24a135 insert fragment and the *Eco*RI(744)-*Xba*I pET24a135 vector fragment (see Figure 3D4.1a).

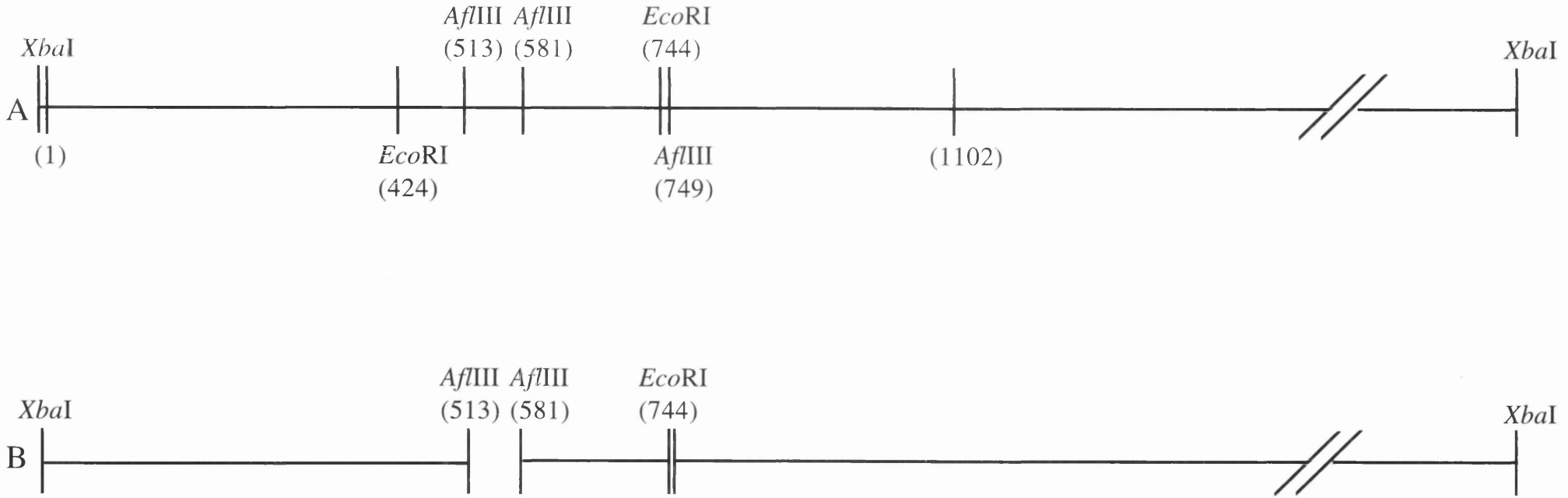


Figure 3D4.1a: Construction of the x513-581 HAUSP deletion mutant.
Part A: Shows a linearised plasmid map of pET24a135, in which all the restriction sites have been indicated for the enzymes used in the construction of the x513-581 HAUSP deletion mutant. The restriction sites above the line are the ones which were directly used for the cloning procedure. Positions 1-1102 represent the HAUSP coding sequence which has been drawn to scale, unlike the vector sequences marked by the // sign.
Part B: Shows the three pET24a135 fragments used in the ligation reaction for the construction of the x513-581 HAUSP deletion mutant.

The plasmid x744-861pET24a135 was constructed in a ligation reaction of three fragments isolated from relevant digestion mixes: these were the *Hind*III-*Bsu*36I(441) pET24a135 vector fragment, the *Bsu*36I(441)-*Eco*RI(744) pET24a135 insert fragment (in which the *Eco*RI site was blunt-ended) and *Bgl*II(861)-*Hind*III pET24a135 insert fragment (in which the *Bgl*II site was blunt-ended) (see Figure 3D4.1b).

The plasmid x529-576pET24a135 was constructed in a ligation reaction of two fragments isolated from relevant digestion mixes: the *Msc*I(576)-*Nde*I(1) pET24a135 vector fragment and the *Nde*I(1)-*Bst*EII(529) pET24a135 insert fragment (in which the *Bst*EII site was blunt-ended) (see Figure 3A3.2 for the positions of these restriction sites in the HAUSP coding sequence).

3D4.2 Expression of the HAUSP deletion mutants

The HAUSP deletion mutants were transformed into *E.coli* strain BL21 pLysS DE3. Cultures were grown and fusion protein expression was induced from the P_{tac} promoter by the addition of IPTG. Protein extracts were prepared as described in Section 2B4.3, with the exception that Triton-X100 was not used and the cell debris was pelleted by centrifugation at 9,500rpm for 30min. The cells were then resuspended in 2ml PBS(A) containing 1mM DTT (which stabilises deubiquitinase activity).

Expression of the deletion mutants was ascertained by probing a western blot of the bacterial extracts using anti-HAUSP rabbit serum (data not shown). The results showed that for the x513-581 HAUSP and x529-576 HAUSP bacterial extracts, a protein of the expected molecular weight was synthesised and expressed at levels equivalent to full length HAUSP. However, the x744-861 HAUSP deletion mutant bacterial extract produced a band of the expected size, but of a much lower intensity relative to the band corresponding to full length HAUSP. This suggests that x513-581 and x529-576 are relatively stable but x744-861 is not and implies that residues 744-861 are important for maintaining a stable conformation of HAUSP.

3D4.3 The effect of removal of residues from HAUSP on its ability to interact with a C-terminal Vmw110 GST fusion protein

The effect of removing these residues on the ability of full length HAUSP to interact with a C-terminal fragment of Vmw110 was then tested using a modified 'pull-down' assay (an outline of the experiment is given in Figure 3D4.3a and the details of which are provided in

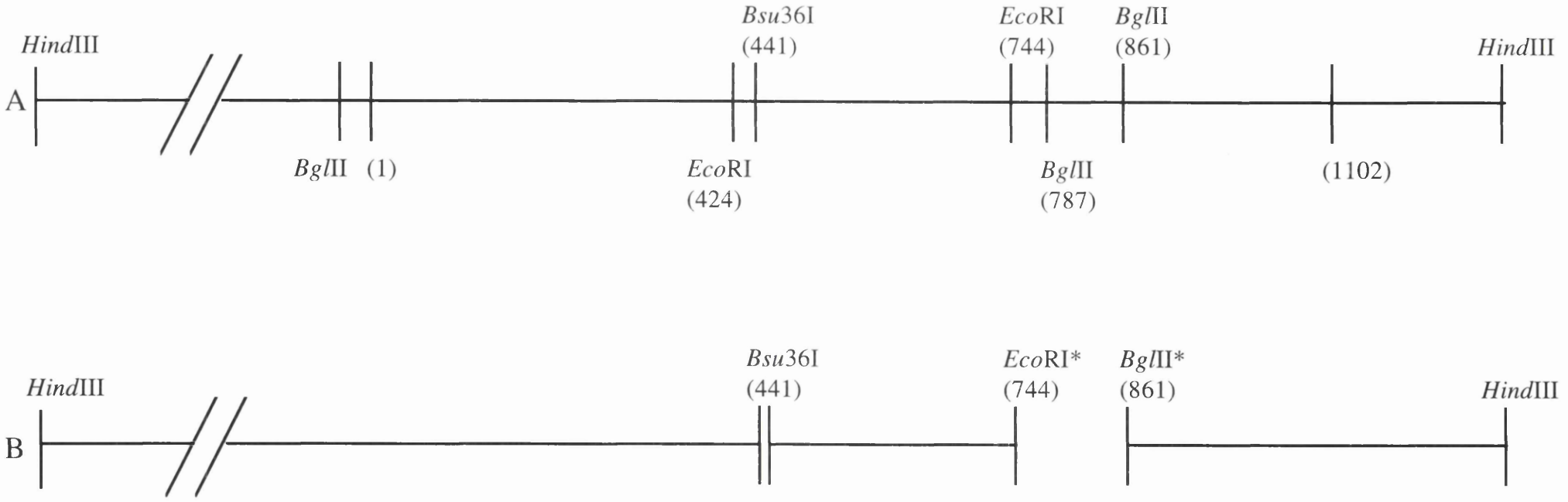
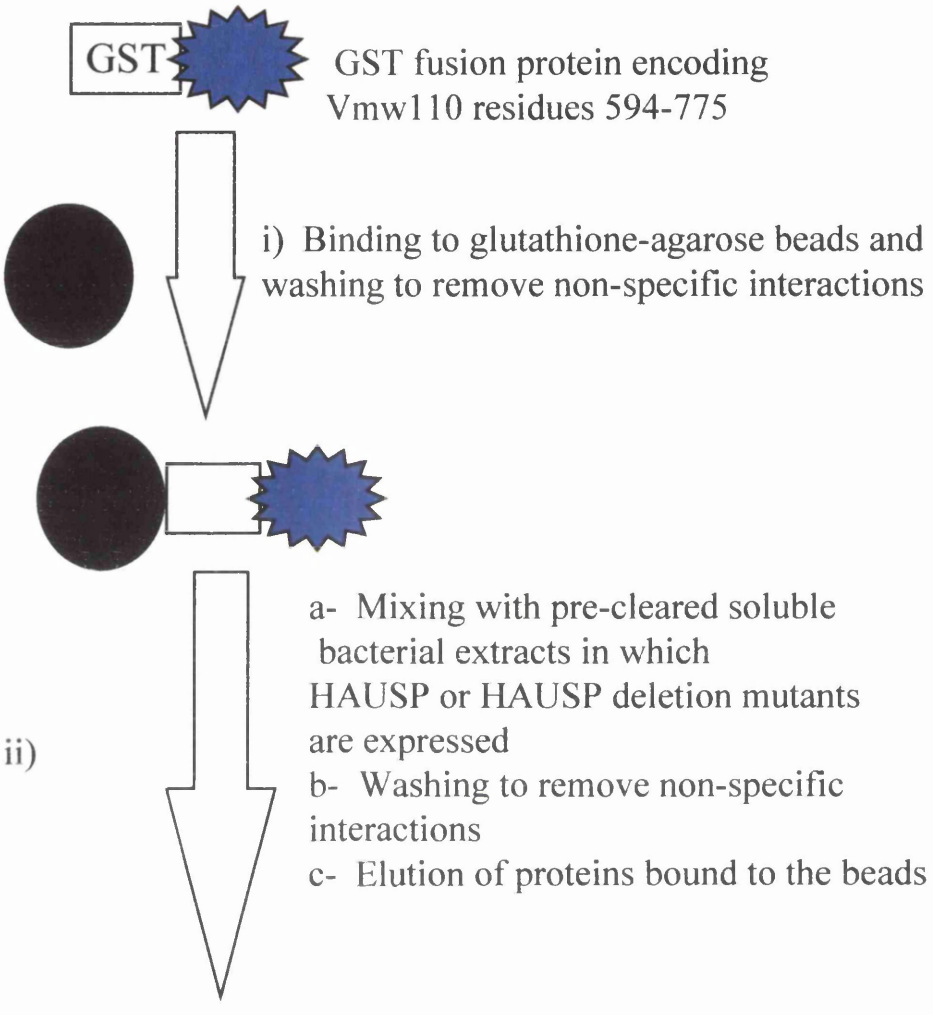


Figure 3D4.1b: Construction of the x744-861 HAUSP deletion mutant

Part A: Shows a linearised plasmid map of pET24a135, in which all the restriction sites have been indicated for the enzymes used in the construction of the x744-861 HAUSP deletion mutant. The restriction sites above the line are the ones which were directly used for the cloning procedure. Positions 1-1102 represent the HAUSP coding sequence which has been drawn to scale, unlike the vector sequences marked by the // sign.

Part B: Shows the three pET24a135 fragments used in the ligation reaction for the construction of the x744-861 HAUSP deletion mutant. Restriction sites which were blunt ended have been indicated by * sign.

Figure 3D4.3a: Outline of the GST 'pull-down' assay, in which the GST fusion protein encoding the C-terminal 594-775 residues of Vmw110 was used as 'bait'.
i) Bacterial extracts in which the GST fusion protein encoding the C-terminal 594-775 residues of Vmw110 had been expressed were mixed with glutathione agarose beads and the bead-fusion complexes were then washed to remove non-specific interactions.
ii) Normalised amounts of beads-fusion complexes were mixed with pre-cleared soluble bacterial extracts in which either full length HAUSP or HAUSP deletion mutants had been expressed. The samples were then washed to remove non-specific interactions. Proteins bound to the beads were eluted by competition with reduced glutathione.
iii) The eluted proteins were run on a SDS-PAGE gel and transferred to a nitrocellulose filter. The blots were analysed by immunodetection for the presence of HAUSP encoding sequences using the r201 antipeptide antibody.



iii) Analysis of eluted proteins for presence of
HAUSP encoding sequences

the legend of Figure 3D4.3b). In order to normalise the amounts of x744-861 HAUSP deletion mutant and full length HAUSP present in the 'pull-down' assay, five times the amount of the deletion mutant extract was used compared to that of full length HAUSP (based on the expression levels observed). This was done to ensure that the potential ability of these proteins to interact with the Vmw110 GST fusion protein was comparable. As can be observed for all three 'pull-down' assays (Figure 3D4.3b), only the full length HAUSP was pulled down by the C-terminal fragment of Vmw110 and the x513-581, x529-576 and x744-861 HAUSP deletion mutants all lost their ability to interact with this protein. This reinforces the data suggesting that residues between x516-837 are essential for interaction with Vmw110 and further suggests that specific residues required for this interaction lie in the regions spanning residues 529-576 and 744-861.

3D4.4 The effect of removal of residues from HAUSP on its ability to interact with full length Vmw110 in an immunoprecipitation assay

Up until now the experiments to define the region of HAUSP required for interaction with Vmw110 had always involved at least one of the proteins being expressed as a fragment fused to GST. Therefore a logical extension of this work was to observe the interaction between two full length proteins by coimmunoprecipitation. The immunoprecipitation experiment designed is outlined in Figure 3D4.4a.

The results from the clearest of a number of immunoprecipitation assays are seen in Figure 3D4.4b. Note, only the x513-581 HAUSP deletion mutant was tested in this assay as the expression of the x744-861 HAUSP deletion mutant was too low for the purposes of this experiment.

The western blot shown in part B is of the immunoprecipitation samples which have been probed to detect Vmw110 and they verify that Vmw110 was immunoprecipitated only in the samples in which the anti-Vmw110 MAb was added. The blot shown in Figure 3D4.4b part A is of the same samples probed against HAUSP. These results show that HAUSP was only coimmunoprecipitated from the sample in which full length HAUSP was present and not from the sample in which the x513-581 HAUSP deletion mutant was present. As such these results imply that HAUSP residues within 513-581 are required for interaction with full length Vmw110.

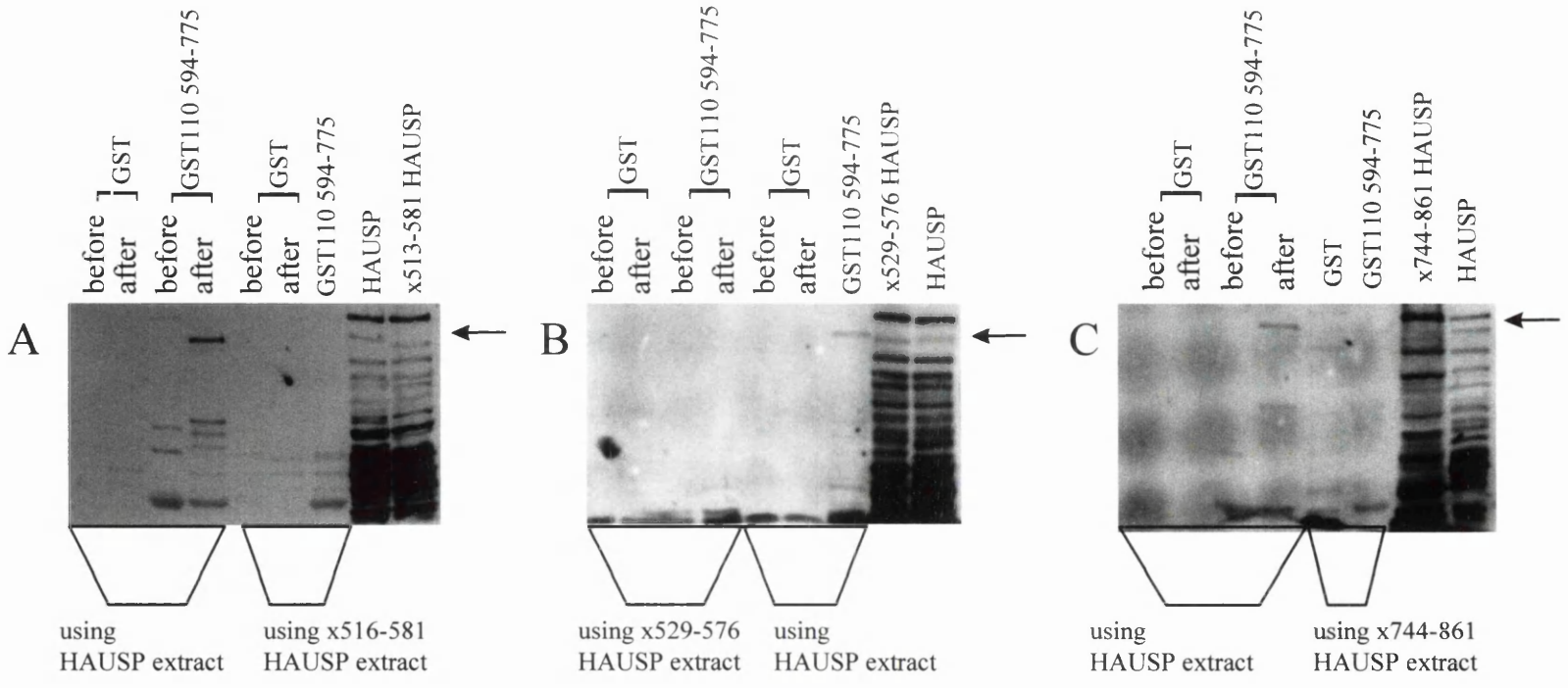
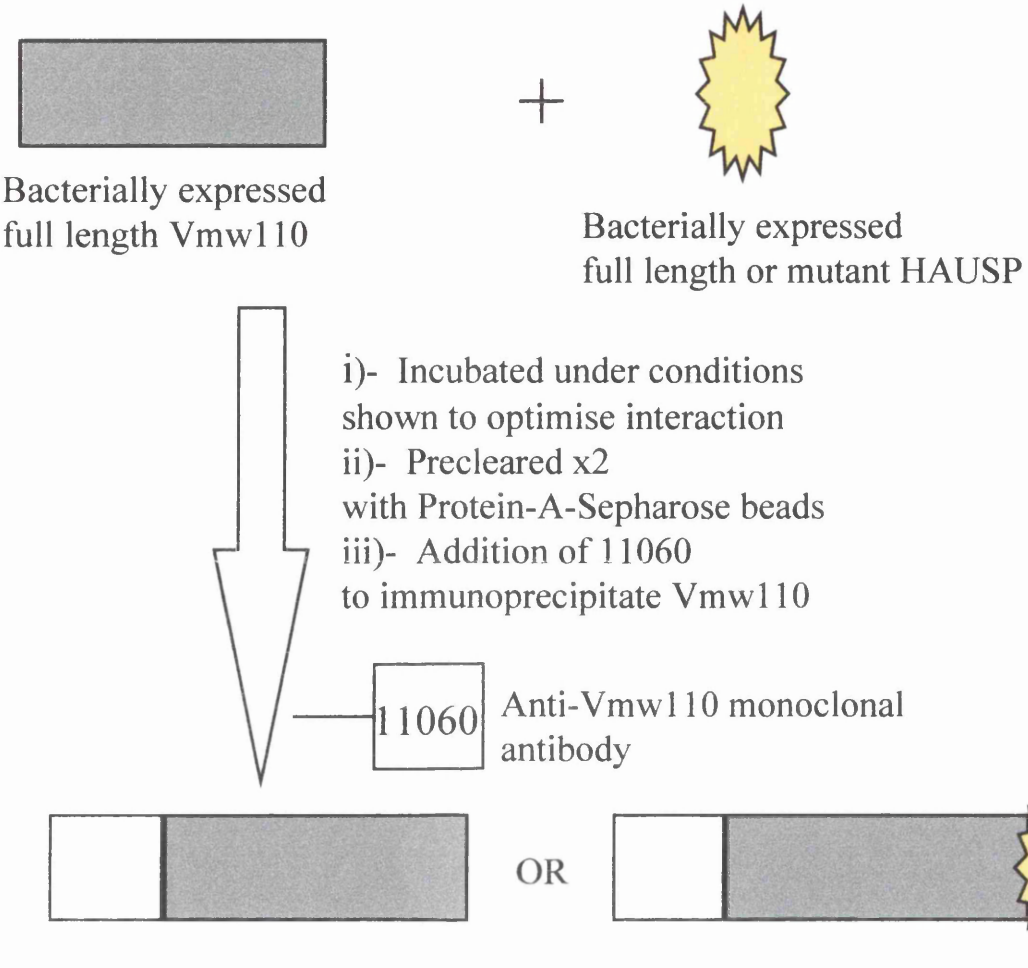


Figure 3D4.3b: GST 'pull-down' assays to investigate the interactions of a C-terminal Vmw110 GST fusion protein with HAUSP deletion mutants x513-581 HAUSP, x529-576 HAUSP and x744-861 HAUSP.

Panels A-C show the 'pull-down' experiments based on the standard assay conditions described in the legend of Figure 3D2, with the exception of the following modifications. Aliquots of beads bound to the control GST protein and the GST fusion protein encoding residues 594-775 of Vmw110 were adjusted in order that equal amounts of these proteins would be present. These beads were then added to 300µl quantities of pre-cleared extracts. The pre-cleared extract consisted of 200µl of bacterial extract (in which either HAUSP protein expression was or was not induced), 64µl dH_2O, 6µl 10% NP40, 30µl 5M NaCl. Furthermore, the blots were analysed by immunodetection for the presence of HAUSP encoding sequences using the r201 antipeptide antibody.

The GST track shows proteins eluted from beads bound to the GST protein. The track labelled GST110 594-775 show proteins eluted from beads bound to the purified GST fusion protein encoding Vmw110 residues 594-775. The before and after has been indicated to represent samples from the 'pull-down' assay obtained before binding to the pre-cleared extract and those which were eluted after binding to pre-cleared extract. The HAUSP, x513-581 HAUSP, x529-576 HAUSP and x744-861 HAUSP tracks show 5µl samples of the bacterial extracts expressing these proteins. The type of bacterial extract used as the source of HAUSP for the 'pull-down' experiments has been indicated below the gels . The arrows denote the position of the bands which represent either wild type HAUSP or a HAUSP deletion mutant.

Figure 3D4.4a: Outline of an immunoprecipitation to investigate the interaction of full length Vmw110 with HAUSP deletion mutants.
i-ii) Bacterial extracts in which full length HAUSP or HAUSP deletion mutants had been expressed were mixed with a bacterial extract in which full length Vmw110 had been expressed and then pre-cleared twice with Protein-A-Sepharose beads.
iii) The samples were then divided into two. To one half only sheep anti-mouse immunoglobulin (SαM IgG) was added and to the other half SαM IgG and the Vmw110 MAb 11060 was added.
iv) Protein-A-Sepharose beads were added next to precipitate 11060 and the interacting proteins.
v) The samples were washed to remove non-specific interactions. Proteins bound to the beads were eluted into 1X SDS loading buffer.
vi) The eluted proteins were run on an SDS-PAGE gel and transferred to a nitrocellulose filter. The blots were analysed by immunodetection for the presence of HAUSP and Vmw110 encoding sequences.



vi)- Analysis of eluted proteins for presence of
HAUSP and Vmw110 encoding sequences

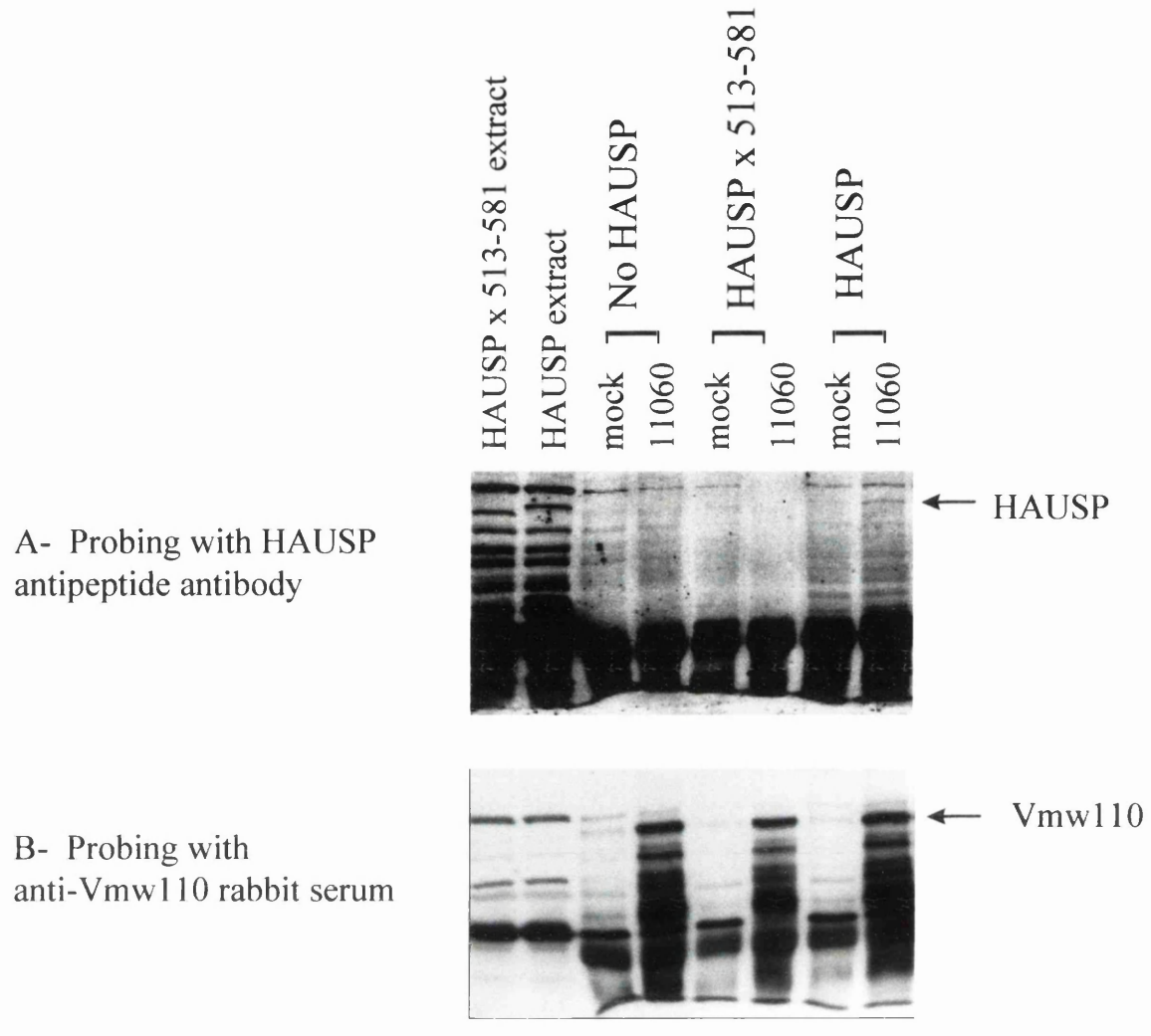


Figure 3D4.4b: Immunoprecipitation assay to investigate the interaction between Vmw110 and a HAUSP deletion mutant.

The immunoprecipitation assay was carried out using the standard protocol described in Section 2B4.9, with the following modifications: full length Vmw110 was expressed in bacteria using the same method as described in Section 3D4.2 as for the expression of HAUSP deletion mutants. Samples were prepared consisting of: 400µl bacterial extract in which either HAUSP had or had not been expressed and 400µl bacterial extract in which Vmw110 had been expressed, made up to a total volume of 1ml in which the final concentrations of NP40 and NaCl were 0.05% and 0.5M respectively. These samples were then pre-cleared twice using Protein-A-Sepharose beads. The samples were then divided into two equal portions. One portion received an aliquot (5µl) of SαM immunoglobulin and other portion also received an aliquot (5µl) of the SαM immunoglobulin, plus a 1µl aliquot of the anti-Vmw110 MAb 11060. These samples were left to incubate for 3hrs at 4°C. Then an aliquot (60µl) of Protein-A-Sepharose beads in a 50% slurry was added to each sample and left for 1hr at 4°C. The samples were then washed three times with a wash buffer (consisting of 50mM Tris, 0.5M NaCl, 1mM EDTA, 0.5% NP40, 1mM PMSF, 1µg/µl leupeptin, 15µg/µl Bestatin). After removal of the supernatant from the final wash an aliquot (20µl) of 1X SDS loading buffer was added to elute the samples. Samples were boiled for 2min prior to loading on a 6% SDS-PAGE gel. Proteins were then transferred to a nitrocellulose filter and analysed by immunodetection for HAUSP encoding sequences using the r201 serum. This blot was then stripped and reprobed for the presence of Vmw110 encoding sequences using the r95 antibody.

Aliquots (5µl) of bacterial extracts were run in the far left hand lanes of each gel. The 'Mock IP' track shows proteins that were immunoprecipitated when SαM immunoglobulin alone was used and the '11060' track shows the proteins that were immunoprecipitated in the presence of both SαM immunoglobulin and 11060. The labels above each track indicate which type of HAUSP was present in the samples prepared for immunoprecipitation. Panel A shows the blot probed using r201 and panel B shows the same blot reprobed using r95. Bands corresponding to HAUSP and full length Vmw110 have been indicated by arrows to the right of the appropriate gels.

3D5 An investigation to identify precise residues required for interaction with Vmw110

3D5.1 Targeting of residues required for interaction with Vmw110

Since the region of HAUSP required for interaction with Vmw110 had been mapped to within residues 529-576, the next stage was to try and more precisely identify which of these residues are essential.

In order to do this sequences C-terminal of the USP histidine domain were compared between HAUSP and other members of the USP family. The most significant results of this work can be seen in Figure 3D5.1. These results show that there is a high degree of homology retained between this region in human HAUSP and the equivalent regions in the two yeast homologues (Section 1E.2).

Furthermore, this region shows strong similarity to the 'KEKE' motif, which is also present in subunits of the 20S and 26S proteasome, and an activator of the 20S proteasome (Realini *et al.*, 1994). The 'KEKE' motif is defined as being greater than 12 amino acids in length, devoid of W, Y, F or P residues, consisting of more than 60% K and E/D residues and lacking five positive or negatively charged residues in a row. It has been proposed that they promote association between protein complexes and may contribute to the selection of peptides presented on MHC class 1 receptors.

It was predicted that these conserved residues have some functional significance. The exact role of these residues for the cellular function of HAUSP is debatable. However, based on the observation that viral proteins often use such pre-existing motifs for their own purposes, it seemed plausible that Vmw110 interacted with HAUSP through these residues. As such, residues E546, E547, K556, E557 and H562 were targeted for mutagenesis.

3D5.2 Construction of E/E546/547P/P, K/E556/557P/P and H562L pET24a135 mutants

The E/E546/547P/P, K/E556/557P/P and H562L pET24a135 mutants were synthesised in several stages. The first stage was to perform site-directed mutagenesis using the pET24a135 plasmid as the template and the E/E546/547P/P, K/E556/557P/P and H562L oligonucleotides as the mutagenic primers (Section 2B1.10 and Table 2B1.10). The successful incorporation of the mutations was checked by restriction analysis using

	513							582
HAUSP	AYMLVYIRES	KLSEVLQAVT	DHDIPQQLVE	RLQEEKRIEA	QKRKERQEAH	LYMQVQIVAE	DQFCGHQGND	
Q09879	AYMLLYLRKD	KLDELMNPVS	ADEIPEHLKE	ALNPSIQLAE	LRRKERLESH	LYTKVQLITP	EFYSEHHEFD	
Sc99552X6	AYMLVYIRQE	QEEDLLRPVL	ESDVPKHVIT	RVREEIKERE	TKEKEIREAH	LYVTLRLHSI	KEFIHYEGFD	
Consensus	AYMLvYiR	kl e l V	diP l e	rl **ee**	kr**KE**r Ea**H**	LY vq	f h g D	

Figure 3D5.1: Comparison of sequences C-terminal of the USP histidine domain. The *Schizosaccharomyces pombe* protein [GenBank accession number Q09879] and *Saccharomyces cerevisiae* protein [GenBank accession number Sc99552X6] were detected by homology to HAUSP using the Blast program. Using Pileup (GCG) the sequences C-terminal of the histidine domain were aligned. The consensus sequence below shows residues that are conserved in all three proteins in capitals and those only conserved between HAUSP and one other protein are shown in lower case. The positions targeted for mutagenesis have been indicated in bold.

restriction sites introduced in each mutagenic primer which did not affect the coding potential. Then in each case for the E/E546/547P/P, K/E556/557P/P and H562L pET24a135 clones a small region surrounding the inserted mutation was subcloned into the original wild type pET24a135 plasmid DNA. This was done in order to remove any additional mutations which may have arisen as part of the site-directed mutagenesis procedure.

The E/E546/547P/P, K/E556/557P/P and H562L pET24a135 subclones were synthesised in ligation reactions with three fragments isolated from the relevant digestion mixes: these were the *Bst*EII-*Bst*EII(529) pET24a135 fragment, the *Bst*EII(529)-*Msc*I(576) fragment from the relevant mutant pET24a135 clone and the *Msc*I(576)-*Bst*EII (dephosphorylated) pET24a135 fragment (see Figure 3D5.2). Sequencing (Section 2B1.9) was then performed using the KEKE sequencing primer (Table 2B1.9) on these mutants to ensure the correct incorporation of the mutations.

3D5.3 Expression of the HAUSP site specific mutants

The HAUSP site specific mutants were then expressed in the *E.coli* strain BL21 pLysS DE3 and protein extracts made as described for the expression of the HAUSP deletion mutants (Section 3D4.2). Expression of the site specific mutants was ascertained by probing a western blot of a SDS-PAGE gel of the bacterial extracts using anti-HAUSP rabbit serum r201. The results of this can be observed in Figure 3D5.3 and they show that proteins of the expected molecular weight have been synthesised and are expressed at levels equivalent to full length HAUSP.

3D5.4 Effect of the E/E546/547P/P, K/E556/557P/P and H562L substitutions on the ability of HAUSP to interact with the C-terminal of Vmw110 in a 'pull-down' assay

The effect of these substitutions on the ability of HAUSP to interact with Vmw110 was then tested. This was achieved by performing a 'pull-down' assay similar to the one described in Section 3D4.3, in which the 594-775 C-terminal Vmw110 GST fusion protein was used as 'bait' to 'pull-down' HAUSP mutants (experimental details are the same as those given in the legend of Figure 3D4.3b). The results of this experiment are shown in Figure 3D5.4 which shows that the substitutions have not altered the ability of these HAUSP proteins to be pulled down by the C-terminal fragment of Vmw110. This suggests that the residues: 546, 547, 556, 557 and 562 of HAUSP are not essential for binding to

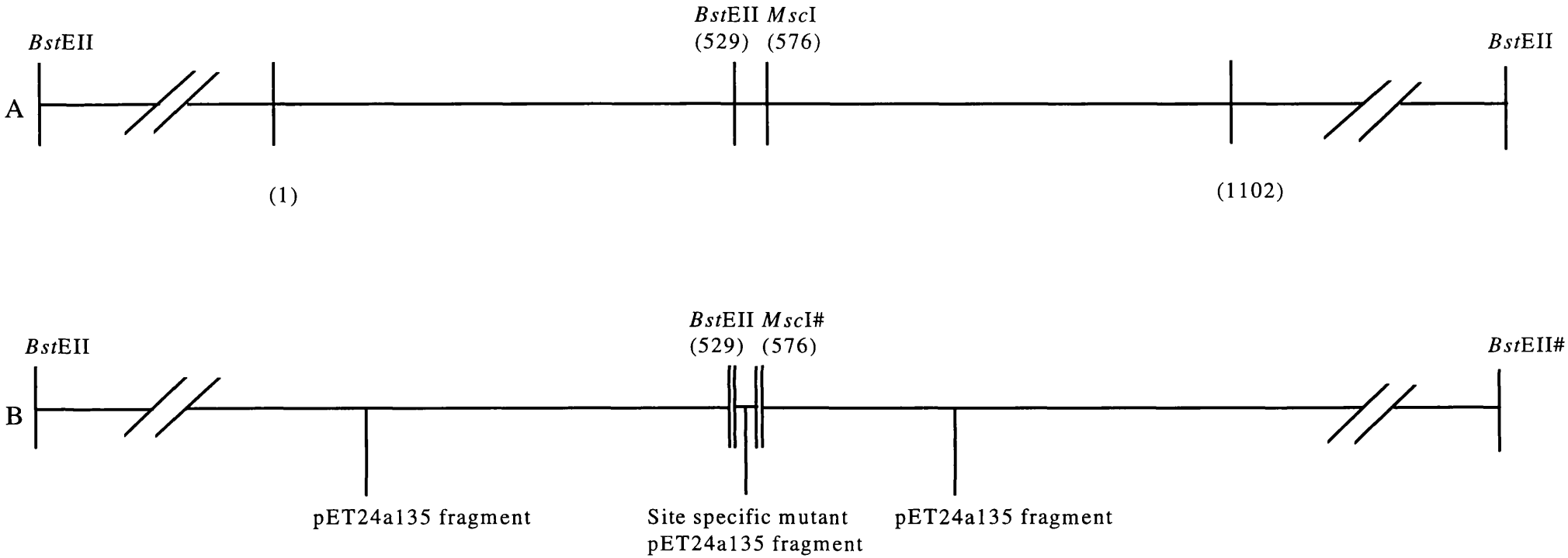


Figure 3D5.2: Construction of the E/E546/547P/P, K/E556/557P/P and H562L pET24a135 subclones.
Part A: Shows a linearised plasmid map of pET24a135, in which all the restriction sites have been indicated for the enzymes used in the construction of the E/E546/547P/P, K/E556/557P/P and H562L pET24a135 subclones. Positions 1-1102 represent the HAUSP coding sequence which has been drawn to scale, unlike the vector sequences marked by the // sign.
Part B: Shows the three pET24a135 fragments used in the ligation reaction for the construction of the E/E546/547P/P, K/E556/557P/P and H562L pET24a135 subclones. The plasmid from which the fragment originated has been indicated underneath. Fragments whose 5' termini were dephosphorylated have been indicated with a # sign.

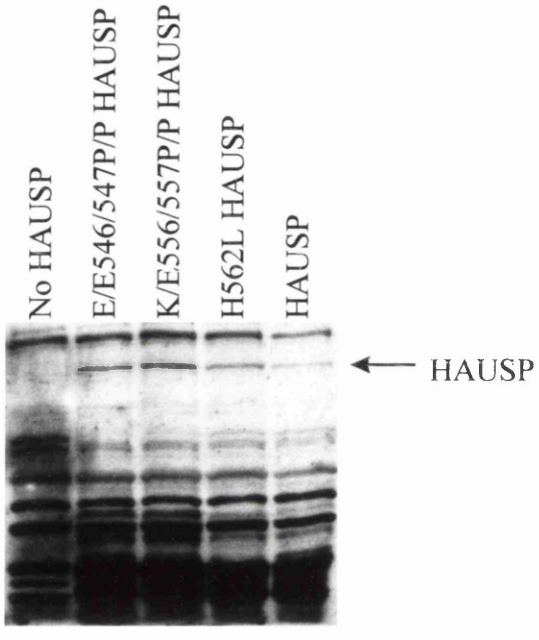


Figure 3D5.3: Western blot analysis of bacterial extracts containing the HAUSP site specific mutants E/E546/547P/P, K/E556/557P/P and H562L.

From 100ml cultures induced with IPTG, 2ml soluble bacterial extracts were prepared and 5µl samples were subjected to SDS-PAGE and the proteins transferred to a nitrocellulose filter and probed for HAUSP encoding sequences using r201 diluted 1 in 1,000 (as described in Section 3D4.2). The label above each track describes the type of bacterial extract loaded in that lane. The arrow represents the position of the bands corresponding to HAUSP.

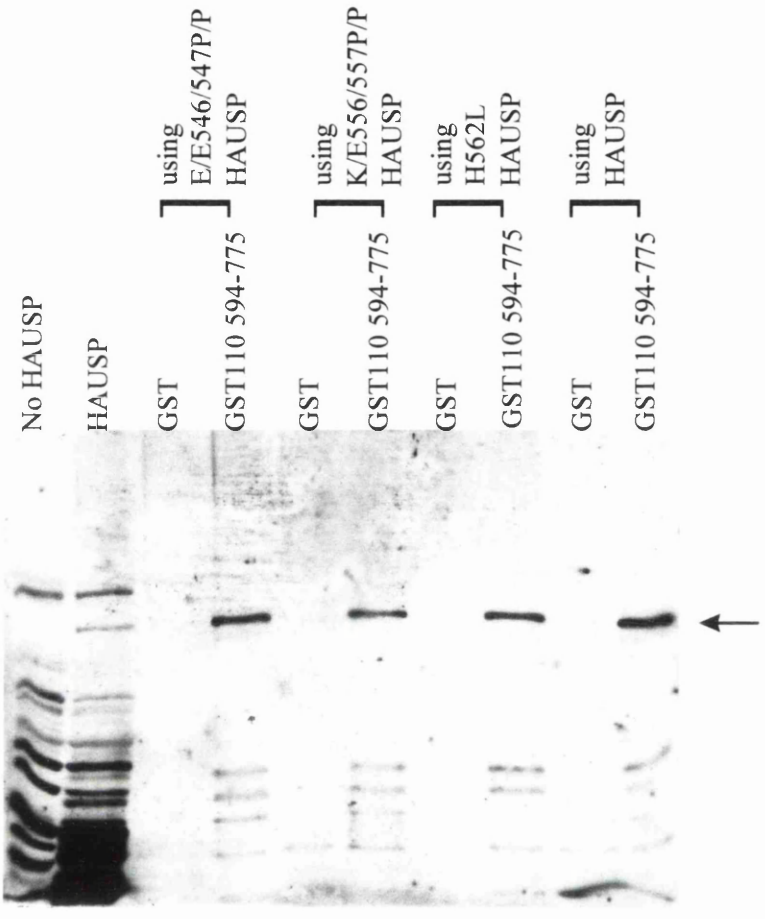


Figure 3D5.4: GST 'pull-down' assay to investigate if the C-terminal Vmw110 GST fusion protein interacts with HAUSP site specific mutants E/E546/547P/P, K/E556/557P/P and H562L.

A GST 'pull-down' assay was carried out using the same protocol as described in the legend of Figure 3D4.3b, with the exception that the HAUSP site specific mutants were included in the experiment in place of HAUSP deletion mutants. The labelling pattern described in the legend of Figure 3D4.3b was also followed here with the following exceptions: the type of HAUSP being tested for interaction with Vmw110 is indicated above the gel and the bands corresponding to HAUSP are indicated by an arrow.

Vmw110. Hence the residues required for interaction with Vmw110 must lie elsewhere within the 529-576 region of HAUSP.

3D6 Summary of studies done to define the region of HAUSP that interacts with Vmw110

- Using the GST 'pull-down' technique in which selected segments of HAUSP were expressed as fragments fused to GST, it was established that residues 26-193 and 516-837 are independently sufficient for interaction with a bacterially expressed C-terminal fragment of Vmw110.

- The interaction of these HAUSP fusion proteins with the Vmw110 fragment were shown to require sequences within Vmw110 which are essential for its interaction with cellular HAUSP.

- Results of GST 'pull-down' assays in which the Vmw110 used was derived from HSV-1 infected cells, implied that HAUSP residues 516-837 and not residues 26-193 interact with full length Vmw110 through sequences within Vmw110 which are essential for its interaction with cellular HAUSP.

- The significance of these residues for interaction with Vmw110 when in the context of full length HAUSP was investigated using HAUSP deletion mutants in which residues within this critical region were removed.

1. Introduction of these mutants in a modified GST 'pull-down' assay showed that HAUSP residues 529-576 and 744-861 were required for interaction with the C-terminal fragment of Vmw110.
2. Introduction of the x513-581 HAUSP deletion mutant in an immunoprecipitation assay showed that these residues are required for interaction with full length Vmw110.

- Investigation into precise residues within the HAUSP region 529-576 which were hypothesised to be important for protein-protein interactions, resulted in the identification of residues that were well conserved with other USPs. However it was shown that these residues are dispensable for interaction of HAUSP with the C-terminal of Vmw110.

3E An investigation into the effects of Vmw110 binding on the biochemical activities of HAUSP

3E1 Introduction

In order to better understand the role of HAUSP in HSV-1 infection, the effects of Vmw110 binding to HAUSP on its normal cellular activities were investigated. For this purpose two strategies were designed.

The first approach was based on the observation that the region of HAUSP required for interaction with Vmw110 was located a few residues downstream of the histidine active site domain (Section 3D). For this reason the effect of Vmw110 binding on the deubiquitinase activity of HAUSP was investigated.

The second strategy was based on the observation that HAUSP residues 516-837 were shown to be required for interaction with both Vmw110 and a ~40kD cellular protein (Sections 3C and 3D). As such it was speculated whether Vmw110 binding to HAUSP affected the ability of the ~40kD protein to bind to the same region.

3E2 An investigation into the effect of Vmw110 on the deubiquitinase activity of HAUSP

3E2.1 An investigation into whether the residues of HAUSP required for interaction with Vmw110 are also important for its deubiquitinase activity

The initial strategy to investigate whether Vmw110 effects the deubiquitinase activity of HAUSP involved establishing if deletion of the HAUSP region required for interaction with Vmw110 affected its enzyme activity. For this purpose the x529-576 HAUSP deletion mutant was tested in the standard *in vivo* deubiquitinase assay, the results of which are shown in Figure 3E2.1. It is worth noting that the conserved residues of the active site domains have been retained in this mutant. As can be seen in the track where expression of the x529-576 HAUSP deletion mutant was induced, no substrate cleavage of Ub-M-β-gal to the lower molecular weight M-β-gal was observed, unlike in the track expressing the wild type HAUSP. This suggests that residues shown to be essential for interaction with Vmw110 also appear to have a function for the deubiquitinase activity of HAUSP. However, the loss of the deubiquitinase activity of this mutant may be the consequence of conformational changes that result from deletion of these residues. Therefore bacterial

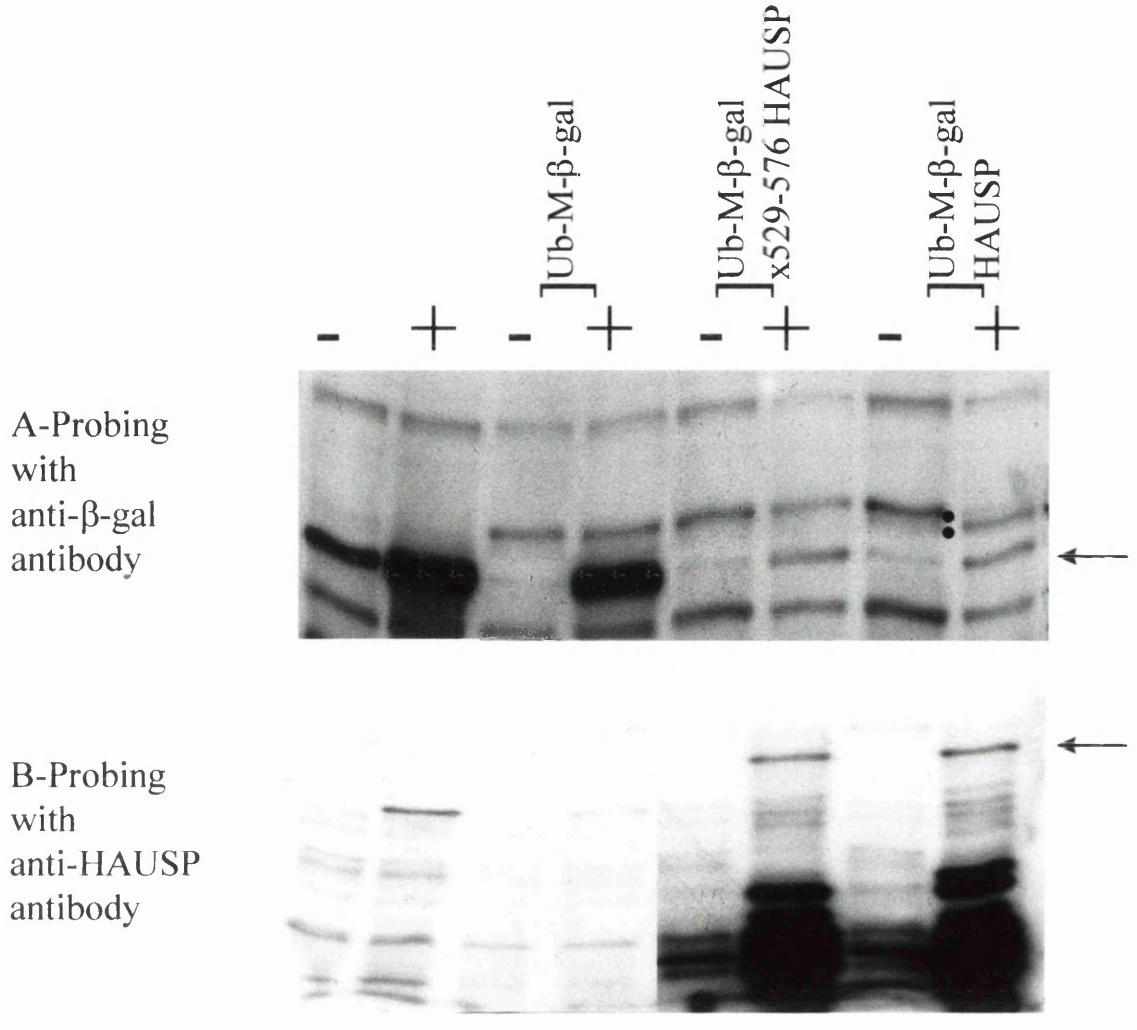


Figure 3E2.1: Assay for deubiquitinase activity in which the effect of removing the x529-576 residues of HAUSP was investigated.

A standard *in vivo* deubiquitinase assay was carried out as described in Section 2B2 using bacteria harbouring plasmids expressing the Ub-M-β-gal model substrate (pAC-M-β-gal) and either x529-576 HAUSP, or wild type HAUSP (pET24a135).

A-The western blot of the bacterial cultures probed with anti-β-gal antiserum r12741. The labels on top of the tracks indicate which proteins were expressed in the cultures and whether their expression had been induced by the addition of IPTG or left uninduced (as indicated by + and - respectively). The upper dot between lanes 7 and 8 indicates the uncleaved substrate and the lower dot indicates the correctly cleaved M-β-gal product. The endogenous truncated β-gal expressed by the NovaBlue bacteria is indicated by the arrow on the right of the gel.

B- The western blot was stripped and reprobed for HAUSP using the r201 anti-HAUSP antipeptide antibody in a 1 in 1,000 dilution. The arrow on the right of the gel indicates the position of HAUSP.

extracts used for the deubiquitinase assay were probed for the presence of HAUSP, to verify that there was not a problem with its expression (Figure 3E2.1 part B). The results indicated that the x529-576 HAUSP mutant expressed was produced at levels comparable to the full length protein.

3E2.2 An investigation into whether expression of HAUSP with Vmw110 effects its deubiquitinase activity

The next step was to investigate the direct effect of Vmw110 binding on the deubiquitinase activity of HAUSP. The initial strategy involved first coimmunoprecipitating Vmw110 and HAUSP and then testing this complex for deubiquitinase activity using an *in vitro* cleavage assay. However, as described in Section 3A attempts to establish such an assay for HAUSP proved difficult.

An indirect approach was used instead. This involved using the standard *in vivo* deubiquitinase assay to test whether expression of Vmw110 affected HAUSP enzyme activity. As such a construct was made in which HAUSP and Vmw110 were placed under the control of the same T7 promoter, in order that they could be expressed simultaneously. A second construct was also prepared in which Vmw110 was again inserted next to the HAUSP coding DNA, but this time in a 3' to 5' antisense orientation. This latter construct was designed to see if the presence of Vmw110 encoding sequences next to the HAUSP coding region, by themselves without protein expression, interfered with the deubiquitinase activity of HAUSP.

3E2.2a Construction of plasmids expressing HAUSP and Vmw110 (either in a sense or antisense orientation) under the control of the same promoter

The bi-cistronic pT7110(sense)-HAUSP and pT7110(antisense)-HAUSP plasmids were constructed in a ligation reaction made from three fragments isolated from the relevant digestion mixes (Figure 3E2.2aa).

The PCR amplified *Bst*EII-*Xba*I fragment encoding residues 709-775 of Vmw110 was prepared in order to introduce a *Xba*I site at the 3' terminus of the Vmw110 open reading frame. Its synthesis firstly involved isolating a fragment from an *Eco*RI-*Eco*RI digest of pGEXE52 plasmid containing the C-terminus of Vmw110. A part of this fragment was then PCR amplified from a reaction set up in 50µl total consisting of: 0.5ng pGEXE52 *Eco*RI fragment, 50pmoles *Bst*EII primer (Figure 3E2.2ab) and 50pmoles *Xba*I primer

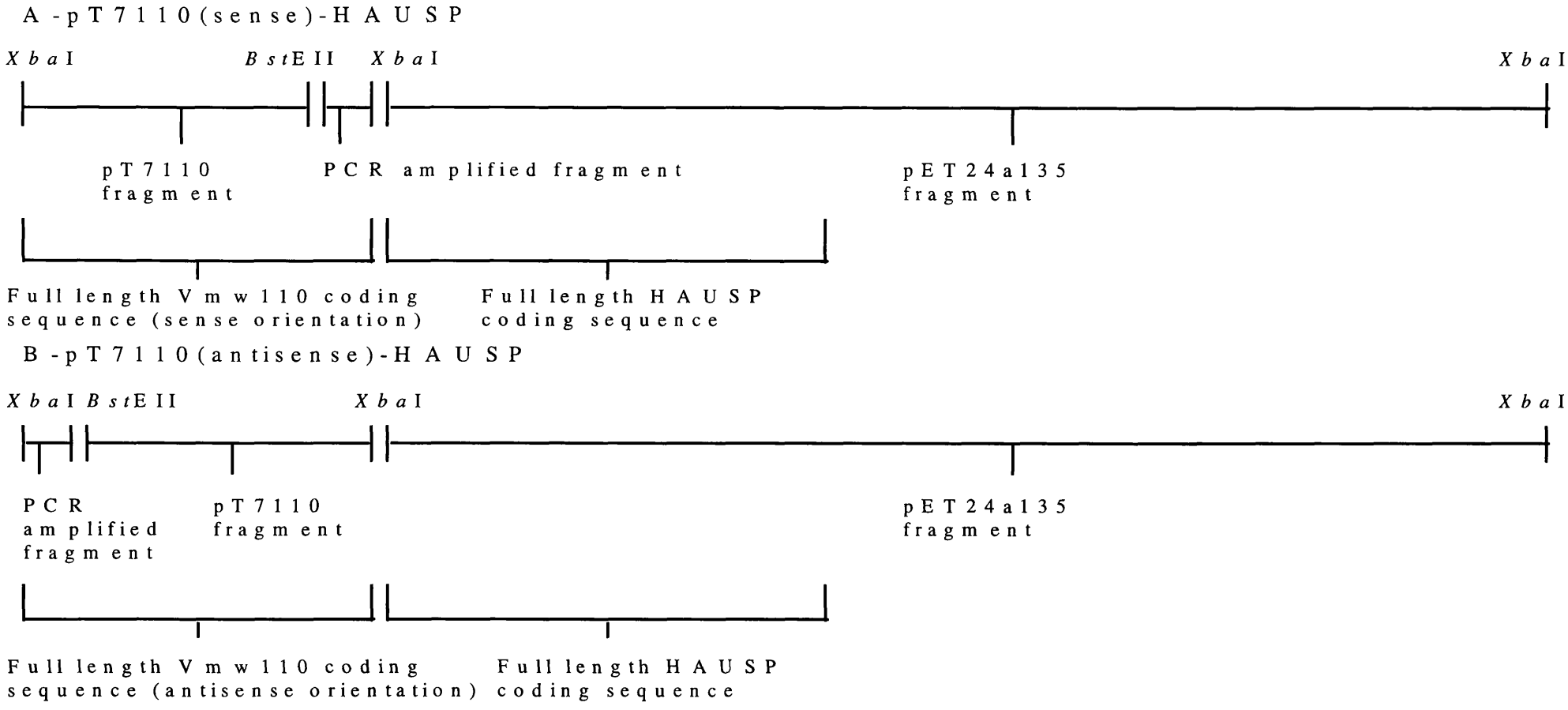


Figure 3E2.2aa: Plasmids pT7110(sense)-HAUSP and pT7110(antisense)-HAUSP are shown with all the fragments relevant to cloning in parts A and B respectively. The fragments from which they were composed were isolated from the relevant digestion mixes: digestion of the pET24a135 plasmid with *Xba*I, followed by dephosphorylation; *Xba*I-*Bst*EII digestion of the pT7110 plasmid and *Bst*EII-*Xba*I digestion of the PCR amplified Vmw110 C-terminal fragment (preparation of which is explained in the text). The orientation in which Vmw110 was inserted into pET24a135 was then deciphered by screening the miniprepared DNA with *Bst*EII.

(Figure 3E2.2ab), 2µl 10mM $MgCl_2$, 1µl 10mM dNTPs, 5µl 10X PCR buffer (supplied with the enzyme) and 0.5 units Taq polymerase. The following cycling conditions were then used to amplify the fragment: 5min initial cycle at 95°C (denaturing), then 2min at 72°C (annealing), followed by 25 cycles at 95°C for 30sec and 72°C for 2min, and then finished by a 30sec cycle at 95°C and a 10min cycle at 72°C. The reaction product was then purified by the isolation of a band of the correct molecular weight from an agarose gel using the GENECLEANII kit (Section 2B1.4b). The final step was to then isolate the DNA from a *Bst*EII-*Xba*I digestion mix of this PCR amplified fragment.

```
709                                 716
Vmw110 sequence (5' to 3'): GAG ACC GCG GGT AAC CAC GTG ATG
BstEII primer   (5' to 3'):   G ACC GCG GGT AAC CAC GTG A

                                    772              STOP CODON
Vmw110 sequence (5' to 3'): GAG GGA AAA CAA TAAG
XbaI primer     (3' to 5'): CTC CCT TTT GTT ATTCAGATCTCGCCG
```

Figure 3E2.2ab: Sequences of the *Bst*EII and *Xba*I primers. The primer sequence has been aligned with the relevant sequence within Vmw110. The codon position of the Vmw110 sequence has also been indicated. The *Bst*EII restriction site (in the *Bst*EII primer) and *Xba*I restriction site (in the *Xba*I primer) have been underlined.

3E2.2b Introduction of the pT7110(sense)-HAUSP and pT7110(antisense)-HAUSP plasmids into the standard *in vivo* deubiquitinase assay

The effect of introducing either the pT7110(sense)-HAUSP or pT7110(antisense)-HAUSP plasmids into the standard *in vivo* deubiquitinase assay can be seen in Figure 3E2.2b. It is clear from the track in which the pT7110(sense)-HAUSP was expressed, that the simultaneous expression of Vmw110 did not detectably alter the ability of HAUSP to cleave the Ub-M-β-gal substrate. Indeed it displayed 100% cleavage of the model substrate, which is comparable to that seen with the control pT7110(antisense)-HAUSP plasmid and with the positive control in which HAUSP alone was expressed.

The blot of the bacterial extracts from the deubiquitinase assay were also probed for the presence of Vmw110 (shown in Figure 3E2.2b part B), these results verified Vmw110 had been expressed by the pT7110(sense)-HAUSP plasmid and not the pT7110(antisense)-HAUSP plasmid. Hence, these results indicated that the presence of Vmw110 does not affect HAUSP deubiquitinase activity.

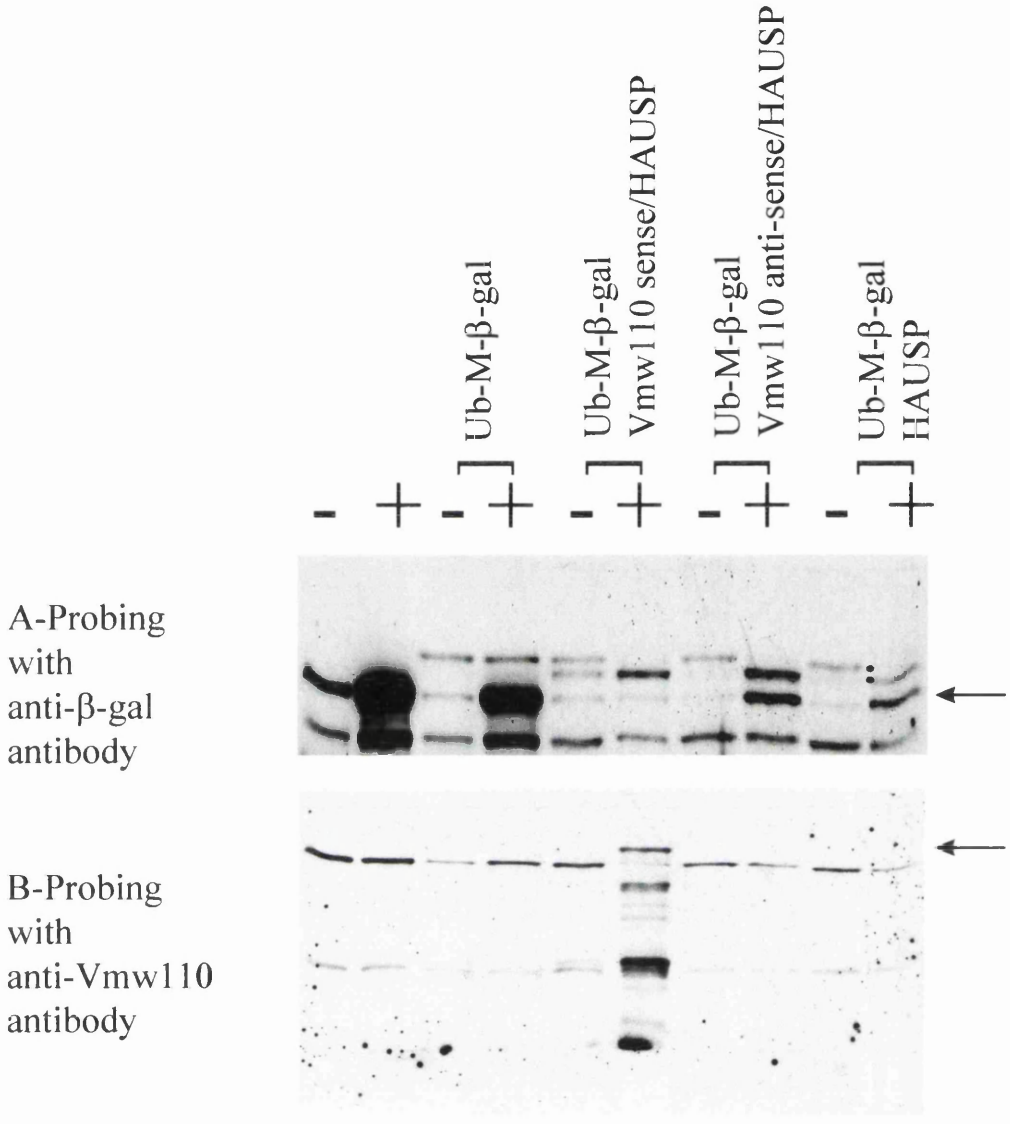


Figure 3E2.2b: A deubiquitinase activity assay to investigate the effect of simultaneously expressing full length Vmw110 and HAUSP on HAUSP enzyme activity.

A standard *in vivo* deubiquitinase assay was carried out as described in Section 2B2 using bacteria harbouring the plasmids: pAC-M-β-gal and either pT7110(sense)-HAUSP, pT7110(antisense)-HAUSP, or pET24a135.

A-The western blot of the bacterial cultures probed with anti-β-gal antiserum r12741. The labels on top of the tracks indicate which proteins were expressed in the cultures and whether their expression had been induced by the addition of IPTG or left uninduced (as indicated by + and - respectively). The upper dot between lanes 9 and 10 indicates the uncleaved substrate and the lower dot indicates the correctly cleaved M-β-gal product. The endogenous truncated β-gal expressed by the NovaBlue bacteria is indicated by the arrow on the right of the gel.

B- The western blot was stripped and reprobed for Vmw110 using the 11060 MAb in a 1 in 10,000 dilution. The arrow on the right of the gel indicates the position of Vmw110.

3E2.2c Limitations of using the indirect *in vivo* approach

This strategy only tested the effect of the presence of Vmw110 on HAUSP enzyme activity. However, whether HAUSP was complexed to Vmw110 when it cleaved the model substrate is debatable. A particular drawback of this approach was that expression of full length Vmw110 in bacteria under this promoter only occurs at low levels. Hence uncomplexed HAUSP may have been available to cleave the model substrate.

3E2.2d Construction of pT7C110(sense)-HAUSP and pT7C110(antisense)-HAUSP plasmids

Therefore, in order to overcome the potential problem of insufficient Vmw110 being expressed (to saturate all the free HAUSP) new constructs were designed. The plasmids were designed to encode HAUSP and the C-terminal 594-775 residues of Vmw110 (either in the sense or antisense orientation) under the control of the same promoter. The idea being that this C-terminal 594-775aa Vmw110 fragment has a high level of expression when placed under the control of the T7 promoter and as such decreases the chances of unbound HAUSP being available to cleave the substrate.

The pT7C110(sense)-HAUSP and pT7C110(antisense)-HAUSP plasmids were constructed in a ligation reaction made from three fragments isolated from their relevant digestion mixes (Figure 3E2.2d).

3E2.2e Introduction of the pT7C110(sense)-HAUSP and pT7C110(antisense)-HAUSP plasmids into the standard *in vivo* deubiquitinase assay

The effects of introducing the pT7C110(sense)-HAUSP and pT7C110(antisense)-HAUSP plasmids into the standard *in vivo* deubiquitinase assay are shown in Figure 3E2.2e. In the track in which the C-terminal region of Vmw110 and HAUSP were expressed simultaneously it is clear that no alteration of HAUSP enzyme activity has occurred.

The blot of the bacterial extracts from the deubiquitinase assay was probed for Vmw110 encoding sequences (Figure 3E2.2e part B), these results show that the C-terminal region of Vmw110 was efficiently expressed. Hence, these results appear to support the idea that Vmw110 does not alter the deubiquitinase activity of HAUSP.

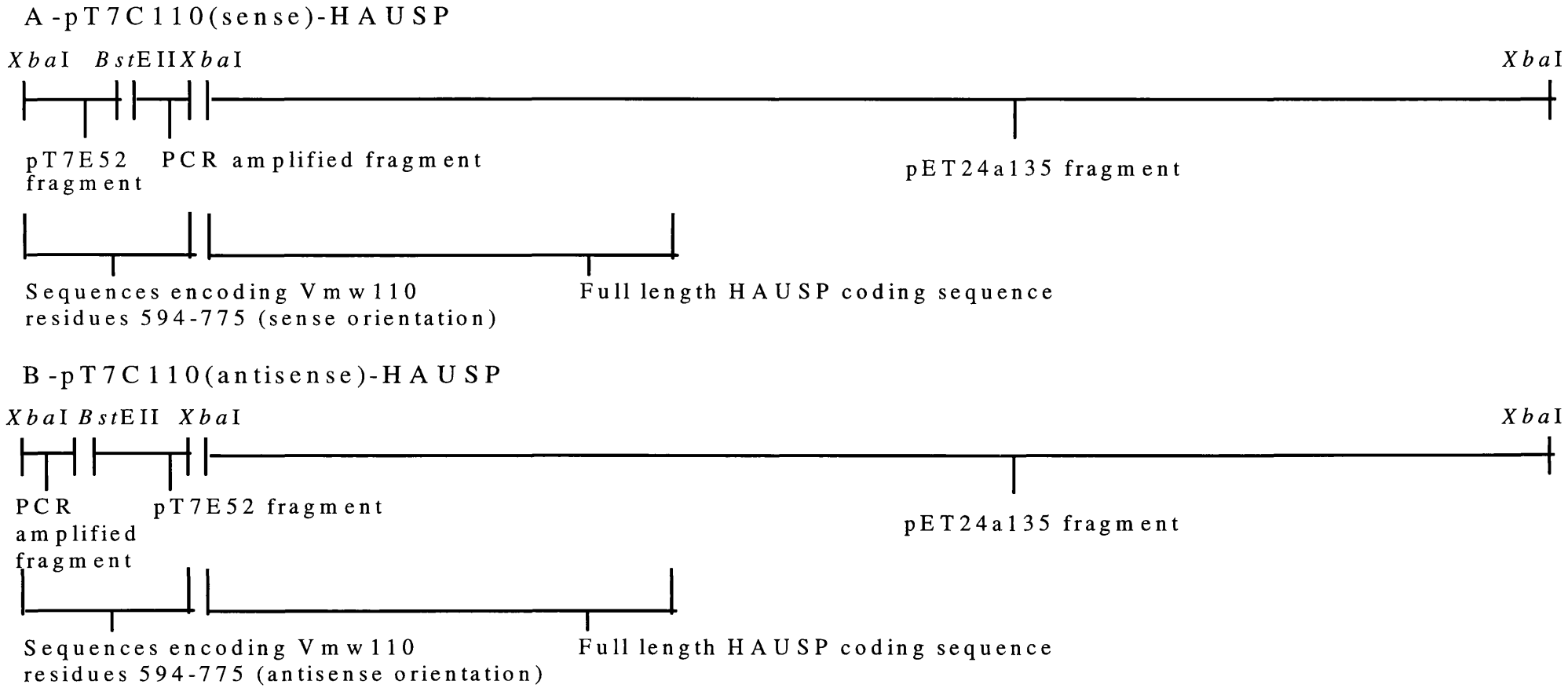


Figure 3E2.2d: Plasmids pT7C110(sense)-HAUSP and pT7C110(antisense)-HAUSP are shown with all the fragments relevant for cloning in parts A and B respectively. The fragments from which they were composed were isolated by the relevant digestion mixes: digestion of the pET24a135 plasmid with *Xba*I, followed by dephosphorylation; *Xba*I-*Bst*EII digestion of the pT7E52 plasmid and *Bst*EII-*Xba*I digestion of the PCR amplified Vmw110 C-terminal fragment (preparation of which is explained in Section 3E2.2a). The orientation in which the C-terminal of Vmw110 was inserted was then deciphered by screening the DNA (prepared from ligation reaction transformed bacterial colonies), with the restriction enzyme *Bst*EII.

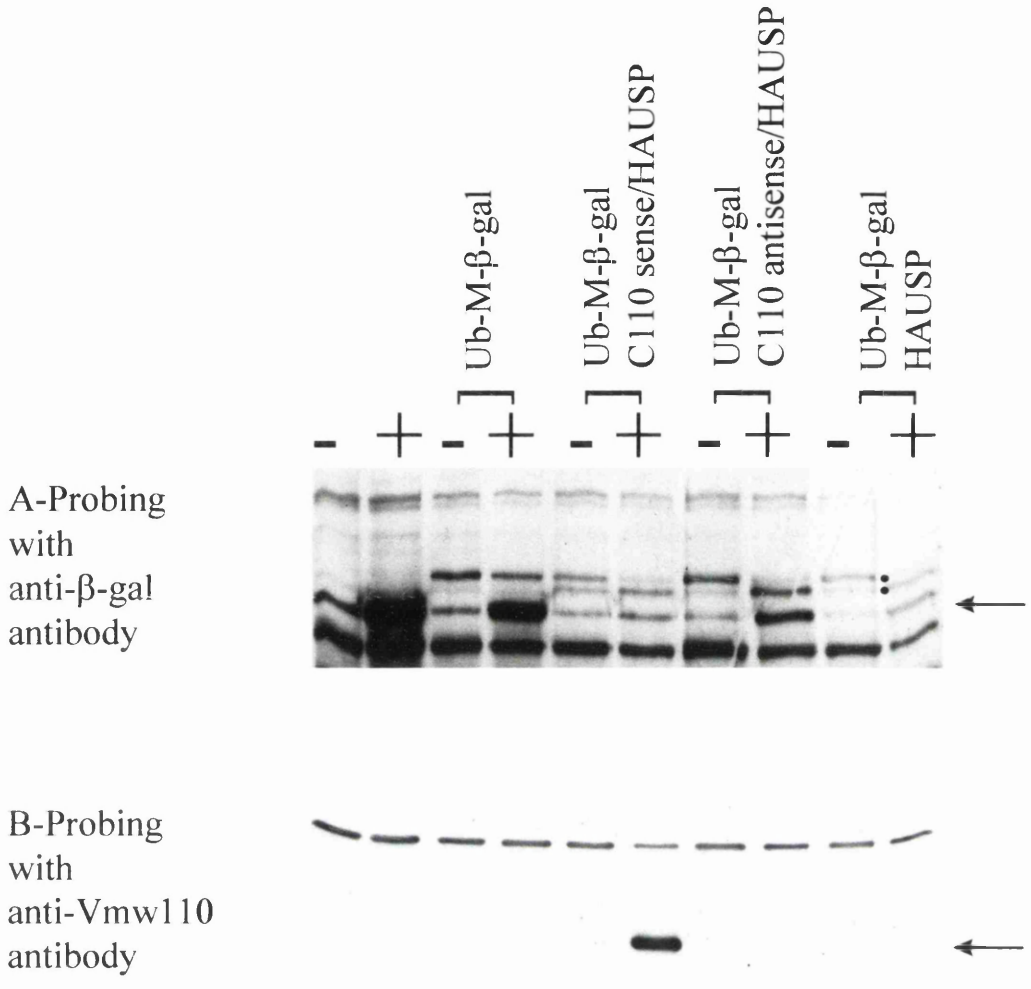


Figure 3E2.2e: A deubiquitinase activity assay to investigate the effect of simultaneously expressing HAUSP and the C-terminal 594-775aa region of Vmw110 on HAUSP enzyme activity.

A standard *in vivo* deubiquitinase assay was carried out as described in Section 2B2 using bacteria harbouring the plasmids pAC-M-β-gal and either pT7C110(sense)-HAUSP, pT7C110(antisense)-HAUSP, or pET24a135.

A-The western blot of the bacterial cultures probed with anti-β-gal antiserum r12741. The labels on top of the tracks indicate which proteins were expressed in the cultures and whether their expression had been induced by the addition of IPTG or left uninduced (as indicated by + and - respectively). Expression of the plasmids encoding the C-terminal 594-775aa region of Vmw110 are indicated by the C110 sense and C110 anti-sense label. The upper dot between lanes 9 and 10 indicates the uncleaved substrate and the lower dot indicates the correctly cleaved M-β-gal product. The endogenous truncated β-gal expressed by the NovaBlue bacteria is indicated by the arrow on the right of the gel.

B- The western blot was stripped and reprobed for Vmw110 using the 10503 monoclonal antibody in a 1 in 5,000 dilution. The arrow on the right of the gel indicates the position of Vmw110.

3E3 An investigation of whether the binding of Vmw110 to HAUSP affects its ability to bind to the ~40kD cellular protein

3E3.1 Strategy

The alternative approach used to investigate potential roles for the interaction between Vmw110 and HAUSP involved testing whether Vmw110 binding to HAUSP affected the ability of the ~40kD cellular protein to interact with the same region. The experimental strategy used to test this theory required several stages and has been described in the legend of Figure 3E3.1. The first stage was to 'pull-down' the C-terminal region of Vmw110 using the 516-1102 HAUSP GST fusion protein. The second stage was to take the Vmw110/GST HAUSP bound-beads and use them in an another 'pull-down' using radiolabelled WS HeLa cell extract. In this manner it could be checked if the complex of Vmw110 fragment/HAUSP bound to the beads prevented the ~40kD cellular protein from being pulled down.

Parallel samples were set up in which either the Vmw110 fragment encoding residues 594-775 was used (which interacts with cellular HAUSP) or one encoding residues 633-775 (in which residues essential for interaction with HAUSP were absent). An excess of these Vmw110 C-terminal fragments was used, relative to the amount used normally, in an attempt to saturate all the available binding sites on the HAUSP GST fusion proteins.

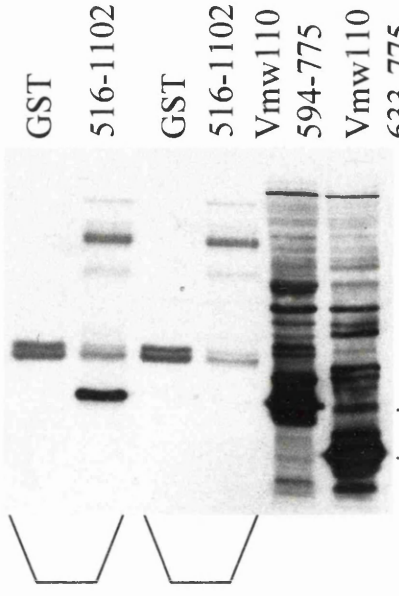
The results of this experiment are shown in Figure 3E3.1. From the first blot probing for Vmw110 encoding sequences (part A) it can be seen that the 594-775aa Vmw110 fragment, but not the 633-775aa fragment was pulled down by the 516-1102 HAUSP GST fusion protein as expected (Section 3D). Figure 3E3.1 part B shows the results of the second 'pull-down' which indicates that in the track in which the Vmw110 fragment encoding residues 594-775 was complexed to the HAUSP GST fusion protein the ~40kD cellular protein was still pulled down. Therefore this implies that interaction of the C-terminal of Vmw110 with HAUSP does not occlude the ~40kD protein from binding.

3E3.2 Limitations of using this approach

This strategy also has limitations that should be taken into account when interpreting the results. The main point being that the HAUSP GST fusion protein was expressed at extremely high levels. Thus even though an excess of Vmw110 was used in the primary 'pull-down' experiment it is unlikely that all the HAUSP GST fusion proteins would have

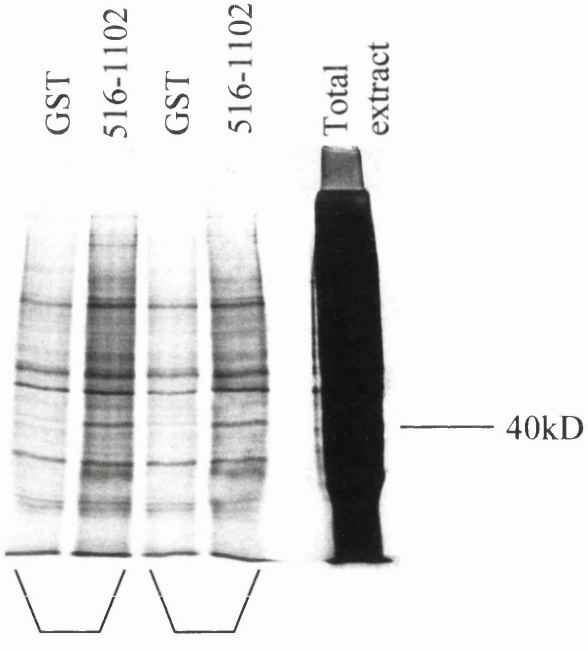
Figure 3E3.1: A GST 'pull-down' assay to investigate if the presence of the C-terminal region of Vmw110 inhibits the binding of the ~40kD cellular protein to a HAUSP GST fusion protein.
Panels A-B show results of a double 'pull-down' experiment, which was based on the standard assay conditions described for GST 'pull-down' assays in Section 2B4.8. Aliquots of beads bound to control GST protein and GST fusion protein encoding HAUSP residues 516-1102 were adjusted in order to provide normalised levels of all the proteins, and each sample was divided into two aliquots. Next the aliquots of each GST protein beads sample were mixed with either a T7E52, or T7E58 pre-cleared extract and samples incubated for 1hr at 4°C. The T7E52 pre-cleared extract consisted of 253µl bacterial extraction buffer, 27µl NaCl, and 20µl T7E52 bacterial extract and the T7E58 pre-cleared extract consisted of 257µl bacterial extraction buffer, 27µl NaCl, and 16µl T7E58 bacterial extract. Beads were then washed three times with 1ml of cold 0.5M NaCl wash buffer (consisting of 50mM Tris, 0.5M NaCl, 1mM EDTA, 0.5% NP40, 1mM PMSF, 1µg/µl leupeptin, 15µg/µl Bestatin) and after the final wash they were left in a 50% slurry of PBS(A). These beads were stored at -70°C overnight. An aliquot of 300µl of pre-cleared ^{35}S-radiolabelled WS HeLa cell extract (the production of which is described in Section 2B3.4) was added to each beads sample and samples incubated for 1hr at 4°C. The samples were then washed three times with 1ml 0.5M NaCl wash buffer C (described in Figure 3C6). After elution of the bound proteins by reduced glutathione, 3X SDS loading buffer was added and samples boiled for 2min. Samples were then loaded on 10% and 12.5% SDS-PAGE gels. Proteins were then transferred to a nitrocellulose filter by western blotting. The blot of the 12.5% SDS-PAGE gel was then analysed by immunodetection (Section 2B4.7), for the presence of Vmw110 encoding sequences using the 10503 MAb and once dried the blot of the 10% SDS-PAGE gel was exposed to film (Section 2B4.7).
A-The blot of the 12.5% SDS-PAGE gel probed with the 10503 anti-Vmw110 MAb. The 'GST' track shows proteins eluted from beads bound to the GST protein. The 516-1102 labelled tracks show proteins eluted from beads-bound to the purified 516-1102 HAUSP GST fusion protein. The 'Vmw110 594-775' and 'Vmw110 633-775' tracks show 5µl samples of T7E52 and T7E58 bacterial extracts respectively. The bands corresponding to these proteins have been appropriately indicated and which of these two extracts was used as the source of Vmw110 has also been indicated below the relevant tracks.
B-The blot of the 10% SDS-PAGE gel dried and exposed to film. A similar labelling scheme as for Panel A has been used. The track labelled 'total extract' shows a 2µl sample of ^{35}S-methionine radiolabelled WS HeLa cell extract. The position of the ~40kD cellular protein has been indicated on the right of the gel.

A



Fragment of Vmw110
used for primary
GST 'pull-down' assay : 594-633aa 633-775aa

B



Fragment of Vmw110
used for primary
GST 'pull-down' assay : 594-633aa 633-775aa

been saturated. Hence, the ~40kD cellular protein could have bound to free HAUSP GST fusion protein.

3E4 Summary of the studies done to investigate the effects of Vmw110 binding on the cellular activities of HAUSP

- Deletion of HAUSP residues required for interaction with Vmw110 result in the loss of its deubiquitinase activity.

- Coexpression of HAUSP with Vmw110 did not alter the enzyme activity of HAUSP observed using the standard *in vivo* deubiquitinase assay. This suggests Vmw110 does not affect HAUSP deubiquitinase activity in the experimental system used.

- The interaction of Vmw110 with HAUSP residues 516-1102 does not appear to inhibit the ~40kD cellular protein from binding to this same region of HAUSP.

CHAPTER 4 DISCUSSION

4A The biological role of HAUSP

4A1 What do we understand so far about the cellular activities of HAUSP?

The biological significance of HAUSP within eukaryotic cells was partially characterised by investigations carried out as part of the requirements of this thesis. The most significant finding was the confirmation that HAUSP has deubiquitinase activity, cleaving the bond between the C-terminal glycine residue of ubiquitin and the N-terminal residue on a model substrate protein. Furthermore, the presence of specific cysteine and histidine residues was shown to be essential for the catalytic activity of the enzyme.

Additionally, studies in which HAUSP was transiently expressed in eukaryotic cells suggested that the levels of intracellular HAUSP may be tightly controlled and that increases in HAUSP expression might be toxic for the cell, which implies that it plays a role in an important cellular regulation pathway. In addition it was suggested that localisation of HAUSP to the ND10 domains was limited by protein-protein interactions.

Results derived from GST 'pull-down' experiments identified several proteins of approximately 40kD, 100kD and 105kD that strongly and specifically interact with HAUSP. The regions of HAUSP required for interaction with these cellular proteins were shown to lie between residues: 1-26 for interaction with the 105kD cellular protein; 26-193 for interaction with the 100kD cellular protein and 516-837 for interaction with the ~40kD cellular protein. Immunoprecipitation analysis supported the interaction of wild type HAUSP with cellular proteins of approximately 40kD and 105kD. Furthermore, it was shown that the ~40kD cellular protein, that interacted with the C-terminal of HAUSP, was a target for proteasome degradation and as such may be a substrate for HAUSP deubiquitinase activity.

Collectively these results suggest that HAUSP plays a role in an as yet unidentified ubiquitin dependent pathway. However, as neither the identity of the HAUSP interacting proteins or the pathway(s) in which HAUSP is involved are defined as yet, the assignment of a specific biological role to HAUSP at this preliminary stage would be highly speculative.

4A2 Future work to assist in our understanding of HAUSP cellular function

The key to understanding HAUSP cellular function lies in identifying the cellular proteins with which it interacts and defining the pathways in which it is involved. Therefore, perhaps the most critical investigation for the future of this project would be to reveal the identity of the cellular proteins that interact with HAUSP. This would first involve isolating high enough quantities of the ~40kD, 100kD and 105kD cellular proteins that interact with HAUSP using large scale GST 'pull-down' assays. Peptide sequencing could then be performed on these purified protein samples from which degenerate oligonucleotides could be synthesised. The next stage would be to use these degenerate oligonucleotides to screen cDNA libraries from WS HeLa cells and in this manner clone the cDNAs encoding these proteins. The yeast two hybrid system could also be used to identify cellular proteins that interact with HAUSP and the results of this investigation may support the findings from the GST 'pull-down' assays.

The yeast homologues in *S.cerevisiae* and *S.pombe* described in Section 1E2 are the most closely related genes to HAUSP, apart from the exact homologue in mice. Therefore, as the manipulation of yeast genetics is relatively simple compared with that of human genetics producing a yeast knockout strain of this gene should be possible. The results generated from the analysis of this yeast strain should give us some insights into the cellular function of this protein. The greatest problem with this idea is that yeast pathways may be different from the human cellular pathways and as such the phenotypes observed from such a yeast knockout strain might not give us a true indication of the function of HAUSP itself.

Another idea which follows on from the one mentioned above is to search for a *Drosophila* homologue of HAUSP. If such a gene is identified then a *Drosophila* knockout fly could be constructed and, as *Drosophila* is higher up the evolutionary scale than yeast, it is more likely that any phenotypes observed from the removal of this gene would have more relevance for HAUSP function. Such a knockout strain could also be used to investigate the role, if any, of a *Drosophila* HAUSP homologue in the silencing phenomena called PEV which, as discussed later in this section, is a system in which HAUSP could potentially be involved.

Studies in which HAUSP was transiently expressed raised the possibility that increases in HAUSP expression in some cells might induce apoptotic pathways. As such it would be interesting to investigate the significance of this result and further investigate if HAUSP has a role in growth control and development.

As many enzymes involved in the ubiquitin dependent pathways play a role in the cell cycle regulation and are themselves cell cycle regulated (Peters *et al.*, 1998), it would be curious to see if HAUSP is similarly cell cycle regulated. Such an idea is supported from the work in which HAUSP was transiently expressed. There are two methods by which this can be tested. The first involves starving the cells from serum which would result in the cells being arrested in G_0. The cells could then be induced into G_1 by the addition of serum. In this manner the cells would be synchronised in the cell monolayer and a cell cycle dependent effect could be monitored either by western blotting of cells synchronised at the different stages of the cell cycle, or by indirect immunofluorescence techniques of the same cells, in both cases using antibodies raised against HAUSP.

Further investigation into the different isoforms of HAUSP which are most likely generated though alternative splicing (described in Section 1E3) would be interesting. In particular, this might be possible by first studying the HAUSP encoding gene and then characterising the alternatively spliced cDNAs and protein products. Furthermore, it might be of interest to investigate if expression of these alternative transcripts differs in non-neuronal and neuronal cells.

Further investigation into the semi-conserved KEKE motif of HAUSP (described in Section 3D5.1) would also be interesting. This region has been implicated to be of importance for protein-protein interactions and has been conserved in a significant number of proteins involved in the ubiquitin-dependent degradation system. Therefore, it would be interesting to investigate if this region plays a similar role in HAUSP. As such, the three mutants already synthesised of this region could be used in similar GST 'pull-down' experiments, as described in Section 3C, to test the significance of these residues for interaction of HAUSP with cellular proteins. The same three mutants could also be tested in the deubiquitinase assay to assess the significance of these residues for the enzymatic activity of HAUSP.

4B The role of HAUSP for HSV-1 infection

4B1 What do we understand so far about the interaction of HAUSP with Vmw110?

The interactions between HAUSP and Vmw110 are likely to be of great importance to the understanding of HSV-1 lytic growth and reactivation from latency. A number of investigations described in this thesis were initiated in order to improve our understanding of the mechanics of this interaction and its role in HSV-1 infection. Firstly, studies using the GST 'pull-down' technique defined the regions of HAUSP required for interaction with Vmw110 as those between residues 529-576 (which are directly downstream of the conserved histidine box) and residues 744-861. Furthermore, immunoprecipitation experiments confirmed the requirement of HAUSP residues 513-581 for this interaction.

Investigations into whether Vmw110 binding to HAUSP affected its normal cellular activities implied firstly that the presence of Vmw110 does not appear to decrease the catalytic activity of HAUSP and secondly that Vmw110 binding to the C-terminal of HAUSP does not affect the ability of a ~40kD cellular protein to interact with the same region.

4B2 What affect might Vmw110 binding have on HAUSP function?

Results presented in this thesis suggest that the interaction of Vmw110 with HAUSP does not inhibit its deubiquitinase activity, however alternative roles for this interaction can be envisioned. For example, Vmw110 may be sequestering HAUSP and targeting its deubiquitinase catalytic activity to other substrates bound to alternate sites on Vmw110, for example the RING finger domain or the multimerisation sequence, both of which are potentially important for protein-protein interactions (see Figure 4B2). This would result in the stabilisation of such Vmw110 bound ubiquitinated-substrates. Secondly, although interaction of HAUSP with Vmw110 may not directly inhibit its deubiquitinase activity it is possible that the act of sequestering HAUSP to ND10 domains may prevent it from deubiquitinating its natural substrates localised elsewhere in the cell. This in turn would prevent those ubiquitinated-substrates from being protected from proteasome-dependent degradation.

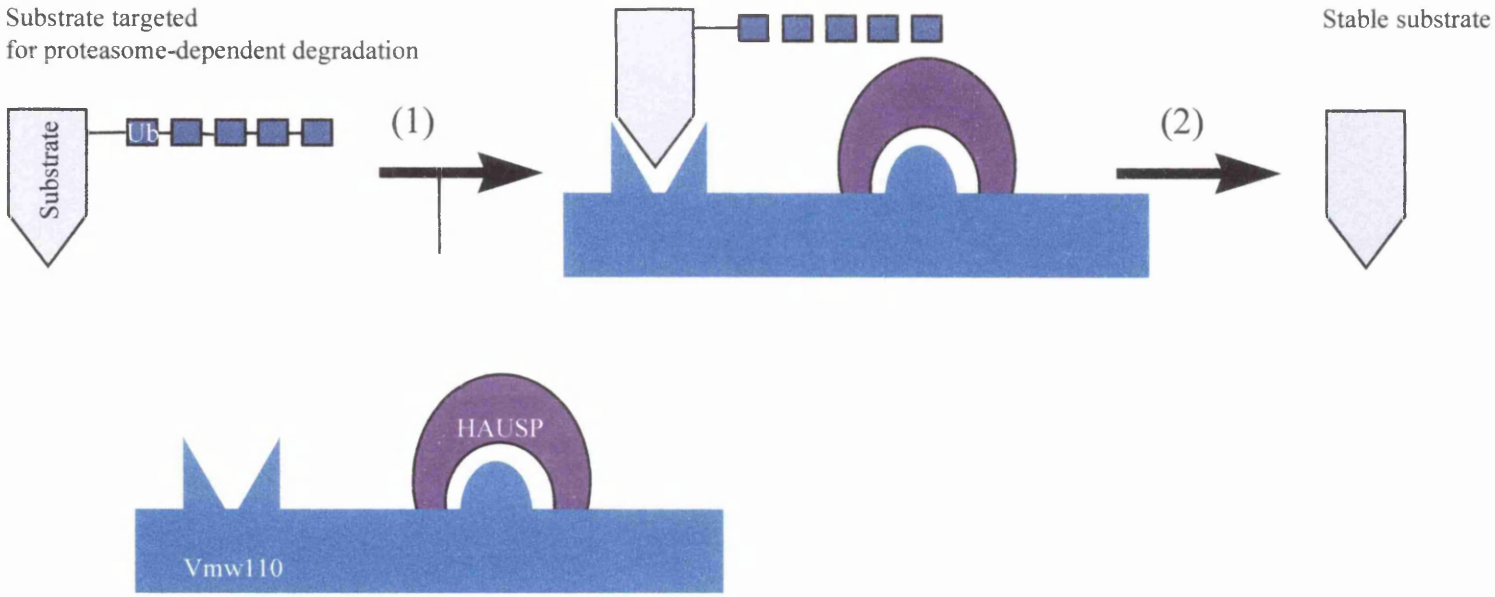


Figure 4B2: Diagrammatic illustration of how sequestration of HAUSP by Vmw110 may redirect its deubiquitinase activity to ubiquitinated-substrates also bound to Vmw110.

1- Vmw110 may bind to both ubiquitinated-substrates and HAUSP.

2- This colocalisation may result in the removal of ubiquitins from the substrate and prevent its degradation by the proteasome.

4B3 Mechanisms by which the Vmw110/HAUSP interaction could affect HSV-1 infection

One of the central aims of this thesis was to try and decipher the role of HAUSP in HSV-1 infection. Using evidence which has accumulated within the field, during the course of this study (see Introduction chapter) and also during the production of this thesis, a number of plausible mechanisms can be suggested.

4B3.1 HAUSP interaction with Vmw110 may result in the stabilisation of ubiquitinated viral proteins

Viral proteins including Vmw110 and Vmw175, which are synthesised at the onset of infection, may be recognised as foreign proteins by the cells and targeted for degradation by the proteasome. Loss of such viral proteins would result in a reduced efficiency in the initiation of lytic replication. However, if Vmw110 sequestered HAUSP and redirected its deubiquitinase activity to these ubiquitinated viral proteins this would result in these proteins being protected from degradation by the proteasome. This is consistent with the finding that Vmw110 is required to stimulate viral growth only at low moi, as at higher moi enough non-ubiquitinated viral proteins would be present to initiate viral replication. However, until the question of whether viral proteins are targeted for degradation by ubiquitination has been answered, this theory remains extremely hypothetical.

4B3.2 HAUSP may function to directly enhance the activities of Vmw110

The region of Vmw110 required for interaction with HAUSP has been shown to play a significant role in the ability of Vmw110 to transactivate gene expression, as well as stimulate virus growth in cultured cells (Everett, 1988a, Everett *et al.*, 1999, Meredith *et al.*, 1995). As such, it has been suggested that the role of HAUSP in HSV-1 infection is to directly aid Vmw110 in eliciting its many effects. Therefore, in order to understand the role of HAUSP one must first understand the mechanism by which Vmw110 is functioning in HSV-1 infection.

4B3.2a Vmw110 functions through the ubiquitin-proteasome pathway

Interaction of HAUSP with Vmw110 was the first indication that Vmw110 may be functioning by altering the stability of proteins. However, recently several other lines of evidence have emerged which suggest that the mechanism by which Vmw110 functions

involves ubiquitin-dependent processes. In particular, Vmw110 has been shown to be required for the proteasome-dependent degradation of several cellular proteins including PML, DNA-dependent protein kinase, CENP-C and several uncharacterised PIC-1 conjugated proteins (Everett *et al.*, 1998a, Lees-Miller *et al.*, 1996, Parkinson *et al.*, 1999, R.Everett, personal communication). Furthermore, it has recently been shown that proteasome activity is required for Vmw110-dependent activities which result in an increased efficiency in viral replication, activation of gene expression and efficient reactivation of quiescent genomes (Everett *et al.*, 1998b). Therefore the alteration of protein stability by Vmw110 appears to be central to the mechanism by which it functions in HSV-1 infection.

- HAUSP may assist this effect of Vmw110 by stimulating ubiquitin-dependent degradation processes

Interaction of HAUSP with Vmw110 could enhance these activities by simply stimulating ubiquitin-dependent degradation processes. For example, Vmw110 could sequester HAUSP and redirect its deubiquitinase activity to cleave ubiquitin precursors which would increase the amount of free ubiquitin. Interestingly, the observation that Vmw175 upregulates the expression of the human ubiquitin precursor Ubi B gene provides an explanation of how Vmw110 and Vmw175 may be acting synergistically to transactivate gene expression (Kemp & Latchman, 1988). Another method by which interaction of HAUSP with Vmw110 could stimulate these activities would be if sequestration of HAUSP by Vmw110 redirected its deubiquitinase activity to stabilise enzymes involved in ubiquitin-dependent degradation.

4B3.2b Vmw110 may be acting to reverse or inhibit the silencing of viral genomes in HSV-1 infection

Recently a more specific and rather elegant mechanism by which Vmw110 may be eliciting its effects has been suggested (Everett *et al.*, 1998b). It has been proposed that viral genomes which are deposited adjacent to ND10 domains after entry into the nucleus may be repressed in a similar manner to silenced heterochromatin DNA. Vmw110 may be acting to reverse or inhibit such silencing processes and as such encourage the onset of lytic infection. There is much circumstantial evidence that lead to the creation of this hypothesis. For example the heterochromatin protein HP1, which is a protein involved in the silencing phenomena PEV, has been shown to interact with a constituent of ND10

called Sp100 and interaction of these proteins has been suggested to have a role in silencing of genes (Section 1D1.1a). Vmw110 is known to be responsible for dispersion of Sp100 from the punctate ND10 domains during HSV-1 infection and PIC-1 conjugated Sp100 has been shown to be degraded in a Vmw110-dependent manner (Section 1D1 and 1D2; R.Everett, personal communication). This theory also provides an explanation for the observation that Vmw110 is only required at low moi, in that if the genomes are present in high moi then the proteins involved in silencing would be swamped and therefore many genomes would be present that are not silenced from which lytic replication could be initiated.

- Sequestration of HAUSP by Vmw110 may prevent it from enhancing silencing of the viral genome

One way in which HAUSP interaction with Vmw110 could inhibit the silencing of HSV-1 genomes is if the natural role of HAUSP in the cell was to enhance silencing processes. Under these circumstances sequestration of HAUSP by Vmw110 may act to prevent HAUSP from enhancing the silencing of the viral genome. This is consistent with the findings that other members of the USP family have roles in silencing. For example, the yeast protein Ubp3 has been shown to interact with Sir4 and regulate silencing both at telomeres and mating-type loci in yeast (Section 1C3.6). Furthermore, the *Drosophila* USP D-Ubp-64E has been shown to suppress PEV in a dose-dependent manner (Section 1C3.6).

- Interaction of HAUSP with Vmw110 may stimulate ubiquitin-dependent degradation of cellular proteins including those involved in silencing of the viral genome

As explained in Section 4B3.2a Vmw110 appears to elicit much of its effects through ubiquitin-dependent pathways, stimulating the degradation of several cellular proteins. As such, it is possible that Vmw110 derepresses quiescent HSV-1 genomes by stimulating the degradation of proteins involved in silencing such as Sp100. The role of HAUSP in such a scenario could simply be to stimulate the ubiquitin-dependent degradation processes by the methods described in Section 4B3.2a.

4B4 Future work to investigate the role of HAUSP in HSV-1 infection

A direct strategy to answer the question of HAUSP function in HSV-1 infection would be to identify proteins with which HAUSP interacts during infection. In this manner if

HAUSP interactions during infection differ from those when the cells are not infected the identification of the proteins concerned might lead to an understanding of how HAUSP may be manipulated by Vmw110 to assist HSV-1 infection. There are two methods by which this could be achieved. The first would involve performing similar GST 'pull-down' experiments, as described in Section 3C, in which GST fusion proteins encoding selected segments of HAUSP are used as bait to 'pull-down' proteins in this case from infected cell extracts. A second method would be to use HAUSP as bait in the yeast-two-hybrid system to screen cDNA libraries derived from infected cells.

The U2OS cell line appears to have a cellular function that can compensate for the loss of Vmw110 during HSV-1 infection (Section 1B2.1). The PC12 neuronal cell line when exposed to nerve growth factor or fibroblast growth factor also has a cellular function that appears to compensate for the loss of Vmw110 during HSV-1 infection (Section 1B2.1). As does release of cells from growth arrest state G_0 (Section 1B2.1). The manner by which the cells derived from the above sources by-pass the need for Vmw110 during HSV-1 infection might involve using the same pathways as Vmw110. As such identification of the proteins which interact with HAUSP in these cells might give us some insight into possible mechanisms by which Vmw110 is acting.

It has long been hypothesised that Vmw110 has an important role in the switch between the lytic and latent states of HSV-1 infection (Section 1B2.4). It has also been established that factors such as stress, cytokines and heat shock are known to affect the intracellular distribution of ND10 domains in a similar manner to Vmw110 (Section 1D1.2). Furthermore, similar factors are known to cause the reactivation of quiescent genomes into the lytic state (Section 1A2.4b). Therefore, as HAUSP interacts with Vmw110 and is also a transient member of ND10 domains it would be interesting to see if the cellular localisation or level of HAUSP expression is altered in cells exposed to such factors.

As mentioned previously HAUSP may be working in HSV-1 infection to stabilise viral proteins targeted for degradation by ubiquitination. To test this hypothesis first it must be established whether viral proteins are ubiquitinated. However there are technical problems with simply performing a western blot and probing HSV-1 infected cell extracts using anti-Ub serum. In particular, there is high background using such serum and secondly a high number of cellular proteins are also conjugated to ubiquitin and so it would be hard to distinguish whether the ubiquitinated-proteins were cellular or viral. However, by

transfecting cells with plasmids expressing either Vmw110 or Vmw175 in the presence of a proteasome inhibitor it should be possible to detect by western blotting techniques, using antibodies to these proteins, if higher molecular weight isoforms of these proteins are generated which correspond to ubiquitinated-conjugates. Furthermore, constructs could be synthesised in which Vmw110 or Vmw175 encoding sequences are fused at their N-terminal to sequences encoding ubiquitin via a sequence encoding the same natural bond that is found in other ubiquitinated-substrates. These constructs could then be used in a modified deubiquitinase assay in which they are cotransfected alongside Vmw110 and HAUSP to see if under these circumstances they are recognised as substrates for deubiquitination.

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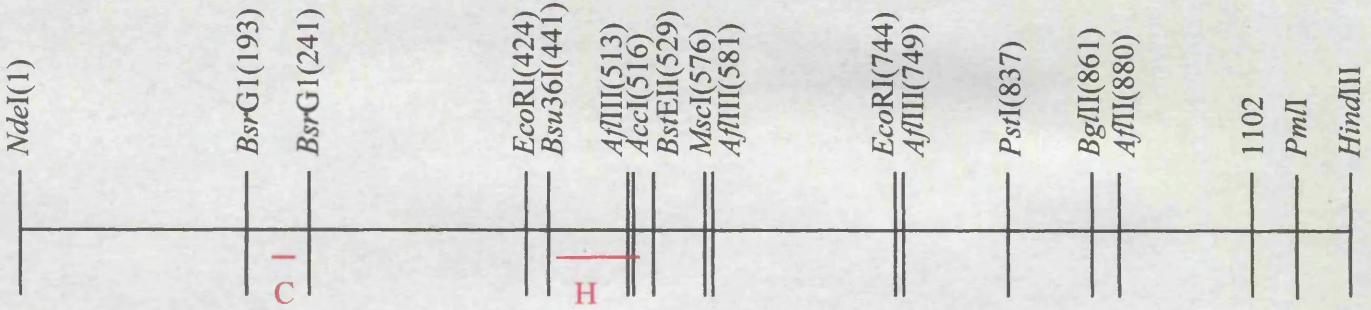

Scale:

—— =50 HAUSP residues

Figure 3A3.2: Restriction map of the sequence encoding HAUSP. The positions of restriction sites within the HAUSP open reading frame which are relevant to the construction of plasmids made for the purposes of this thesis have been indicated and their codon position marked. A scale has been given to indicate the length of line representing 50 codons. The red lines indicate the approximate positions of the cysteine (C) and histidine (H) active site domains.
N.B. Where in the text a restriction site has been followed by a number in parentheses, the number is an indication of the codon position within the HAUSP open reading frame at which the fragment had been digested.