CLONING AND PURIFICATION OF THE ADENOVIRUS PROTEIN pVIII AND ITS CLEAVAGE PRODUCTS

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DECLARATION

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Abbreviations

A	Adenine
a.a.	Amino Acid
Ad2	Adenovirus serotype 2
Ad5ts	Adenovirus serotype 5, temperature sensitive
Amp	Ampicillin
BSA	Bovine serum albumin
C	Cytosine
CIAP	Calf intestinal alkaline phosphotase
CM	Carboxymethyl
CRB	Cambridge Research Biochemicals
Da	Dalton
DAPI	4',6'-Diamidino-2-phenylindole
dATP	Deoxyadenine triphosphate
DBP	Deoxyribosenucleic acid binding protein
dCTP	Deoxycytosine triphosphate
DEAE	Diethyl-aminoethyl
dGTP	Deoxyguanine triphosphate
DMF	Dimethylformamide
DNA	Deoxyribosenucleic acid
dNTP	Deoxynucleotide triphosphates
DTT	Dithiothreitol
dTTP	Deoxythymine triphosphate
E1-4	Early (genes or proteins) 1-4
EDS	Egg drop syndrome virus
EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal calf serum
Fmoc	Fluorenyl methoxycarbonyl
FPLC	Fast Protein Liquid Chromatography
G	Guanine
GST	Glutathione S-transferase

HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
HSA	Human serum albumin
IPTG	Isopropyl-b-D-thiogalactopyranoside
L1-6	Late (gene or protein) 1-6
L-Agar	Luria agar
L-Broth	Luria broth
MEM	Eagle's minimal essential medium
mp	Map position
mRNA	Messenger ribonucleic acid
NBCS	New born calf serum
NEB	New England Biolabs
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
p.f.u.	Plaque forming units
pTP	Pre-terminal protein
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RNAse	Ribosenucleoase
SAPU	Scottish Antibody Production Unit
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPDP	N-succinimidyl 3,2(pyridyl-dithio)propionate
T	Thymine
TAE	Tris-acetic acid-ethylenediaminetetraacetic acid buffer
TE	Tris-EDTA buffer
Tet	Tetracycline
TEMED	N,N,N',N' tetramethylethylenediamine
TFA	Trifluoroacetic acid
TNF	Tumour necrosis factor

TP	Terminal protein
ts1	Adenovirus serotype 2, temperature sensitive 1
TTBS	Tris buffered saline containing Tween 20
UV	Ultra-violet
VA RNA	Virus associated ribonuleic acid
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside

Single letter code and Standard abbreviations for amino acids

A	Alanine	Ala
C	Cystine	Cys
D	Aspartic acid	Asp
E	Glutamic acid	Glu
F	Phenylalanine	Phe
G	Glycine	Gly
H	Histidine	His
I	Isoleucine	Ile
K	Lysine	Lys
L	Leucine	Leu
M	Methionine	Met
N	Asparagine	Asn
P	Proline	Pro
Q	Glutamine	Gln
R	Arginine	Arg
S	Serine	Ser
T	Threonine	Thr
V	Valine	Val
W	Tryptophan	Trp
Y	Tyrosine	Tyr

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Abstract

The properties of the adenovirus structural protein precursor VIII (pVIII) were investigated by cloning the gene coding for pVIII into the vector pRSETA via the Bam HI restriction enzyme site. The fusion protein (pVIII) was expressed in BL21(DE3) cells by inducing with iso-propyl-thiogalactoside (IPTG) and pVIII purified via a novel method which included the use of a nickel affinity column.

Cleavage of pVIII by the adenovirus 23 kDa protease was observed to produce results consistent with cleavage occurring between 111/112 residues, 131/132 residues and 157/158 residues. Cleavage between 131/132 residues and 157/158 residues was confirmed by N-Terminal sequencing of cleavage products. An antiserum raised against a synthetic peptide of the N-Terminal of pVIII detected VIII, a 15 kDa cleavage product of pVIII, from mature adenovirus 2 indicating that VIII may be formed by the amino acid sequence 1-131.

Immunofluorescence studies of HeLa cells 28 hours post-infection indicate that pVIII may be co-localising with the protease in the cytoplasm forming rod-like structures. Studies with pVIII immobilised on a Sepharose CL column indicate that the adenovirus protease only binds to the column after being activated with the C-Terminal fagment of pVI.

The gene coding for the EDS L3 23 kDa protease was sequenced and the protein expressed in BL21(DE3) cells using the vector pRSETA. The DNA sequence of the protease gene was in agreement with the sequence previously published. An initial purification procedure for the protease was developed using anion exchange chromatography.

Contents

1. Introduction

1.1 General Background

The human adenovirus, which belongs to the family adenoviridae, was first isolated from adenoids grown in tissue culture by Rowe *et al.* (1953). Since 1953 the human adenovirus has been identified as having forty-nine distinct serotypes (Schnurr and Dondero, 1993), and has been isolated from many mammals, marsupials, birds and amphibians (Wadell, 1984). Some human adenovirus serotypes have been shown to be capable of inducing tumours in rodents (Lewis and Cook, 1984).

The Adenoviridae family of viruses is composed of two genera, the Mastadenovirus and Aviadenovirus (Norrby *et al., 1*976). The Aviadenovirus genus is restricted to the avian adenoviruses and the Mastadenovirus genus incorporates human, simian, bovine, canine, murine and other mammalian adenoviruses. There is no common antigen to all adenoviruses but, there is an antigenic cross-reactivity among viruses of each genera due to conserved epitopes in the hexon proteins of the adenovirus capsids.

Recently, with the advent DNA sequencing it has been proposed by Harrach *et al.* (1997) that a third genera exists based on the homology of the viral proteases, see figure 1. The phylogenetic tree evidence places the egg drop syndrome virus (EDS), bovine 7 and ovine 287 viral strains in a third cluster which is removed further from the mastadenoviruses than the aviadenoviruses. As there is no common host for the third cluster of viruses it is unlikely that the similarities are a result of recombination but, instead they diverged from a common ancestor (Harrach *et al.,* 1977).

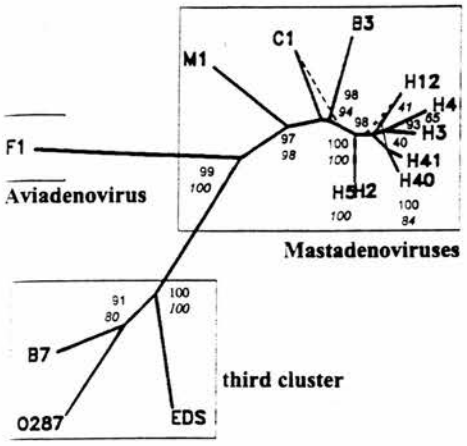


Figure 1: Phylogenetic tree of adenovirus protease sequences showing the homology of EDS, Bovine 7 and Ovine 287, and their distinctiveness from all other members of the mastadenoviruses or aviadenoviruses. The length of the branches indicates the phylogenetic distance between the different viruses. The tree was generated by distance matrix analysis (Dayhoff's PAM 001 scoring matrix, FITCH) and by parsimony analysis (shown by broken line where it differs from the earlier). Taken from Harrach *et al* (1997).

Subgenus	Serotype	DNA Homology(%)	G+C(%)	Haemaggl-utination Pattern[b]	Oncogeni-city in hamsters
A	12, 18, 31	48-69 (8-20)	48	IV	High
B	3, 7, 11, 14, 16, 21, 34, 35	89-94 (9-20)	51	I	Weak
C	1, 2, 5, 6	99-100 (10-16)	58	III	Nil
D	8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 36, 37, 38, 39	94-99 17)	58 (4-	II	Nil
E	4	100 23)	58 (4-	III	Nil
F	40	- (-)	-	IV	Unknown
G	41	-	- (-)	IV	Unknown

Table 1. Classification of Human Adenovirus. Percentage homology is that displayed within the subgenus except where figures are in brackets where homology is with members of other subgenera. Haemagglutination pattern: I, complete agglutination of monkey erythrocytes; II, complete agglutination of rat erythrocytes; III, partial agglutination of rat erythrocytes (fewer receptors); IV, agglutination of rat erythrocytes discernible only after addition of heterotypic antisera. Adapted from Wadell (1984) and Horwitz (1990a).

The forty-nine species of human adenovirus are classified into seven subgenera A-G (see table 1), with the subgenera being determined by the homology of DNA digest fragments among the adenovirus species. Members of the same subgenera show at least 50% DNA homology and approximately 10% between subgenera (Wadell, 1984). The characteristics of an adenovirus infection vary with each subgenus. Members of subgenus A infect infants and cause diarrhoea (Wadell, 1984), subgenus B serotypes cause conjunctivitis or respiratory difficulties with a fever, which can be fatal in infants (Wadell, 1980). Subgenus C viruses infect the adenoids and tonsils persistently, infection mainly occurs in young children (Schmitz *et al.*, 1983). The virus infections of subgenus D are characterised by severe eye infections (Schmitz *et al.*, 1983). Subgenus E virus infections result in either a respiratory infection, or in conjunctivitis (Wadell, 1984).

Egg Drop Syndrome 1976 virus (EDS) is characterised by the production of soft-shelled and shell-less eggs, these symptoms were first observed in the Netherlands in flocks of laying hens (Van der Eck *et al., 1*976). A similar outbreak was also reported in Northern Ireland in 1976 by McFerran *et al. (*1978). The virus was first isolated in Northern Ireland by McFerran and Adair (1977) and was identified as agent responsible of egg drop syndrome 1976. The EDS virus was classified as an adenovirus as a result of its morphology, replication and chemical composition.

The outbreaks in the Netherlands and Northern Ireland were both considered to be independent of any factors previously described to affect egg production. The birds appeared to be clinically healthy, but the symptoms appeared when the flocks of hens were between 29 and 31 weeks old. A previously uncharacterised adenovirus was isolated from the affected flocks that strongly agglutinated chicken haemoglobin. Hens showing depressed egg development also showed production of antibodies to the newly

isolated strain of adenovirus. Similar syndromes were later reported across Europe and in Japan (Baxendale 1978; Meulemans *et al., 1*979; Yamaguchi *et al., 1*981) and similar viruses isolated.

The viruses isolated from the geographically different locations were used to inoculate adult female birds in order to test whether the viruses were the agent responsible for the egg drop syndrome. After one week post infection there was an observed loss in egg-shell pigmentation and followed by soft-shelled or shell-less eggs. Between 13 and 16 days post infection 25-40% of all eggs laid were abnormal; it was only 24 days post infection that the hens began to lay normal eggs. Overall egg production was not greatly affected and the hens appeared healthy throughout the seventeen weeks the experiment lasted (McCracken and McFerran 1978). Similar experiments with the Japanese isolate confirmed these results (Yamaguchi *et al., 1*981)

Baxendale (1978) used an English strain of the virus to infect 23 and 29 week old hens to produce the egg drop symptoms. Baxendale also showed that hens which had been injected with an inactivated viral vaccine at 19 weeks of age failed to develop the egg drop 1976 symptoms when infected at 23 and 29 weeks of age. Studies by McFerran and Adair (1977) found that transmission of the virus from chicken to chicken was rare and therefore the method of transmission most likely to by egg transmission. It is thought that the sudden and widespread appearance of EDS76V in Europe was because of a contamination of a vaccine with the virus.

It was found that EDS grew more efficiently in cells of duck origin than in those of chickens. This prompted studies by Baxendale (1978) and Calnek (1978) into duck flocks, in which it was found that 20 out of 24 duck flocks in England had antibodies to the virus. In New York all seven White Peking flocks examined were seropositive.

These findings indicate that EDS76V is indigenous in the duck population and that it could be classified as a duck virus. Further studies by Kaleta *et al. (*1980) detected antibodies against EDS76V in owls, storks, swans and wild geese.

1.2 Adenovirus Structure

Adenoviruses are nonenveloped viruses, which have a characteristic icosahedral capsid, with twelve protruding fibres (Horne *et al.,* 1959). Within the icosahedral capsid there is a single copy of the double stranded viral DNA. The virus has a diameter of 140 nm,

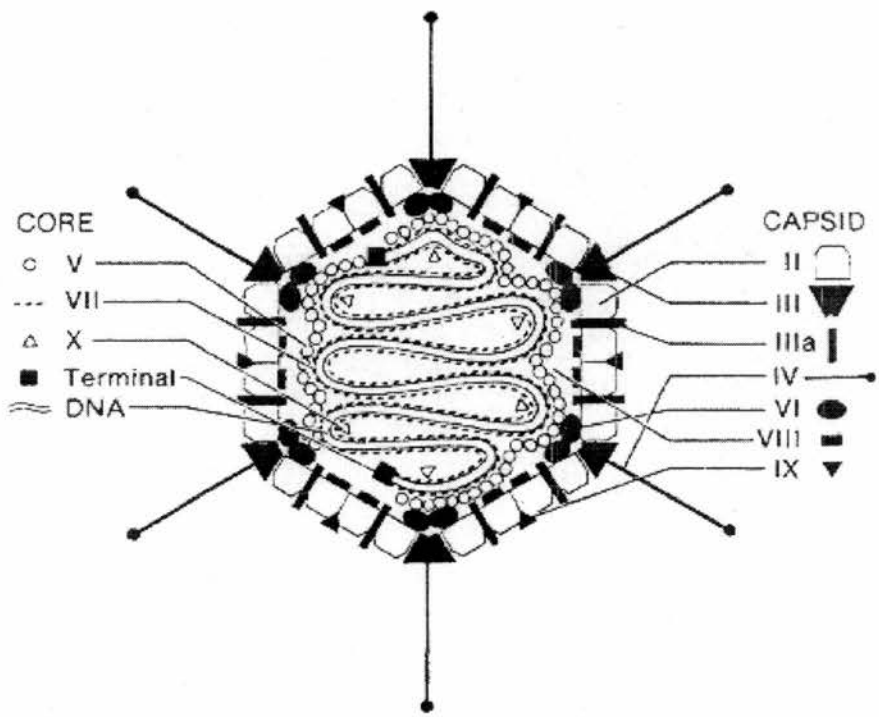


Figure 2 : A stylized section of the adenovirus particle summarizing current structural knowledge of the polypeptide components and the viral DNA. No real cross section of the icosahedral virion would contain all components. (Stewart and Burnett, 1993)

including fibres, and a mass of 150,000 kDa (van Oostrum *et al.,* 1985; Stewart *et al.,* 1991). The mature viral capsid requires the production of 15 structural proteins, which

account for over 80% of the adenovirus DNA coding capacity (Philipson, 1983). See figure 2 for location of structural proteins.

1.3 Structural Proteins

Adenovirus virions are composed of between 11 and 15 distinct virus-encoded polypeptides (Horwitz, 1990), which form the capsid and the core. The outer capsid is composed of 252 subunits. The penton complex is located at the 12 vertices of the icosahedral capsid, and 240 polypeptide II (hexons) form the 20 facets and 30 edges (van Oostrum and Burnett, 1985). Other minor proteins in the capsid are IIIa, VIII and IX. The core is composed of proteins V, VII, mu, the terminal protein and a single copy of double stranded DNA.

1.3.1 Hexon

The crystal structure of the Ad 2 hexon (see figure 3) was originally obtained by Roberts *et al. (*1986) and resolved to 2.9 Å by Athappilly *et al. (*1994). The adenovirus type 2 hexon is a trimer containing three polypeptide chains 967 amino acids long, the 109 kDa hexon monomer forms two eight-stranded ß-barrels and three extended loops (Roberts *et al.*, 1986). It is the loops of three different subunits that intertwine in the hexon trimer and are exposed on virion surface. Construction of the hexon trimer does not occur spontaneously and requires the presence of the L4 100 kDa protein (Frost and Williams 1978). From gel filtration studies it appears that the ratio between 100 kDa protein and hexon monomer associate in a 1:1 complex. It is thought that the 100 kDa protein functions as a scaffolding protein but, to date, the protein interaction site has not

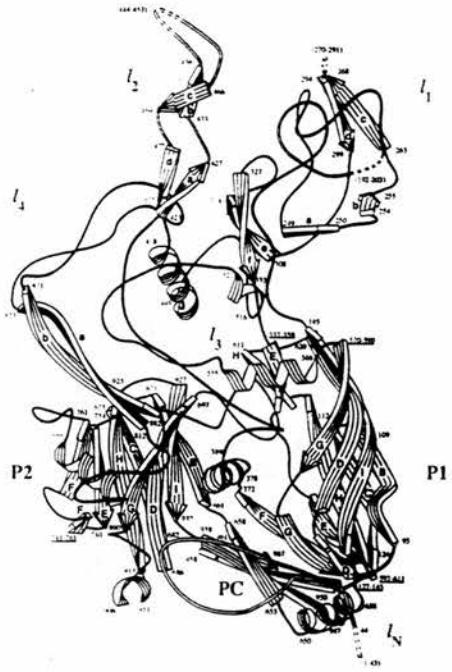


Figure 3. Ribbon representation of the hexon subunit showing its secondary structure from Athappilly *et al* (1994). The loops l1, l2 and l4 protrude from the 8 strand b-barrels (P1 and P2) and form 3 towers which project from the main body of the virion. The l3 loop is sandwiched between the P1 and P2 domains.

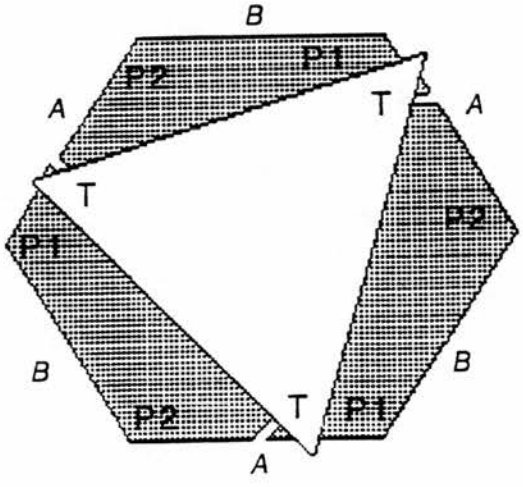


Figure 4. The hexon trimer from Burnett (1985). The hexon is made up of three copies of protein II and contains two distinct regions: the base and the tower. The base of the hexon is shaded and the arrangement of the P1 and P2 b-barrels means that each hexon has two distinct faces (A and B). Projecting out from the base are three towers, with each A face lying under an apex of the triangular tower.

been identified. After the formation of the hexon trimer the 100 kDa protein dissociates from the unit.

Structurally the hexon is composed of two parts; a triangular top containing three towers, and a pseudo-hexagonal base, see figure 4. The hexons are tightly packed and form a protective shell that is 33 Å thick. Each loop or tower which projects away from the base are supplied by each of the three subunits. It has been demonstrated by Toogood *et al.* (1989) that the hexon surface loops display the highest variability between adenovirus serotypes and contain most of the type-specific epitopes.

1.3.2 Penton Base and Fiber (Penton Complex)

Protein III is composed of 571 amino acid residues and is the secondary capsid protein with an approximate molecular weight of 63 kDa . The penton base is a pentamer composed of five 63 kDa subunits of protein III which interact non-covalently to form a ring like structure. The penton base is approximately 95Å in diameter and 110 Å high and the 30 Å core of the penton base is filled by the fiber, a trimer of protein IV forming the penton complex. (Stewart *et al.*, 1993, see figures 5 and 6).

Each of the penton bases is surrounded by five hexons, which are termed peripheral hexons. It is thought that one of the two hexon ß-barrels fits into a grove on the side of the penton base. One strand of the ß-barrel is adjacent to an acidic sequence of 16 consecutive aspartate and glutamate residues. Wohlfart (1988) predicted that a pH mediated structural change occurs when the virion enters the endosome. A drop in pH could cause a shift in the position of the ß-barrels and thus the disassociation of the penton base from the capsid.

Figure 5. Diagram of the penton complex, comprising the penton base and fiber. The penton base is made up of five copies of protein III and the fiber of three copies of protein IV.

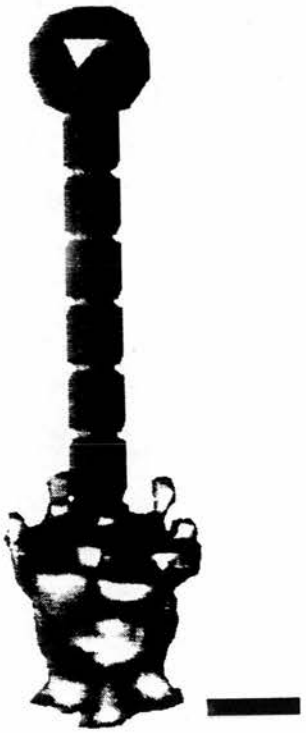


Figure 6. The penton complex is shown as a composite of the penton base from the three-dimensional image reconstruction and the fiber modeled using dimensions obtained from negatively stained electron microscopy images. Taken from Stewart and Burnett (1995).

The fiber is a trimer of three 62,000 Da subunits of protein IV (van Oostrum and Burnett, 1985) and has a length of 373 Å in Ad2. It can be split into three sections the tail, shaft and head. The N-terminal is located in the tail and binding of the fiber to the penton requires the first seventeen N-terminal amino acids. Weber (1989) showed that epitope for an antibody which recognised a peptide 30 residues from the N-terminus was masked which indicates that at least the first thirty residues fill the hole in the penton ring. The shaft of the Ad2 fiber is 28 Å wide and composed of a pseudo repeat sequence of 15 residues (Green *et al.*, 1983). The head is box shaped 50 Å x 50 Å structure that contains the C-terminus of the subunits and contain the recognition sites that bind to cellular receptors. It is possible that the head binds either by one site per head as rhinovirus 14 or by one site on each of the subunits that form head like the tumour necrosis factor (Xia, 1995).

1.3.3 Polypeptide IIIa

Protein IIIa is the 63,000 Da phosphoprotein cleavage product of pIIIa a 65,000 Da protein (Boudin, 1980). There are approximately 60 copies of IIIa in a mature virus particle, and it exists as a monomer that spans the adenovirus capsid (Stewart *et al.*, 1993), see figure 7. It is thought that because the Ad protease cleavage site in pIIIa is located at the C-terminus of the protein and the C-terminal end of the protein is located in the interior of the virion. Originally it was thought IIIa was located in the vertex region (Everitt *et al.*, 1975) but, it was later found that it possesses a stronger affinity for the capsid than the vertex proteins (van Oostrum and Burnett 1985). The role of IIIa in the adenovirus may possibly be to link hexons of adjacent facets together (Stewart *et al.*, 1993) but, the significance of the phosphorylation of IIIa remains unknown. However, it has been suggested that the phosphorylation of IIIa is carried out by a virion associated

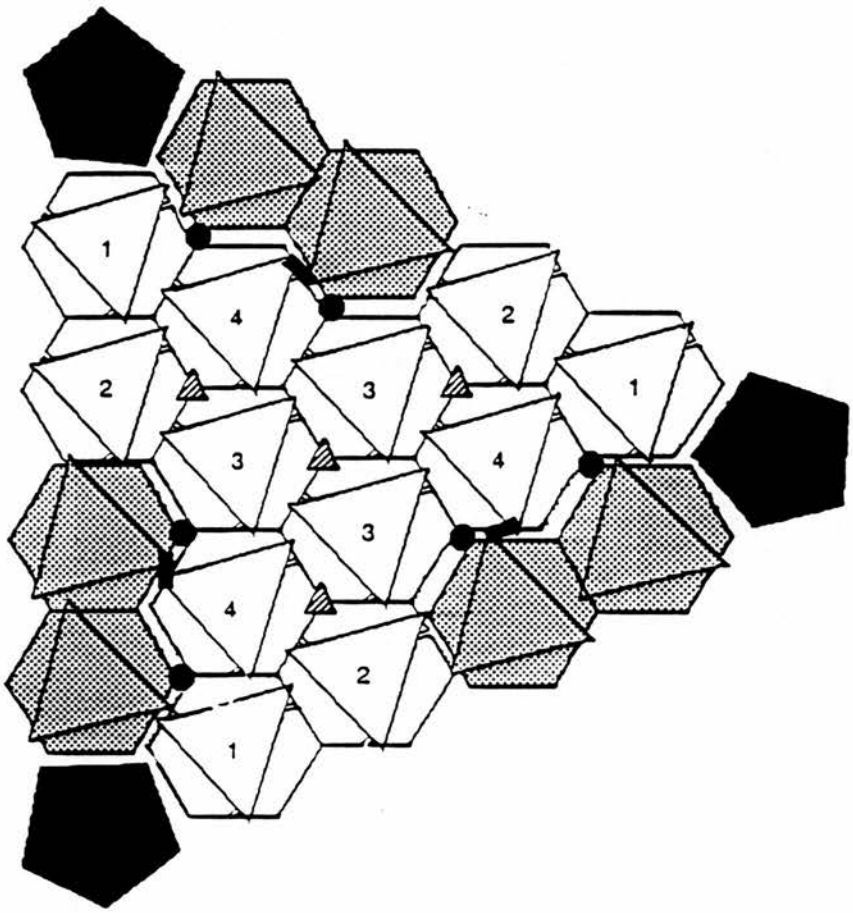


Figure 7. Schiematic diagram showing the arrangement of capsomers and associated proteins on one face of the icosahedral capsid of the adenovirus. The hexons are shown as hexagons, with a superimposed triangle representing the towers, and the pentons as pentagons. The shaded hexons are from adjacent faces of the capsid. The positions thought to be occupied by proteins VI ●, IIIa ▬, and IX ◬ are also shown. Adapted from Stewart *et al* (1991).

kinase and that it takes place early in infection (Blair and Russell, 1978; Tsuzuki and Luftig, 1983)

1.3.4 Proteins VI, VIII and IX

Protein VI is a 22,000 Da cleavage product of the precursor (Edvardsson *et al.*, 1976). Protein VI is a hexon associated protein (Everitt *et al.*, 1973), which is thought to bind to the capsid internally (Stewart *et al.*, 1991) and has DNA binding properties (Russell and Precious, 1982). In the virion, Stewart *et al.* (1993) have suggested that protein VI is present as a hexamer, each hexamer is composed of a trimer of ordered VI and a trimer of disordered VI, and is located between the hexons adjacent to a penton base. There is no evidence that VI exists in a disordered state in the capsid, but Stewart *et al.* (1993) claim this theory fits the observed results . The function of VI may be to anchor the viral DNA to the capsid. The protease activating peptide is derived from the C-terminus of the protein VI precursor, molecular weight 27,000 Da (Webster *et al.*, 1993). The protease is discussed later in section 1.4.

For details about Protein VIII see section 1.9.

Protein IX is present in the adenovirus capsid, as three 14,000 Da subunits (Stewart *et al.*, 1993). Twelve copies of IX are present on each facet, located as four trimers between the towers of three hexons (Furcinitti *et al.*, 1989). Protein IX stabilises the central nine hexons in each facet, which produces the group of nine hexons observed when the capsid dissociates (Furcinitti *et al.*, 1989). Although IX has a stabilising effect upon the hexons, it is not required for viral assembly as a partially deleted IX gene in an Ad5 mutant was able to produce virions, which did not give groups of nine upon disassociation (Colby and

Shenk, 1981). However, it appears that IX is essential for the packaging of the entire viral genome into a capsid (Ghosh-Choudhury *et al.*, 1987).

1.3.5 The Core

Protein X was thought to be a cleavage product of pX (11,000 Da protein). However, protein X has been redefined as Mu (Horwitz, 1990a). Protein Mu is the 3,000 Da cleavage product of pX, and is located within the adenovirus nucleosome. Protein Mu was found by Chatterjee *et al.* (1985) to bind to protein V, or to V in protein complexes.

Protein V has a weight of 42,000 Da, and is synthesized as the mature viral protein. Phosphorylation of V occurs during incorporation into the virus core, and while DNA is being inserted into the core V is dephosphorylated (Weber *et al.*, 1983). The adenovirus nucleosomes, which bind non-covalently to the DNA, are separated by a variable length of DNA that contains one molecule of V (Horwitz, 1990), which also binds non-covalently to the adenovirus DNA. Protein V can bind to III (penton base), which may serve as an anchor for packaging the viral DNA in the core.

Protein VII has a molecular weight of 18,500 Da and is a cleavage product of pVII, a 22,000 Da protein. Protein VII is a core protein, which forms a complex with V to surround the viral DNA (Newcomb, 1984). Protein VII is present in the virus particle as three dimers, which are positioned within the nucleosome and binds non-covalently with the viral DNA (Horwitz, 1990b). The binding of IIIa to VII may play a role in anchoring the nucleosome to the capsid (Everitt *et al.*, 1975).

The terminal protein is a 38,000 Da cleavage product of the 75,000 Da terminal protein precursor, and is covalently bound to the 5' ends of viral DNA molecules (Rekosh *et al.*,

1977). The linkage between the DNA and terminal protein is via a phosphodiester bond formed between a serine residue in the terminal protein and the terminal deoxycytosine residue in the viral DNA (Smart and Stillman, 1982). The attachment of the terminal protein is essential for transcription of adenovirus DNA and possibly also for DNA replication (Schaak and Shenk, 1989). The presence of the terminal protein prevents access of 5' exonucleases to the viral DNA and increases the ability of viral DNA to replicate over DNA lacking the terminal protein (Pronk and Vliet, 1993).

1.4 Adenovirus Protease

Highly specific proteases play an important role in the maturation of many viruses. Such proteases are highly specific in their recognition sites in order to prevent unnecessary degradation, and early maturation. The adenovirus requires the proteolysis of precursor proteins to occur at various stages in the replication cycle of the virus for the production of infectious viral particles. The use of synthetic peptides has established that the adenovirus protease cleaves at (M,L,I)XGG-X and (M,L,I)XGX-G motifs (Webster *et al.*, 1989). Six of the twelve major virion proteins (pIIIa, pVI, pVII, pVIII, pTP and the L2 11K protein) undergo processing by the adenovirus protease to form mature virion components. Failure of the protease to cleave precursor proteins result in an immature virion, which is unable to infect cells (Hannan *et al.*, 1983).

Isolation of a temperature-sensitive adenovirus type 2 (Ad2ts1) defective in protease activity at non-permissive temperatures (Weber 1976) and the mapping of the mutation led to the suggestion that the virus encoded L3 23 kDa protein is the protease (Yeh-Kai *et al.*, 1983). Expression of the protein in *E.coli* and baculovirus systems has lent support to this proposal (Webster *et al.*, 1993). The gene coding for the adenovirus protease is located in the L3 region of the genome between the hexon and the DNA-binding protein

genes. To date the sequence of seventeen protease genes have been determined and submitted to EMBL/GenBank. The length of the proteases vary from 201 to 214 residues including the initiating methionine, the sequences having been derived from the genome sequence. Only a partial sequence of Ad2 protease has been determined by direct verification (Anderson, 1990).

The sequence of the 23 kDa protein contains no recognisable protease motifs (Anderson 1990; Houde and Weber 1990) although the protease sequence is well conserved in the serotypes for which sequence information is available (Cai *et al., 1*992). Homology between each of the proteases sequenced vary from as high as 93.6% between Ad40 and Ad41 and as low as 54.5% between Ad4 and Adb7. Only 17.6% of the residues remain conserved across the entire genome (Weber 1995).

The adenovirus protease was initially classified as a serine protease based upon inhibition by diisopropyl fluorophosphate (Tremblay *et al., 1*983). However, extensive inhibitor studies indicated that it was a member of the cysteine class that are related to trypsin (Webster *et al., 1*989) and that replacement of Cys 122 with an alanine residue results in the loss of activity (Jones *et al., 1*996). Recombinant protease is activated by the peptide GVQSLKRRRCF, which is identical to the sequence of the C-Terminal 11 amino acids of the viral protein pVI (Webster *et al., 1*993b). This probably represents the *in vitro* manifestation of an *in vivo* mechanism whereby pVI-CT, or possibly pVI itself, is responsible for the regulation of protease activity. The importance of viral protease regulation was shown by Krausslich (1991) who demonstrated that premature activation of the HIV-1 protease, an aspartyl protease activated by dimerization (Navia *et al., 1*989), is toxic to cells and prevents virus assembly. Analysis of the crystal structure of the adenovirus protease with the activating peptide reveals that the pVIc-t forms a disulphide bond with Cys 104.

The crystal structure of the adenovirus protease, see figure 8, has confirmed that it possesses a cysteine active site (Ding *et al., 1*996). Typically cysteine proteases have an active site composed of a histidine, cysteine and aspartic acid residue. The crystal structure of the Ad2 protease showed that the catalytic site was composed of the only conserved histidine at residue 54, Cys 122 and a glutamic acid residue at position 71. papain and subtilisin, a cysteine protease and serine protease respectively, display an alpha helix and several ß-strands within their central region as does the adenovirus protease. When the structures are aligned the similarities between papain and the adenovirus protease are increased. The active Cys 122 residue of the protease is in an identical position to the nucleophilic Cys 25 of papain. The protease residues his 54 and Glu 71 are in identical positions to those of His 159 and Asn 175 which constitute the two other residues that compose the charge-relay system of papain. Another pairing revealed by the alignment of the two active sites are Gln 19 of papain with the conserved Gln 115 of the adenovirus protease. The Gln 19 of papain is thought to participate in the formation of an oxyanion hole with the active-site Cys 25 (Drenth *et al., 1*976). The similarity in the position of the catalytic elements of the protease with papain suggests that the protease employs the same catalytic mechanism as papain.

The active site of the protease lies within a 25 Å long, bent grove that is approximately 8 Å wide, Cys 122 and His 54 lying in the middle of this grove (Ding *et al., 1*996). These two residues are conserved among all the known sequences of the adenovirus protease. A 3.6Å hydrogen bond is formed between atoms S of Cys 122 and Nd of His 54. These two amino acids probably form a Cys-His ion pair because a thiolate anion in the adenovirus protease can be titrated at pH 5.0 with dithiodipyridine (Mangel *et al., 1*996). Glu 71, probably the third member of the charge-relay system lies on the other side of the imidazole ring of His 54 from Cys 122. A 2.7 Å hydrogen bond is formed between the

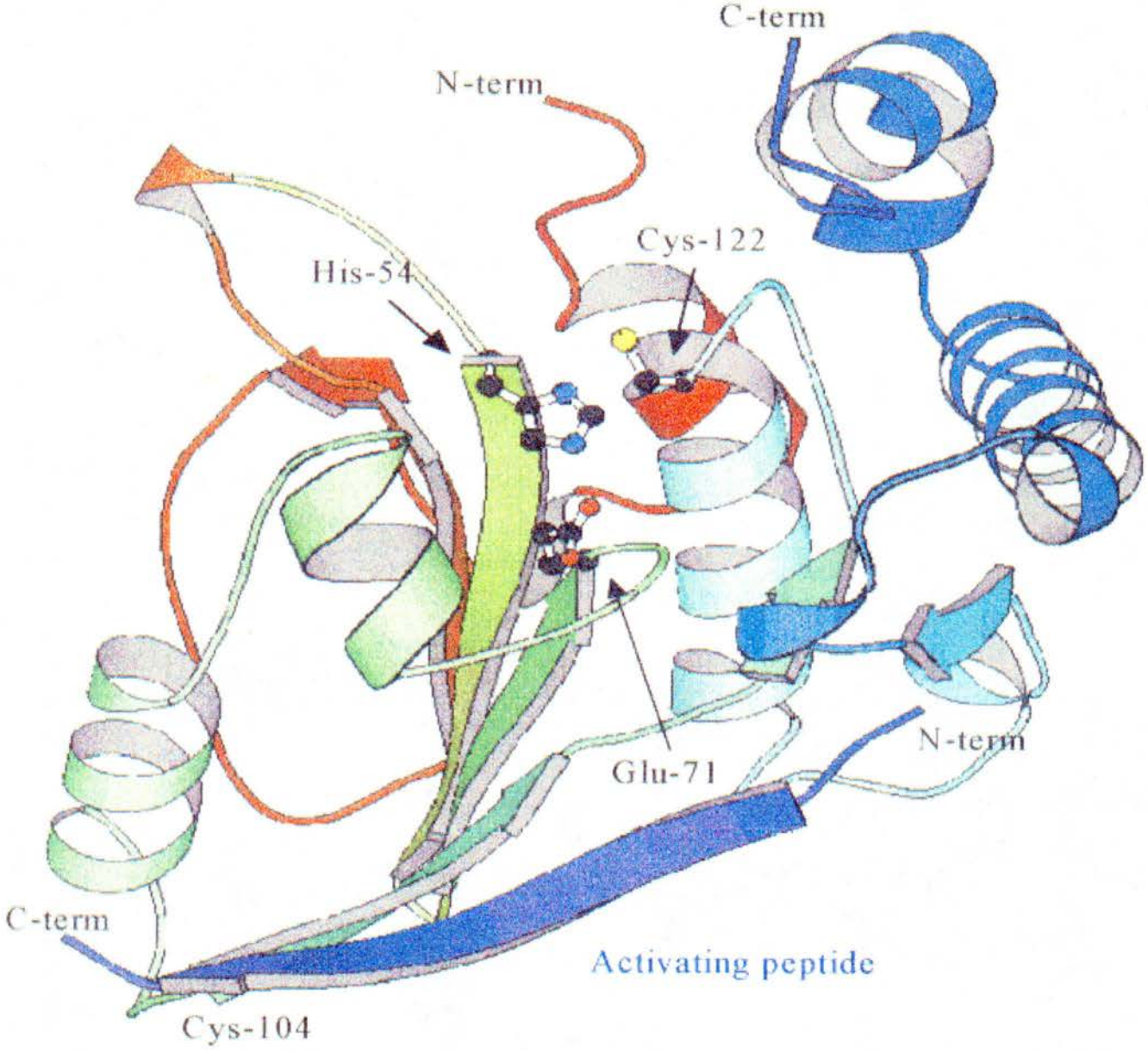


Figure 8. The three dimensional structure of the human adenovirus protease serotype 2 with its 11 amino acid cofactor, pVIct, as determined at 2.6 Å resolution by X-ray crystallographic analysis. The termini of the protease are denoted as N and C, those of pVIct as n and c. The path of the polypeptide is colour-coded according to the visible spectrum, red at the N-terminus to violet at the C-terminus. Taken from Ding *et al* 1996.

Oe of Glu 71 and Ne of His 54. Glu 71, is replaced by an Asp residue in two of the seventeen adenovirus proteases serotypes that have been sequenced to date.

Despite the similarities of the adenovirus protease with papain the sequential order of the amino acids residues involved in the catalytic triad is different. In the protease the triad is His 54, Glu 71 and Cys 122 whereas in papain the order is Cys 25, His 159, Asn 175. Therefore the protease would be in a different class from the papain superfamily and the viral cysteine proteinases with chymotrypsin-like folds. The protease would belong to the subclass His/Cys and because of the unique fold the adenovirus protease would represent a fifth group (Mangel *et al., 1*996).

The L3 23 kDa protease expressed by the adenovirus requires the presence of an 11 amino acid co-factor for maximum activity (Webster *et al., 1*993, Mangel *et al., 1*993). DNA has also been implicated in the activation of the adenovirus (Mangel *et al., 1*997) but, this effect has not been widely confirmed. Evidence from Webster *et al. (*1993) suggested that intact viral pVI was capable of activating the adenovirus protease and Matthews and Russell (1995) showed that recombinant pVI was capable of activating the 23 kDa protease. This would suggest that pVI has a role in the regulation of protease activity in the infected cell.

The crystal structure of the adenovirus protease with the pVI-CT reveals that residues 1-3 and 4-10 interact with two non-continuous regions on the protease (Ding *et al., 1*996). The first three residues bind on or near β-sheet S7 while residues 4-10 of pVI-CT interact with β-sheet S5, a disulphide bond is formed between Cys 10 of pVI-CT and Cys 104 of the viral protease. Cabrita *et al. (*1996) showed that pVI-CT lacking the Cys 10 residue failed to form complexes with the protease and that the absence of the first two residues

of pVI-CT resulted in weakly bound peptides which were readily displaced by full length pVI-CT.

1.5 Genome Organisation

The adenovirus genome is composed of a single copy of double stranded DNA approximately 36 kbp in length and contains two identical origins of DNA replication. The same general organisation has been observed in all adenovirus serotypes that have been sequenced to date, with the exception of the avian CELO virus which has the VA RNA gene positioned differently from the mammalian adenoviruses. The genome is organised into early and late transcription units, see figure 9, all of which are transcribed by RNA polymerase II. The six early transcription units generate mRNA which codes for polypeptides required to establish viral replication, transformation, and viral latency in infected cells. The late phase of adenovirus infection begins at around 5 to 6 hours after infection with the onset of viral DNA replication. It is the initiation of DNA replication that activates the major late promoter which generates five families of late mRNAs. Most of the late mRNAs encode structural polypeptides of the virion and proteins involved in the packaging of the genomic DNA.

1.5.1 Early Viral Gene Expression

Six regions of the viral genome are transcribed during the early phase of Ad2 infection, these are E1A, E1B, E2A, E2B, E3, E4 and L1. Each early transcription unit generates a variety of spliced mRNAs from a single promoter. The E1A and E1B regions, located at map position (mp) 1.3-4.5 and mp 4.6-11.1 respectively, have been investigated as it is the products of these genes which are necessary for viral oncogenesis. It is thought that the E1A products have the major role in the production of tumours (Gallimore *et al.*,

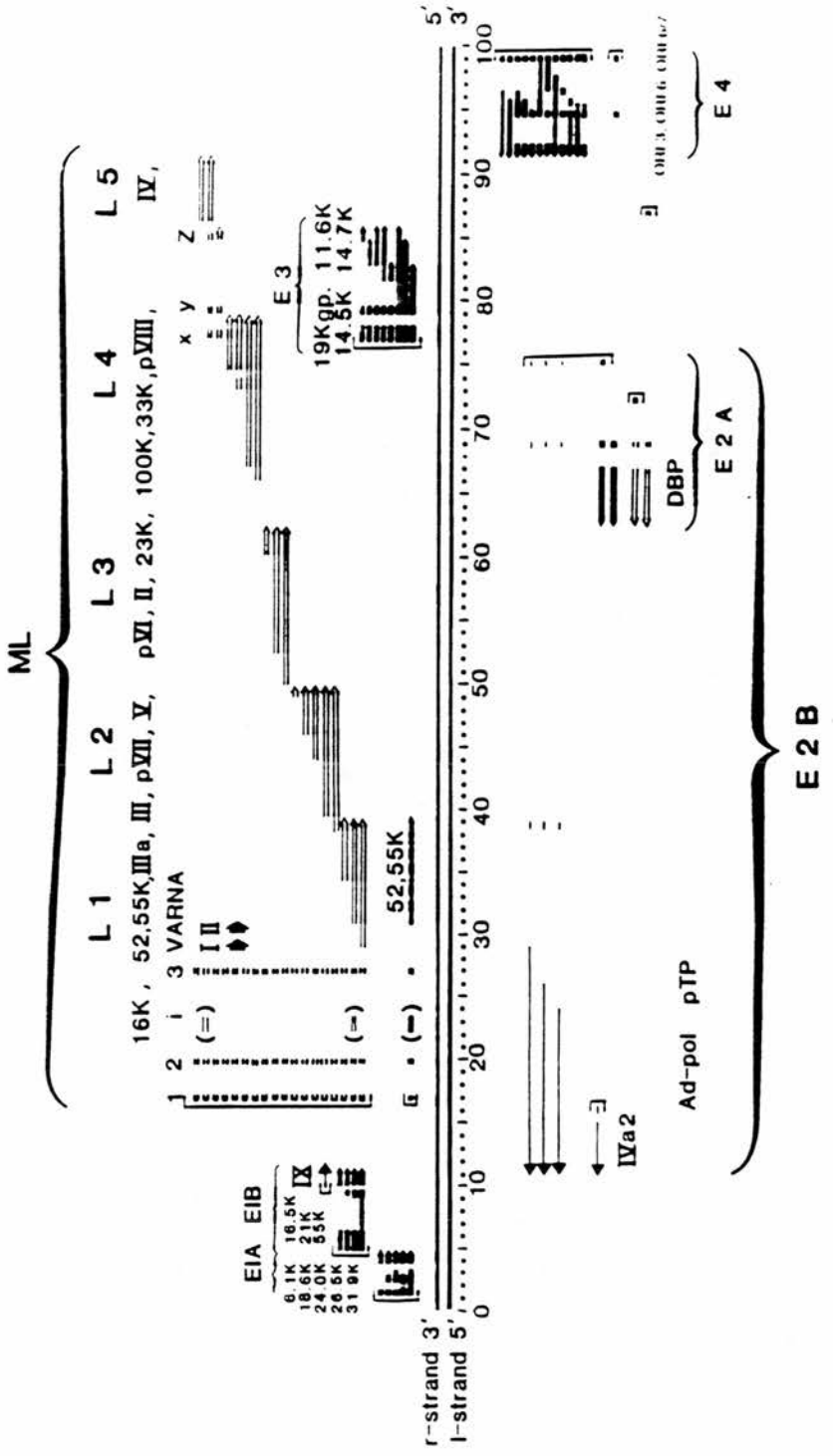


Figure 9. Schematic map of genes and transcription units encoded by the adenovirus type 2 genome. Thick lines indicate early mRNAs and the thin lines indicate mRNAs expressed at intermediate times of infection. Open arrows show mRNAs expressed late after infection. Gaps in the arrows denote the position of introns. Arrowheads show the locations of 3' ends of mRNAs, and the promoter sites are indicated by brackets. ML, major late transcription unit; ORF, open reading framw; VARNA, Virus-associated RNA; gP, glycoprotein; DBP, DNA-binding protein; Ad-pol, Adenovirus DNA polymerase; pTP, preterminal protein. Taken from Imperiale *et al* (1995).

1984). The E1A gene produces five mRNA species with common 5' and 3' termini. The two largest species 13S and 12S are present early in infection but, the three smaller species (11S, 10S and 9S) are only present later in infection (Stephens and Harlow 1987). Translation of the 13S and 12S species of mRNA reveals that both produce proteins of identical sequence apart from some internal residues which are missing in the smaller product.

DNA sequence analysis of the larger E1A proteins, produced from 13S and 12S mRNA has revealed that the proteins contain three conserved region named CR1, CR2 and CR3 (Kimelmann *et al., 1*985; van Ormondt and Galibert 1984). The E1A protein functions that influence transcription repression and activation map to the first two conserved regions and the last respectively (Jones 1992). The function significance of the E1A proteins is unknown but, it is thought that because the two larger proteins are produced early in infection and the other proteins are produced later that the smaller proteins increase and/or decrease individual E1A functions at different times in the viral replication cycle (Williams *et al., 1*995).

The E1B gene is located adjacent to the E1A gene and transcripts copied directly from this region are differentially spliced to generate two major mRNA species of 22S and 13S and two minor species of 14.5S and 14S all of which have identical 5' and 3' termini (Saito *et al.*, 1983; Virtanen and Pettersson 1985). The two major products of the E1B gene are the 19K and 55K proteins which have been identified as playing a role in transformation (Rao *et al., 1*992). Immunoprecipitation of E1B 55K from adenovirus transformed cells resulted in the coprecipitation of p53 (Sarnow *et al., 1*982). Protein p53 which is normally localised in the nucleus translocates to a perinuclear aggregate when complexed with E1B 55K suggesting that direct physical sequestration of p53 by 55K may contribute to inhibition of p53 function (Zantem *et al.*, 1985). The 19K protein

produced by the E1B gene has a variety of functions including transformation, tumour necrosis factor -α (TNF- α) and inhibition of Fas antigen mediated apoptosis (Rao *et al., 1*992; Hashimoto *et al., 1*991). Studies using missense mutants of the E1B 19K protein have indicated that this multifunctional activity is derived from the single function of inhibiting apoptosis as transforming activity was linked with resistance to TNF- α mediated apoptosis and Fas antigen mediated apoptosis (Chiou *et al.,* 1994; White *et al.,* 1992).

The E2A and E2B genes, located at mp 75.4-61.5 and mp 75.4-14.2 are both very important for successful completion of virus infection as they encode proteins vital to viral DNA replication. Encoded, by the E2A region, is the 72 kDa DNA binding protein (DBP) which, as well as playing a role in DNA replication is also involved in a multitude of functions which include repression of E4 transcription (Nevins and Winkler, 1980), mRNA stability (Babich and Nevins, 1981), assembly of the virus particles (Nicolas *et al., 1*983), and transformation (Ginsberg *et al., 1*974). E2B encodes an 80-kDa precursor terminal protein and a 140 kDa DNA polymerase. Regulation of the E2 regions fall under the control of two promoters, the early E2 promoter, located at mp 76 and is regulated by E1A. The second promoter is termed the late E2 promoter, located at mp 72, and is activated at intermediate times after infection.

The E3 region, located at mp 75-86, expresses a number of proteins that appear to reduce the effects of the host's immune response. These include a 19 kDa glycoprotein that has been shown to bind to the major histocompatibility complex class I complex preventing its transport to the cell surface and so lowering the cytotoxic T-cell response (Rawle *et al.,* 1989). It has also been proposed that the E3 14 kDa protein inhibits lysis of adenovirus infected cells by the TNF- α (Gooding *et al., 1*988).

The E4 region is located at mp 99.1- 91.3 and encodes a large number of mRNAs with the potential to produce at least seven proteins (Virtanen *et al., 1*984). The E4 proteins are involved in regulation of DNA replication and late gene expression (Bridge *et al., 1*991; 1993) Phenotypic analysis of E4 open reading frame (ORF) 3 and the E4 ORF 6 proteins appear to have mutually redundant activities during infection as expression of either one appears to be sufficient to establish an infection with the same characteristics as a wild-type infection (Hemstrom *et al., 1*988).

1.5.2 Intermediate Transcription

The structural protein pIX, despite being inside the E1B coding region of the adenovirus, is produced at 6-8 hours post infection. The gene is switched on independently of the E1B region and pIX coded for by a 9S mRNA which is unspliced (Alestrom *et al.,* 1980). The reason why pIX is the first structural protein synthesised is unknown but its role in the virus capsid is described in section 1.3.4.

Protein IVa2 is also produced during the intermediate stage of viral infection and is located between mp 11.3 and 16. IVa2 is coded for by a primary mRNA strand that is spliced to join two exons (Crossland and Raskas, 1983). The function of IVa2 is unknown but, it does binds to viral DNA and was originally thought to be a scaffolding protein as it was not detected in the mature virion. However, it was identified in the mature virion by Winter and D'Halluin (1991) and therefore its presence may indicate that it has another role other than assembly.

1.5.3 Late Transcription

The L1 region produces two cytoplasmic mRNAs coding for the L1 proteins 52/55K and IIIa. The 52/55K mRNA is expressed at early and late transcription periods whereas the IIIa mRNA is only produced late in infection (Shaw and Ziff 1980). Protein IIIa is a structural component of the mature capsid, see section 1.3.3 for further details, and the L1 52/55K protein is not a structural protein but, it is required for viral assembly (Hasson *et al.*, 1989).

The L2 transcription unit produces a family of mRNAs that code for proteins III, V, pVII an the 11 kDa precursor of protein mu. All the proteins coded for by the L2 region are located in the viral core, see section 1.3.5 for details, except for protein III, which forms the penton base, see section 1.3.2 for details.

The genes coding for protein II, pVI and the 23 kDa protease are located in the L3 region of the adenovirus genome. Protein II forms the hexon and pVI is the precursor protein of VI, see sections 1.3.1 and 1.3.4 respectively. The L3 23 kDa protease is essential for virus maturation and is described in greater detail in section 1.4.

The L4 region codes for pVIII, a 100 kDa protein, and a 33 kDa protein. The L4 100 kDa protein is required for efficient viral protein synthesis during late infection (Hayes *et al.*, 1990). Protein pVIII is the precursor of the viral protein VIII and is described in greater detail in section 1.9. The L5 region of the viral genome contains the gene for protein IV which forms the fiber, see section 1.3.2 for details.

1.5.4 Virus-Associated mRNAs

The virus-associated mRNA (VA RNA) is located within the major late transcription unit at map position 28.8-29.5 and are transcribed by RNA polymerase III to give, in most human serotypes, two transcripts between 150-170 nucleotides in length, the two mRNA transcripts are termed VA RNA_I and VA RNA_{II}. It is thought that the second VA RNA gene originated by gene duplication and that the human serotypes with only one VA RNA gene lost the second VA RNA late in their evolution (Mathews, 1995) Transcription of VA RNA starts in the early phase and accelerates late in infection, when present the VA RNA_{II} is produced in quantities 10-20 fold less than VA RNA_I due to the VA RNA_{II} promoter being weaker and because of promoter competition (Bhat and Thimmappaya, 1984).

The only identified function to date of VA RNA_I is to inhibit the activation of DAI, the double stranded RNA dependent protein kinase which inhibits protein synthesis and is induced by interferon. DAI inhibits protein synthesis by phosphorylating the protein initiation factor eIF-2 on its α subunit, leading to the inhibition of a recycling factor (GEF or eIF-2B) and consequently bringing translational initiation in the cell to a halt (Hershey 1991). To date no role has been identified for VA RNA_{II} however, it is speculated that VA RNA_{II} may inhibit a pathway related to the DAI pathway. It is thought that the enzyme 2'-5' oligoadenylate synthetase, which is induced by interferon and activated by ds RNA (Hovanessian, 1991), may be the target for VA RNA_{II} but, there is no experimental evidence to support this (Mathews, 1995).

1.6 DNA Replication

Viral DNA replication marks the division between early and late transcription events and occurs at approximately 6 hours post infection. The replication process continues until 24 hours post infection resulting in the production of 100 000 copies of the viral DNA in each cell (Flint *et al.*, 1976) but, only about 20% of these genomes are packaged into virions (Pina and Green, 1969; Green *et al.*, 1970).

Electron microscopy studies of replication intermediates, isolated from Ad2 infected cells, led to the proposal of a strand displacement mechanism for replication (Flint *et al.*, 1976). The replication cycle of the adenovirus DNA, which operates by a protein primer mechanism, can be divided into two stages, see figure 10. The first stage of DNA replication is termed initiation and requires the formation of a preinitiation complex. This complex is formed by DBP binding to the viral DNA and bringing about a conformational change that exposes the binding sites for NFI and Oct-1 (Stuiver *et al.*, 1992). The pTP-pol complex is bound tightly by NFI and the resulting pTP-pol-NFI complex binds to the origin DNA, the binding of this complex is further enhanced by the binding of Oct-1. After assembly of the preinitiation complex the origin unwinds to permit the template strand to enter the active site of the polymerase. The mechanism involved in the unwinding of the DNA is unknown but, it is known that the DBP is capable of unwinding DNA (Zijderveld and Van der Vliet, 1994; Monaghan *et al.*, 1994).

The initiation reaction is started by the formation of an ester linkage between the phosphate of a dCMP base and the β-hydroxyl group of Ser_{580} in pTP. This reaction requires the presence of Mg^{2+} but, two other bivalent cations (Mn^{2+} and Ca^{2+}) can substitute for Mg^{2+}. The 3' hydroxyl group of the resulting pTP-dCMP complex acts as a

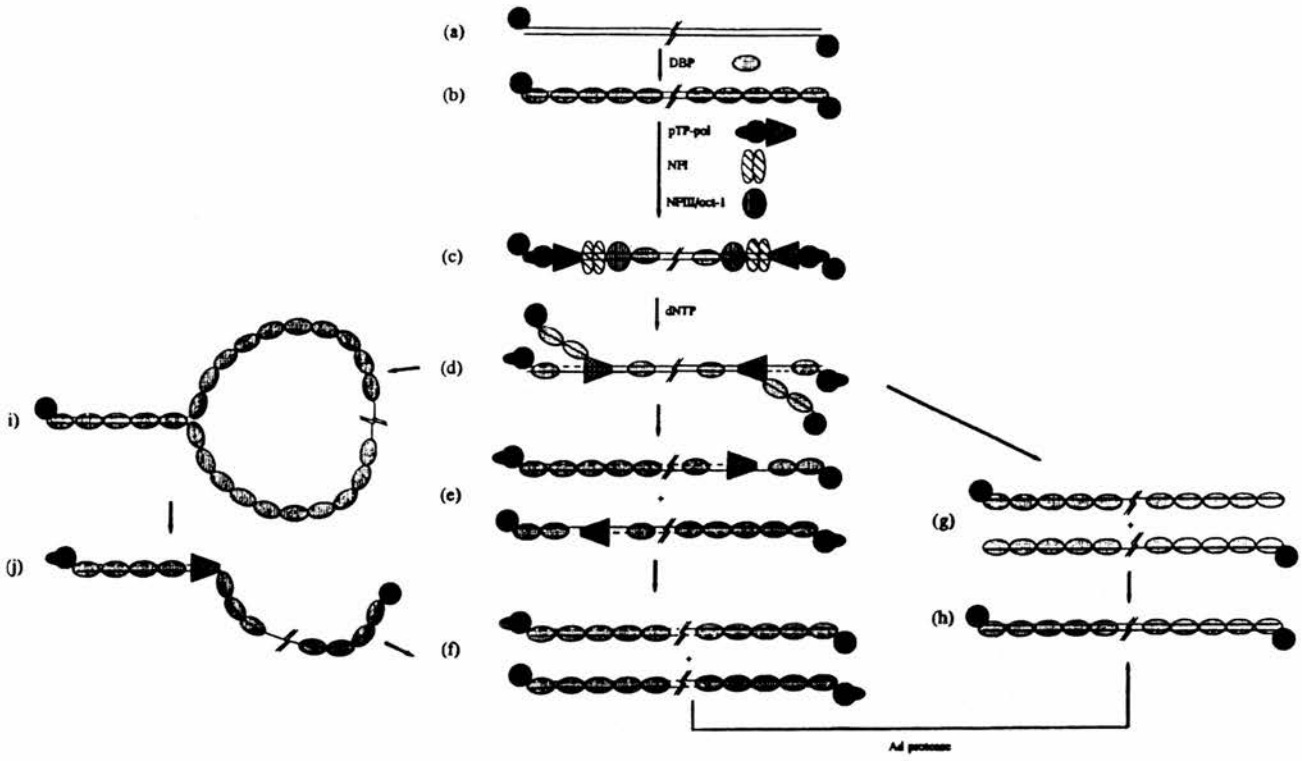


Figure 10. General outline of the first round of adenovirus (Ad) DNA replication. The parental terminal protein (TP) containing template DNA (a) forms a multiprotein-DNA complex with DNA-binding protein (DBP; b) A preinitiation complex (c) is assembled with various replication proteins (not on scale). Initiation occurs by covalent coupling of a deoxycytidine monophosphate residue to precursor TP (pTP). In the presence of deoxynucleoside triphosphate, elongation starts by a displacement mechanism, minimally requiring DNA polymerase (pol) and DBP. Illustrated is a molecule which started replication at both origins simulataneously (d). Replication can proceed from both sides, leading to partially single-stranded replication intermediates (e) and finally duplex daughter strands containing TP at one end and pTP at the other end (f). Alternatively, if replication started at either end in different molecules, single-stranded displaced strands of opposite polarities are formed (g). These can renature, a process enhanced by DBP, to form duplexDNA with two TP molecules (h). Another possibility is that intrastrand renaturation of the inverted terminal repeat occurs, leading to a panhandle structure (i). The regenerated double-stranded origin can be used for protein-primed initiation, leading to a partially duplex intermediate (j) and finally a daughter molecule (f). pTP in these daughter molecules is processed later in infection by the Ad protease. It should be stressed that later in infection daughter molecules will be effectively used as templates for second and further rounds. This will lead to a considerable increase in daughter molecules containing pTP at both ends, while keeping the number of TP-containing molecules constant at the level of the input DNA. Taken from Van der Vliet (1995).

primer for the synthesis of a new strand of DNA using one strand of the parental DNA as a template.

Once started elongation can occur at a high rate provided DBP is present an NFII removes topological stress. The polymerase can synthesise at least 30 000 bp on primed poly-dT in one single association step in the presence of DBP (Field *et al., 1*984). The inverted terminal repeats play a central role in DNA replication with residues 1-18 being required for initiation and the presence of residues 19-50 capable of amplifying the elongation process up to 200 times (Hay *et al., 1*984). Elongation is thought to proceed with the replication fork entering the inverted terminal repeat from the inside and moving towards the end of the molecule. When the polymerase reaches the end of the molecule it probably disassociates from the DNA but, it is possible that the polymerase may remain bound to the DNA molecule and initiate replication of the opposite strand in association with the TP, NFI and Oct-1. If the polymerase dissociates the displaced ssDNA is released as a stable ssDNA-DBP complex. Displaced strands of opposite polarity are thought to renature forming a double-stranded daughter molecule. Finally, the pTP containing templates that are produced during replication are processed during assembly by the viral protease. Cleavage of the precursor section of free pTP, by the protease, removes the capacity of the protein to function as a primer and therefore halts replication.

1.7 Adenovirus Assembly

Assembly of the adenovirus into an icosahedral capsid with a DNA core requires the production of individual polypeptide chains, which are assembled into multimeric units like the hexon, a trimer of II (Van Oostrum *et al.,* 1985), the fibre a trimer of IV (Philipson, 1983), and the penton base a trimer of III (Van Oostrum *et al.,* 1985). This is followed by the generation of a DNA empty capsid (Philipson, 1984). Approximately 10-

20% of the synthesised structural proteins are assembled into virions, and 10% of the DNA synthesised is inserted into the virion (Philipson, 1984). As a result of the low incorporation rate of viral proteins, and because approximately 100,000 viral particles are generated per cell, the viral protein and DNA forms a pool comparable to the total DNA and protein content of the host cell.

Formation of an empty capsid is thought to occur by the grouping of hexons into nonamers, possibly in association with IX, which may automatically assemble into a capsid skeleton (Philipson, 1984). The roles of protein IVa_2, the 32,000 Da protein and 40,000 Da protein may be as scaffolding proteins as after the insertion of DNA into the empty capsid as they are no longer associated with the viral particle (Philipson, 1983). Proteins pVI and pVIII may also play a role in capsid assembly as scaffolding proteins (Edvardsson *et al.*, 1976).

Viral DNA is thought to be inserted into the skeletal capsid with the left hand end of the adenovirus genome entering first (Hammarskjold and Winberd, 1980). The preferential insertion of the left-hand end of viral DNA is due to a recognition signal located between residues 100 and 390 (Horwitz, 1990). The recognition signal mimics the recognition signal at the left hand end of lambda phage DNA which binds to the proteins required to insert the lambda DNA into the head structure (Philipson, 1983). The core proteins (pVII, V and IVa_2) are inserted into the skeletal capsid at around the same time as the DNA, but it is unclear whether this occurs separately or together with the viral DNA (Philipson, 1983).

In the young virion protein IVa_2 and the two scaffolding proteins (32 kDa and 40 kDa proteins) are absent (Philipson, 1983). However, the precursor peptides are uncleaved and the proteins Mu, XI, and XII are not present (Philipson, 1983). Young virions are

very similar in structural characteristics to the mature virion but they are unable to infect cells (Horwitz, 1990). Adenovirus ts1 when grown at restrictive temperatures (above 39°C) produces a pool of young virions, which are unable to mature due to the production of a protease which is deficient in activity and are thus unable to infect cells (Bhatti and Weber, 1979).

Maturation of the viral particle is brought about by the proteolytic cleavage of the precursor proteins. The processing of pIIIa, pVI, pVII, pVIII, pX, pTP results in the appearance of the mature polypeptides IIIa, VI, VII, VIII, Mu, and the terminal protein (Horwitz, 1990). Only the mature virus is capable of infection and replication of the viral DNA.

1.8 Strategy of Infection for Adenovirus 2

The infection of a host cell can only be successful if a virus is able to fulfil three basic requirements. First the virus must be able to transfer its DNA and viral proteins across the cytoplasmic membrane. This is achieved by either, direct penetration through the plasma membrane or, by entry via endosomes. Secondly the DNA and viral proteins must arrive at the targeted cell organelle, usually the nucleus. Finally the viral DNA and protein must be released from their transport carrier.

The adenovirus attaches to a host cell by an interaction between the cell receptors and the multi lengthed fibre protein (Philipson *et al.,* 1968), which forms part of the penton complex. Absorption of the receptor bound adenoviruses into the cell occurs by receptor mediated endocytosis (Greber *et al.,* 1993). After the absorption of the adenovirus into the host cell the dismantling /uncoating of the virus particle begins. Results obtained by Greber *et al. (*1993) indicate that the uncoating of the adenovirus was initiated by the loss

of the fibre and dissociation of VIII and IIIa in the preacidic endosome. In the next stage of the endosome cycle (acidification) Greber *et al.* (1993) propose that protein VI undergoes proteolytic degradation. However, it is unlikely that the cleavage of VI is due to the adenovirus protease as there are no cleavage sites (as defined by Webster *et al.*, 1989 and Anderson, 1990) in the mature protein. The enzyme(s) responsible for cleavage of VI remains unclear as Greber *et al.*, 1993 suggest that lysosomal and endosomal hydrolases were not responsible for cleavage due to data obtained from inhibitor studies. The dissociation of the penton base protein, IX and most of the hexons from the capsid also occurs during acidification. It is at some time in the latter stages of acidification that the capsid escapes from the endosome and penetrates into the cytoplasm. The penetration of the endosome by the adenovirus is thought to involve the penton base as Seth *et al.* (1986) showed that antibodies to the penton base inhibited the release of virus particles into the cytoplasm. Within the cytoplasm the virus particle is formed from the core proteins and associated hexons. The next stage of infection is the transport of the DNA to the nucleus. The characteristics of the transport mechanism are undefined but it is thought, from motif sequences, that either or both the terminal protein and protein V are involved in the nucleus targeting mechanism as both contain nuclear localisation sequences (Russell and Kemp, 1995).

The strategy of infection adopted by adenovirus is extremely efficient. Almost 40% of the viral particles bound to a cell membrane are able to release their DNA (Greber *et al.*, 1993). Only 5% of the surface bound viruses are degraded in the lysozymes. This indicates a highly efficient escape mechanism from endosomes.

1.9 Protein Precursor VIII (pVIII) and Protein VIII

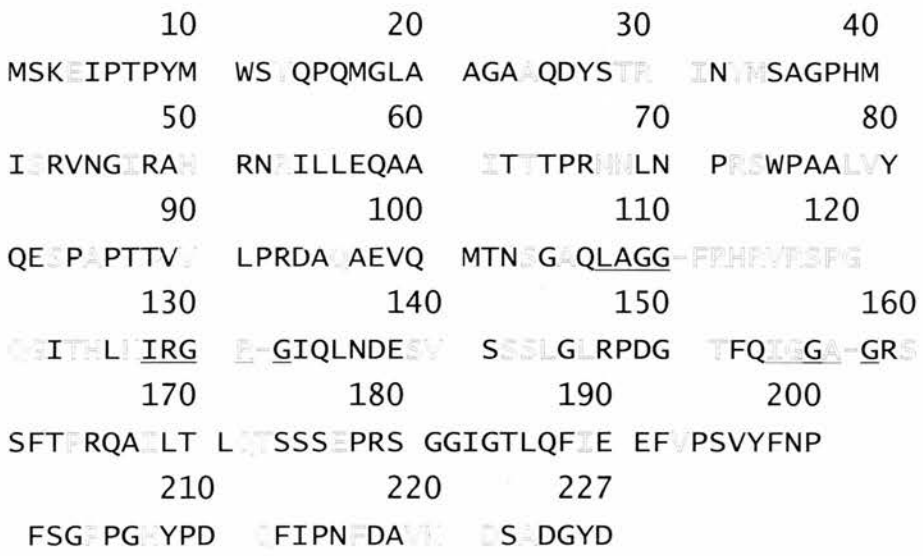


Figure 11. Conservation of Amino Acid Residues in pVIII. Outlined characters represent unconserved amino acid residues; underlined characters form an adenovirus protease recognition site, the cleavage point is represented by the symbol -. The pVIII sequences used : adenovirus 2, complete; adenovirus 5, complete; adenovirus 12, complete; adenovirus 41, complete; adenovirus 3, partial; adenovirus 40, partial.

The precursor of VIII in adenovirus serotype 2 is a 26,000 Da protein (Edvardsson, 1976) which contains 227 amino acid residues (see figure 11 for amino acid sequence). The protein pVIII contains no cysteine residues but contains high levels of serine and proline residues. Radioactive labelling of pVIII showed that between 10 and 20% of the [^{35}S] methionine label is transferred to VIII, indicating that only part of the total population of pVIII in the immature virus forms VIII in the mature virus (Edvardsson *et al.*, 1976). The precursor protein has three theoretical cleavage sites at 111 a.a. (LAGG-F), 131 a.a. (IRGR-G) and, 158 a.a. (IGGA-G) for the adenovirus protease (as defined by the protease recognition sequence).

The similarities between the pVIII sequence produced by various serotypes of adenovirus may aid in determining which segments of the protein have specific functions. Although the average difference in residues among the pVIII sequences compared in figure 11 is three in every ten amino acids there are sections of the protein with higher or lower residue changes. Between residues 1 and 22 there are only two mutations as compared to the expected six mutations. It is possible that the low rate of mutation is due to this region being a functional domain, i.e. a binding domain to other structural proteins, or, recognition sequence for a transport protein during viral assembly.

The protein segment between 112 and 125 may be functionally redundant as only one amino acid remains unmutated in this sequence. The redundancy of this sequence may be unlikely as it would be retained in VIII if it is formed by cleavage at 131 a.a. Therefore this may be evidence that VIII is formed by the sequence 1-111 a.a. The function of the protein segment 131 to 157 is probably as a spacer between VIII and the protein produced by cleavage at 157 a.a. Use of protein homology cannot be relied upon in determining sequence function or redundancy as the protease cleavage sites are not identified by this method alone.

Conservation of the protease cleavage sites by the virus is necessary as loss of one of the three sites may be disadvantageous. Without conservation of the cleavage sites pVIII would either fail to be cleaved or not cleaved in the desired manner and viral stability and/or infection capability could be lost. Examination of the area around the protease recognition sites show a degree of conservation (see table 2), but it is not significant when compared to the overall rate of conservation. However, it is significant that the mutated residues at the cleavage sites are either unnecessary for cleavage, or, have been mutated to amino acids which would still allow cleavage. This indicates that cleavage at the

cleavage sites are essential for the virus. The theoretical cleavage site at 111 a.a. is the only cleavage site in pVIII which does not contain an amino acid change, although it is thought that this protease recognition site is not cleaved (Anderson, 1990).

Cut-Site	Serotype	Subgenera	Start	Amino Acid Sequence	End
111	Ad2	C	100	QMTNSGAQ**LAGG** FRHRVRSPG	120
	Ad5	C	100	QMTNSGAQ**LAGG** FRHRVRSPG	120
	Ad41	G	100	QMTNSGAQ**LAGG** SRHVRFRGR	120
	Ad12	A	100	HMTNAGAQ**LAGG** ARHSFRYKG	120
	Ad3	B	*	QMTNAGVQ**LAGG** SALCRHRPQ	*
131	Ad2	C	121	QGITHLK**IRGR** GIQLNDESV	141
	Ad5	C	121	QGITHLT**IRGR** GIQLNDESV	141
	Ad41	G	127	GPIKRLI**IRGR** GIQLNDEVV	147
	Ad12	A	128	PAIKRVL**IRGK** GIQLNDEVT	148
	Ad3	B	*	QSIKRLV**IRGR** GIQLNDESV	*
157	Ad2	C	150	GTFQ**IGGA** GRSSFTPRQAILT	170
	Ad5	C	150	GTFQ**IGGA** GRPSFTPRQAILT	170
	Ad41	G	156	GVFQ**LGGA** GRSSFTPRQAYLT	176
	Ad12	A	156	GVFQ**IGGS** GRSSFTARQAYLT	176
	Ad3	B	*	GVFQ**IAGC** GRSSFTPRQAYLT	*

Table 2: Theoretical Cut-Sites. *: Data from partial sequence, **Bold type**: Protease recognition site, : Protease cleavage site.

The N-terminal fragment of pVIII is the most highly conserved fragment (76% of Ad2 amino acids conserved) among the adenovirus serotypes. This may suggest that pVIII was formed by the N-terminal fragment as indicated by experiments performed by Tremblay *et al.*, (1983), and Hannan *et al.*, (1983). However, there is a significant difference between the percentage amino acid content of VIII (claimed by Edvardsson, 1976) and the N-Terminal fragment. The C-terminal fragments of pVIII have the best correlation to the percentage amino acid content to VIII thus, indicating the C-terminal of pVIII forms protein VIII. However, there is no evidence to support this theory, and papers which included part of the C-terminal fragment in VIII, i.e. Van Oostrum and

Burnett (1985) were written prior to the identification of the protease recognition site. The available evidence agrees with the hypothesis that the N-terminal fragment forms the mature protein VIII after proteolytic cleavage of pVIII.

A temperature sensitive mutant adenovirus (ts1) is deficient in protease activity when grown at restrictive temperatures (Weber, 1976). The precursor proteins which are not cleaved (pVI, pVII, pVIII, IIIa, pTP and 11 kDa protein) are present as the structural proteins in ts1 as a result. In the temperature sensitive adenovirus ts1 pVIII is located either totally or partially externally as tyrosine residues in pVIII could be iodinated (Hannan *et al.*, 1983). Protein VIII is located internally (Everitt *et al.*, 1974 ; Van Oostrum and Burnett, 1985; Stewart *et al.*, 1993) and although VIII has more tyrosine residues than VI it was iodinated less than VI, which is also located internally (Hannan *et al* , 1983). This indicates that either VIII is less exposed than VI or that the tyrosine residues in VIII are not as accessible as those in VI (Hannan *et al.*, 1983).

An adenovirus 5 temperature sensitive (Ad5ts) mutant was found to possess a mutation at an EcoRI site. The location of the site was identified as being located in the pVIII gene (Liu *et al.*, 1985). The location of the insertion mutation at 27376 and resulting temperature sensitivity of the virus indicates that pVIII and VIII play an important role in the capsid's structural stability. Analysis of the gene products by SDS-PAGE showed that the Ad5ts pVIII did not migrate as far down the gel as wild-type pVIII, and Ad5ts VIII migrated the same distance as wild-type VIII (Liu *et al.*, 1985). This would indicate either a conformational difference in Ad5ts pVIII or the insertion of one or more amino acid residues into Ad5ts pVIII, which is removed after cleavage. Analysis of the DNA sequence shows an insertion of 9 bases in the N-terminal segment of pVIII which is within the section of pVIII thought to form VIII (Liu *et al.*, 1985). Therefore an insertion of 3 amino acids into the pVIII gene would probably result in a change in conformation of

itself and the mature protein VIII. This would result in a reduction or loss of the stabilising properties of VIII and the formation of temperature sensitive viral particles. However, as the mobility of Ad5ts VIII appears to be the same as wild-type VIII it may indicate that VIII is formed from the C-Terminal of pVIII. Cleavage of pVIII at 111 a.a. would result in the formation of a 13.2 kDa protein which is approximately the same weight as the apparent molecular weight of VIII (15 kDa). Should the C-Terminal of pVIII form VIII it would indicate that pVIII has a greater role in the stability of the capsid. This is because wild-type VIII does not correct the capsid instability that the mutated pVIII has caused.

1.10 *E.coli* Strains

1.10.1 The XL1-Blue Cells

E.coli strain XL1-Blue, supplied by Stratagene, provides a host for optimal propagation of both plasmid and lambda phage vectors. The XL1-Blue strain allows blue/white colour screening and single-strand rescue of phagemid DNA. The phenotype also allows preparation of high-quality plasmid DNA, suitable for DNA sequencing. XL1-Blue cells carry a gene coding for resistance to the antibiotic tetracyclin, eliminating the need to select on minimal media plates. The strain has a maximum transformation efficiency of 5 x 10^9 transformants/mg DNA and is therefore is suitable for use in cloning experiments.

1.10.2 BL21(DE3) Cells

E.coli BL21(DE3) cells are designed for high-level protein expression using T7 RNA polymerase-based expression. The BL21(DE3) cells is an all-purpose strain for high-level protein expression and easy induction. In addition BL21 cells can be used for non-

T7 RNA polymerase protein expression systems. The BL21(DE3) cells are lysogenic for a fragment of the phage DE3 which has been inserted into the *E.coli* chromosome. This fragment contains the *lac*I gene, the *lac* UV5 promoter, the start of *lacZ* (beta galactosidase) and the T7 RNA polymerase gene. The *lac* UV5 promoter is responsible for driving the expression of T7 RNA polymerase and is inducible by IPTG (isopropyl beta D thiogalactopyranoside). BL21(DE3) and its derivatives are deficient in the *omp*T and *lon* proteases, which may interfere with isolation of intact recombinant proteins. The BL21(DE3) strain of *E.coli* is recommended for use with established expression constructs that have been cloned and sequenced.

1.10.3 BL21(DE3)pLysE

The *E.coli* BL21(DE3)pLysE strain regulates expression using the DE3 lysogen. The DE3 lysogen encodes T7 RNA polymerase under the control of the IPTG-inducible *lac*UV5 promoter. T7 RNA polymerase drives high-level transcription from the T7 promoter. The BL21(DE3)pLysE strain constitutively express T7 lysozyme, which reduces basal expression of recombinant genes by inhibiting basal levels of T7 RNA polymerase. The BL21(DE3)pLysE strain is therefore recommended when the recombinant protein to be expressed is potentially toxic.

1.10.4 ER2508

E.coli strain ER2508, supplied by New England Biolabs, provides a host for optimal expression and detection of maltose binding protein fusions due to the *mal*B deletion which elimates expression of maltose binding protein. The ER2508 strain is deficient in the lon protease which degrades foreign proteins in the cell cytoplasm. ER2508 cells

carry the genes coding for resistance to the antibiotics tetracyclin and kanamycin, eliminating the need to select on minimal media plates.

1.10.5 JM105

E.coli strain JM105, supplied by Pharmacia, provides a host for expression of recombinant proteins with the P*lac* promoter. The JM105 strain allows blue/white colour screening as it is capable of complementing the *lac* a fragment expressed by selected vectors i.e. pUC19. JM105 cells carry a gene coding for resistance to the antibiotic streptomycin, eliminating the need to select on minimal media plates.

1.11 *E.coli* Expression Vectors

1.11.1 pET Expression Systems

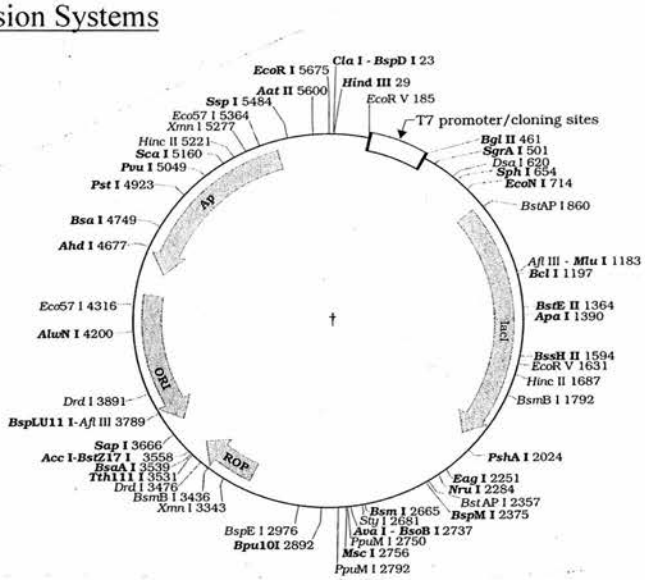


Figure 12. Map of the expression vector pET11c

The pET *E.coli* expression system, see figure 12, is a widely used *in vivo* bacterial expression system due to the strong selectivity of the bacteriophage T7 RNA polymerase

for its cognate promoter sequences, the high level of activity of the polymerase and the high efficiency of translation mediated by the T7 gene 10 translation initiation signals (Studier *et al.*, 1990). *E.coli* cells which have been transformed by pET vectors can selected for using the ampicillin resistance gene contained within the plasmid.

In the pET system, protein-coding sequence of interest is cloned downstream of the T7 promoter and gene 10 ribosome binding site, and then transformed into *E.coli*. Protein expression is achieved by IPTG induction of a chromosomally integrated cassette in which the T7 RNA polymerase is expressed from the lacUV5 promoter ,BL21(DE3) cells. Due to the specificity of the T7 promoter, basal expression of cloned target genes is extremely low in strains lacking a source of T7 RNA polymerase. However, upon induction, the highly active polymerase produces an abundant number of transcripts. This phenomenon together with high-efficiency translation result in such high expression levels that, after only a few hours, the target protein may constitute the majority of the cellular protein.

To achieve stringent control of expression, the *lac* operator has been inserted between the T7 promoter and translation initiation sequences in the pET-11 vectors. This results in repression when IPTG is absent. Addition of IPTG causes de-repression of the T7 promoter and induction of T7 polymerase from the *lac*UV5 promoter in the DE3-containing strains. The *lac*Iq gene is included in the pET-11 plasmids to provide adequate levels of *lac*I repressor protein to shut off T7 polymerase gene expression as well as T7 promoter transcription (Studier and Moffatt, 1986).

1.11.2 pRSET Expression System

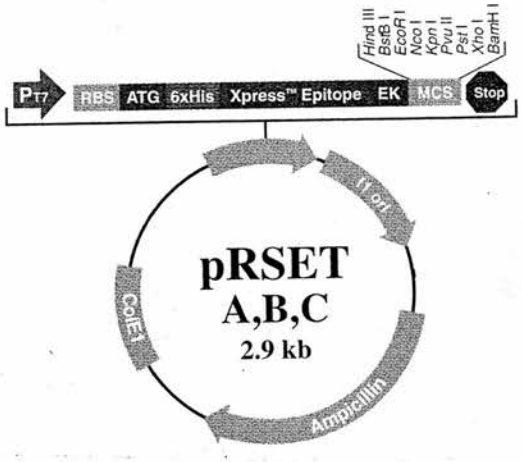


Figure 13. Map of the expression vector pRSETA

The pRSET vectors, see figure 13, are pUC-derived expression vectors designed for high level protein expression and purification from cloned genes in *E.coli* . High levels of expression of DNA sequences cloned into the pRSET vectors are made possible by the presence of the T7 promoter. In addition, DNA inserts are positioned downstream and in-frame with a sequence that encodes an N-terminal fusion peptide. This sequence includes an ATG translation initiation codon, a series of six histidine residues that function as a metal ion binding domain in the translated protein, a transcript stabilising sequence from gene 10 of phage T7, and the enterokinase cleavage recognition sequence.

The metal binding domain of the fusion peptide allows simple purification of recombinant proteins by immobilised metal affinity chromatography, which is made possible with ProBond resin (Invitrogen). The enterokinase cleavage recognition site in the fusion peptide located between the metal binding domain and the recombinant protein allows for subsequent removal of this N-terminal fusion peptide from the purified recombinant protein.

Recombinant pRSET vector is transformed into XL1-Blue *E.coli* cells for propagation and maintenance. To express the recombinant protein from pRSET the construct must be transformed into an *E.coli* strain which contains a lambda lysogen expressing the T7 polymerase under control of the *lac*UV5 promoter. Expression of the T7 polymerase is necessary for transcription of recombinant protein cloned into pRSET. In the presence of IPTG, the cells are induced to express high levels of T7 RNA polymerase and expression of the T7 promoter-driven recombinant protein from the pRSET vector is possible. High levels of recombinant protein can be produced within a short period of time following addition of IPTG to the *E.coli* growth medium.

1.11.3 pMAL Expression System

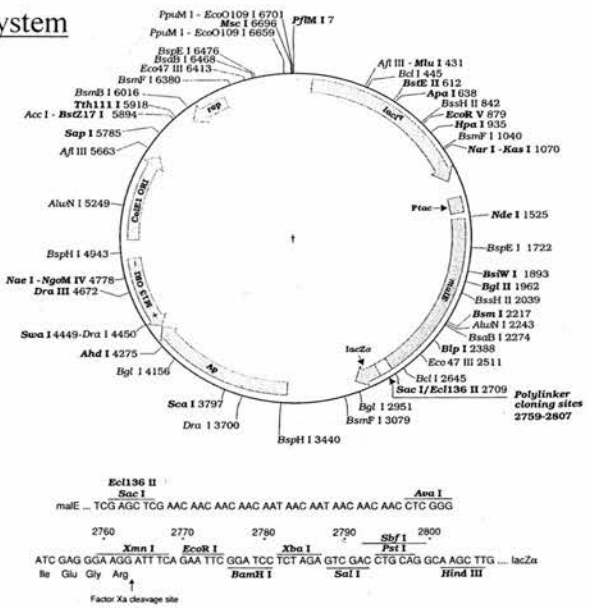


Figure 14. Map of the expression vector pMAL

The pMAL *E.coli* expression vectors, see figure 14, are designed to create fusions between a cloned gene and the *E.coli malE* gene, which codes for maltose binding protein (MBP). The MBP fusion can be expressed and purified, taking advantage of the properties of MBP. The two pMAL vectors pMALc2 is identical to the pMALp2 vector except for a deletion of the *malE* signal sequence (bases 1531-1605). When present the

signal peptide of pre-MBP directs fusion proteins to the periplasm. For fusion proteins that can be successfully transported, this allows folding and disulphide bond formation to take place in the periplasm of *E.coli* as well as allowing purification of the protein from the periplasm (Guan *et al.*, 1987).

The vectors contain the inducible Ptac promoter, positioned to transcribe a *malE-lacZα* gene fusion. The *lac*Iq gene encodes the *lac* repressor, which turns off transcription from Ptac until IPTG is added. The polylinker provides restriction endonuclease sites to insert the gene of interest, fusing it to the malE gene. Insertion inactivates the ß-galactosidase α-fragment activity of the *malE-lacZα* fusion, which results in a blue to white colour change on Xgal plates when construction is transformed into an α-complementing host i.e. XL1-Blue. A portion of the *rrnB* operon containing two terminators, derived from the vector pKK233-2, prevents transcription originating from Ptac from interfering with plasmid functions. The gene coding for ß-lactamase and the origin of replication are from pBR322. The M13 origin is derived from pZ150.

1.11.4 The pGEX Expression System

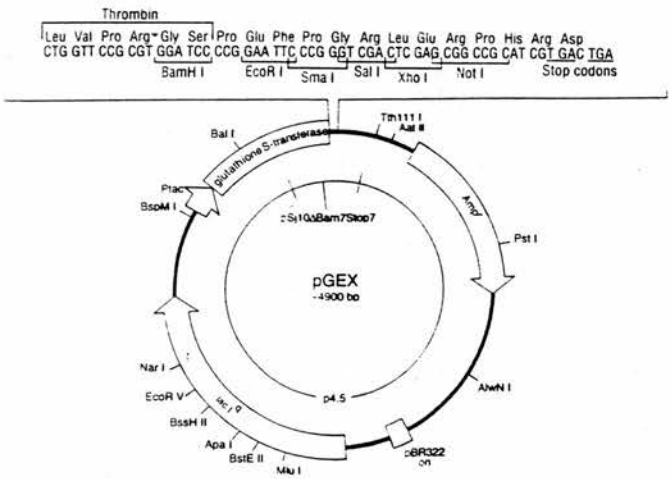


Figure 15. Map of the expression vector pGEX

The pGEX vectors, see figure 15, are constructed so that they direct the synthesis of foreign proteins in *E.coli* as fusions with the C-terminus of Sj26, a 26 kDa glutathione S-transferase (GST). The pGEX-4T1 vector has been engineered so that the coding sequence for a thrombin cleavage site (LVPR-GS) is included, in frame, at the 3' end of the GST gene. The expression of the fusion proteins is under the control of the *lacZ* promoter and since the plasmids also encode for the *lac* repressor (*lacI*), the fusion proteins will only be expressed in the presence of IPTG. Thus, synthesis of a foreign protein, that may be toxic to *E.coli* , can be switched on by the addition of IPTG after the cells have reached the required level of growth. The vectors include an ampicillin resistance gene, enabling the positive selection of transformed cells. Attachment to the carrier molecule GST means that proteins can be readily purified by affinity chromatography on a glutathione Sepharose column and subsequently cleaved from the carrier using thrombin. Smith and Johnson (1988) report that although insolubility is a frequent characteristic of foreign proteins expressed in *E.coli* the majority of GST fusion proteins expressed are at least partially soluble. The origin of replication in pGEX4T1 is derived from the vector pBR322.

1.11.5 pUC18 Expression System

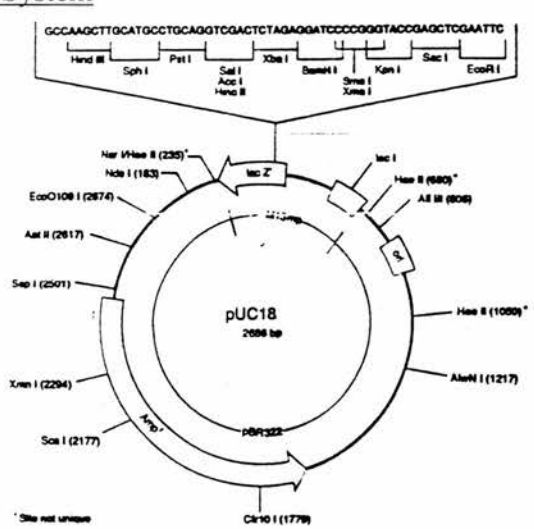


Figure 16. Map of the expression vector pUC19

pUC18 is a small, high copy number, *E.coli* plasmid, 2686 bp in length, see figure 16. It is identical to the pUC19 plasmid except that the multiple cloning sites (MCS) are arranged in opposite orientations. pUC18/19 plasmids contain the pMB1 replicon *rep* responsible for the replication of plasmid from plasmid pBR322. The high copy number of pUC plasmids is a result of the lack of the *rop* gene and a single point mutation in *rep* of pMB1 (Yanisch-Perron *et al.*, 1985). Ampicillin resistance is conferred by the *bla* gene, coding for beta-lactamase and is taken from plasmid pBR322. The region of *E.coli* operon *lac* containing CAP protein binding site, promoter Plac, *lac* repressor binding site and part of the *lacZ* gene encoding the N-terminal fragment of beta-galactosidase is taken from M13mp18/19. This fragment, whose synthesis can be induced by IPTG, is capable of α-complementation when the construct is transformed into an appropriate host i.e. JM105. In the presence of IPTG, bacteria synthesise both fragments of the enzyme and form blue colonies on media with X-gal. Insertion of DNA into the MCS located within the *lacZ* gene (codons 6-7 of *lacZ* are replaced by MCS) inactivates the N-terminal fragment of beta-galactosidase and abolishes alpha-complementation. Bacteria carrying recombinant plasmids therefore give rise to white colonies.

1.12 Immunocytochemistry

Immunocytochemistry was developed by Coons *et al.*, 1941, who bound a fluorescent marker to an antibody and used the complex to identify antigens in tissue culture sections. The basic principle of an antibody linked to fluorescent marker has not changed, but been developed for application to a wide range of histological and electron microscope techniques. The use of fluorochrome as labelling agent has a major advantage over other forms of labelling in that a high level of resolution is possible. However, it does have disadvantages which include quenching and low sensitivity. These disadvantages can be overcome by using anti-quenching agents and indirect labelling to increase the sensitivity.

Fluorochrome-labelled reagents require specially designed microscopes to be viewed and as a result of the low levels of fluorescence produced the microscope must be able to transmit the exciting radiation through the objective lens (epifluorescence). The microscope contains two filters, one to ensure that the specimen is irradiated only by light at the correct wavelength for excitation and the second set in the viewing light path which only transmit light of the wavelength emitted by the fluorochrome. This produces a black background and a high resolution image. Certain fluorochromes have emission spectra which do not overlap and therefore can be observed in the same sample to study the relationship between two different antigens. The most commonly used fluorochromes are fluorescein, which has a green colour, and rhodamine which has a red colour. The fluorochromes can be conjugated to anti-immunoglobulin antibodies and used in indirect labelling, alternatively they can be conjugated to the probe but, this reduces the sensitivity of the technique.

Immunofluorescent techniques can be divided into four steps : cell preparation, fixation, antibody binding and detection. Adherent cells can be grown on microscope slides, coverslips or optically suitable plastic, whereas suspension cells can be centrifuged onto glass slides and bound to the slides using chemical cross-linkers. With yeast the cell wall has to be removed enzymatically, and the cells attached to a solid substrate by a chemical cross-linker. The second step is usually to fix and permeabilise the cells to ensure free access of the antibody although, permeabilisation can be omitted when examining cell-surface antigens. Unbound antibody is removed by washing and the bound antibody either detected directly, if the primary antibody is labelled, or indirectly using a labelled secondary antibody.

There are three major factors that determine how well an antigen is detected. They are the local antigen concentration, the types of fixative and antibody used, and the type of detection method. The most important of these three factors is the local antigen population as diffuse antigens, even when present at high concentrations, are difficult to distinguish from background signals. Consequently the antigens that are easiest to detect have a characteristic structure and present a large number of identical antibody binding sites in a small local environment.

The next important factor that determines how easily an antigen is detected is the type of fixative and the characteristics of the antibodies being used. Perfect fixation would immobilise the antigens, while retaining authentic cellular and subcellular architecture whilst allowing unhindered access of antibodies to all cell and subcellular compartments. Unfortunately no fixation method is capable of providing this as many epitopes are masked or altered by certain fixatives. Some antibodies are unable to recognise the altered epitopes or the epitopes are hidden within subcellular structures.

The final major factor that affects the sensitivity is the method used for antibody detection. Enzyme-labelled antibodies can identify small amounts of antigen but, unlike fluorochrome-labelled antibodies, these cannot be used to gain high resolution.

1.12.1 Antibody Selection

There are three types of antibody preparations that can be used in immunofluorescence. these are polyclonal, monoclonal and pooled monoclonal antibodies. Polyclonal antiserum, raised against a protein, will contain antibodies against various different epitopes on the antigen. Despite the fact that steric hindrance will prevent large numbers of antibodies binding to the same antigen molecule, several antibodies may be capable of

binding to the same antigen resulting in a strong signal. However, as well as the specific antibodies the antiserum will contain a high concentration of antbodies against unknown antigens. Therefore, when using polyclonal antiserum the serum should be preadsorbed and titrated so that the antiserum is used at a concentration which does not reveal any non-specific binding. An alternative to this is to remove the non-specific antibodies by immunoaffinity purification where the antiserum is passed through a column of immobilised antigen. As polyclonal antiserum contain antibodies against a variety of epitopes there is a lower probability of the epitopes being masked or denatured and therefore are a good source of antibodies for observing paraffin sections or paraformaldehyde-fixed samples.

Monoclonal antibodies usually work extremely well as their purity and high specificity yield low backgrounds. Also, they tend to work well in tissue that has been fixed in organic solvents or paraformaldehyde. However, the two problems which can be encountered with monoclonal antibodies are that they occasionally do not work well, presumably this is due to epitope masking, or they may cross-react with cellular components. Usually, it is found that the cross-reaction is a result of binding to cytoskeletal filaments due to the presence of a common epitope.

Pooled monoclonal antibodies can be useful providing the different antibodies can combine with the antigen noncompetitively. This would result in increased signal strength whilst maintaining a low background. However, with pooled monoclonal antibodies each one must be tested individually and with each other in order to establish the optimal concentations and combinations of antibodies.

1.12.2 Fixation and Permeabilisation

Fixation protocols are designed to prevent antigen leakage, permeabilise the cell to allow access of the antibody, retain sufficient structural conformation of the antigen that the epitopes are still recognisable and to maintain the cell structure. The choice of fixative is dependent upon the nature of the antigen being examined and the properties of the antibody being used. Fixatives fall generally into two classes, organic solvents and cross-linking reagents. Organic solvents such as alcohols and acetone remove lipids and dehydrate the cells, precipitating the proteins on the cellular architecture. Cross-linking reagents form intermolecular bridges, normally through free amino groups, thus creating a network of linked antigens.

Fixation with cross-linking agents, e.g. gluteraldehyde and paraformaldehyde, preserves cell structure better than organic solvents but may reduce the antigenicity of some cell components. However, hidden epitopes can be exposed by incubating the sample in proteases i.e. trypsin. The other disadvantage of using cross-linking agents is that after fixing the cells still require permeabilising with an organic solvent or nonionic detergent to allow antibodies access to the antigen. As the main reason for fixing with cross-linking reagents is to preserve cell structure, cells are permeabilised with nonionic detergents such as Triton X-100.

1.12.3 Detection of Antigen

After fixing and permeabilising the cells the antibodies are added to the cells. Detection of the antigen can be direct, using labelled antibodies, or indirect, using a labelled secondary antibody. The detection agents used can be labelled with fluorochromes, enzymes, gold or iodine. Fluorochromes provide a high degree of resolution and allow the

possibility of double labelling however, signal is lost over time. Enzyme labels are highly sensitive but provide poor resolution and double staining is difficult. Iodine is sensitive and produces a low background but, it produces poor resolution and the procedure is time consuming. Gold labels produce high resolution images and is compatible with electron microscopy but needs silver enhancement for high sensitivity.

Direct labelling of the antibody, see figure 17, results in a lower background but has the main disadvantage of producing a weaker signal than indirect labelling. Indirect detection, see figure 18, is usually carried out using anti-immunoglobulin antibodies that have been tagged although, proteins A and G can be used as well. However, indirect detection with a secondary antibody requires the serum to be preadsorbed and titrated.

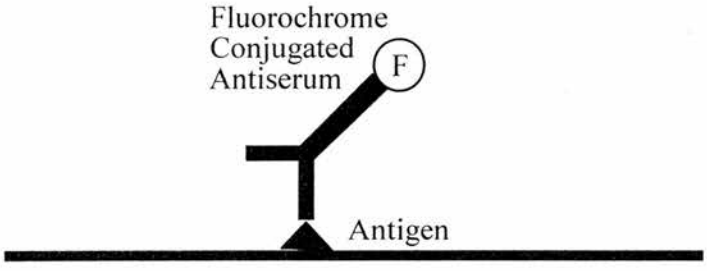


Figure 17. Direct immunolabelling method

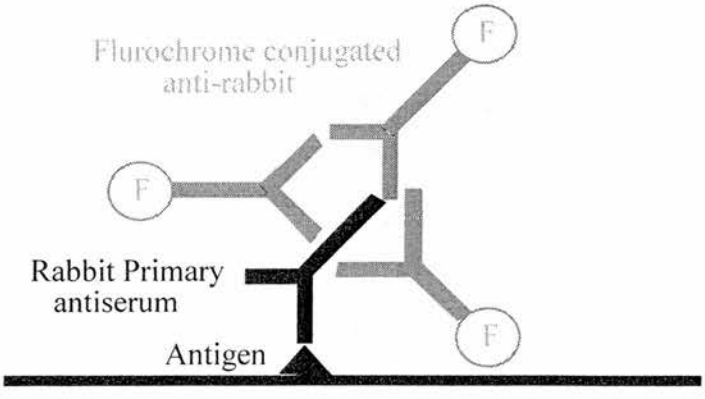


Figure 18. Indirect immunolabelling method

1.13 Aims of Investigation

The aim of this project is to investigate the relationship between the adenovirus precursor protein pVIII and the mature protein VIII. Specific antibodies were raised to the terminal regions of pVIII to identify the fusion protein and pVIII. It is hoped that the gene coding for pVIII in adenovirus serotype 2 can be cloned into an *E.coli* expression vector. After cloning the vector will be transformed into the BL21(DE3) cell line and expression of the fusion protein should occur. Once purified the fusion protein will be digested *in vitro* by the adenovirus protease to determine which of the theoretical cleavage sites are cleaved and which part of pVIII forms VIII. It is also hoped that through use of the terminal antibodies it will be possible to confirm these results using VIII from the adenovirus.

After the generation of a vector containing the adenovirus gene coding for pVIII it is hoped that N-terminal and C-terminal deletion mutants can be generated by site-specific mutation. These deletion mutants would be expressed in *E.coli* and polypeptides produces purified and used to generate antisera in rabbits and sheep. It is hoped that by the use of various different antisera raised against adenovirus proteins, to identify where selected antigens accumulated in the cell. Also it was hoped that it would be possible to identify proteins that may interacted with each other.

It is hoped that it will be possible to determine the nucleotide sequence of the EDS 23k Da protease and to ascertain whether the EDS protease is capable of digesting a structural protein from another adenovirus serotype. It is hoped that the gene coding for the EDS protease can be sequenced using the primer walking technique and the recombinant protease expressed in *E.coli* cells. Once purified it is hoped that the EDS protease can be used to digest the Ad2 structural protein pVIII and the cleavage site of the EDS protease confirmed.

2. Materials and Methods

2.1 Materials

All chemicals used were of highest available grade and were supplied by either Sigma or BDH unless otherwise stated, except for Tris which was supplied by Boehringer Mannheim and was of highest available grade.

2.2. Molecular Biology Methods

2.2.1 Preparation of Agar Plates

L-agar was prepared by adding 15 g of Bacto-agar (Difco) to 1 litre of LB and solution autoclaved. The L-agar was melted in a microwave oven immediately prior to use and cooled to 55°C. The antibiotic(s) required were added at the desired concentration (see table 3), mixed and 20 ml was poured into each 90 mm Petri dish. Any bubbles in the agar were removed by flaming with a Bunsen burner. The plates were allowed to set and were stored for up to 1 week at 4°C.

2.2.2 Preparation of Competent Cells

The strain of *E.coli* normally made competent was BL21(DE3) (Pharmacia). Other Strains of *E.coli* used were XL1-Blue (Stratagene), BL21(DE3)pLysS (Novagen), BL21(DE3)pLysE (Novagen) , JM109 (Pharmacia) , ER2058 (NEB). The selected bacterial strain was made competent, as in Maniatis *et al.* (1982), by growing overnight in 1 ml of L-broth (plus antibiotic) at 37°C. This was used to inoculate 50 ml of L-Broth which was incubated at 37°C until the culture had an absorbance between 0.7 and 0.8 units at 600 nm. The culture was centrifuged for 10 minutes at 3000 rpm and the pellet saved. The pellet was resuspended in 10 ml of filter sterilised

(0.2 μm filter purchased from Gelman Sciences) 0.1 M magnesium chloride, centrifuged as before and resuspended in 4 ml of filter sterilised 0.1 M calcium chloride.

2.2.3 Transformation of Competent Cells with plasmid DNA

The selected strain of *E.coli* was transformed as in Maniatis *et al.* (1982) by adding 100 ng of vector DNA to 200 μl of competent cells, which had been left on ice for 15 minutes. The cells and DNA were vortexed for 5 seconds and left on ice for at least 30 minutes. The cells were heat shocked by incubating at 42°C for 90 seconds before placing on ice for 5 minutes. A sample of 100 μl was spread onto an agar plate (containing ampicillin) and grown overnight at 37°C. Typically, control transformations were set up (a) no DNA added, (b) uncut plasmid, and (c) cut, ligated plasmid (no insert).

2.2.4 Polymerase Chain Reaction

The polymerase chain reaction (PCR) was performed as Maniatis *et al.* (1982). Oligonucleotide primers for the PCR were designed using the computer programme Amplify 1.2. The oligonucleotides were made by Ian Armitt and dissolved in 250 μl of filtered Rathburn HPLC water. In order to carry out the PCR oligonucleotides were added to a concentration of 100 μM to 350 ng of adenovirus 2 DNA, 10 μl of PCR buffer (NEB), 10 μl of 0.5M dNTP's (Pharmacia), 1μl of Vent polymerase (NEB) and the volume made up to 100 μl with water. This was overlaid with 30 μl of paraffin oil. The DNA fragment was amplified by heating the sample at 94°C for 1.5 minutes (melting), cooled to 60°C for 1.5 minutes (annealing), heated to 72°C for 2 minutes (elongation). This was repeated 30 times and the sample heated at 72°C for 7 minutes after the final cycle.

2.2.5 Agarose Gel Electrophoresis

DNA fragments were separated by agarose gel electrophoresis in a Pharmacia GNA-100 submersible rig according to manufacturers instructions. The buffer system used 60 mM Tris/acetic acid, 3 mM EDTA, pH8.

Agarose gels were prepared by adding the required quantity of agarose to 70 ml water and heating in a microwave until the agarose had melted. The agarose was cooled to 50°C by the addition of 20 ml water and 10 ml of 10x TAE (0.2 M Tris, 5 μM EDTA and 0.57% (v/v) acetic acid), poured into a plate and left to set for 45 minutes. DNA samples were prepared by typically adding 2.5 μl of 5x gel loading buffer (0.5mg bromophenol blue, 4 ml glycerol, 5 ml 10x TAE and 1 ml of 0.5 M EDTA) to 10 ml of sample. A 1K base pair ladder purchased from Pharmacia was used as the size marker on gels. Gels were run at 100 volts until the dye front was half-way down the gel, and developed in ethidium bromide (0.5 mg/ml) for 15 minutes and observed under ultra-violet light.

2.2.6 Extraction of DNA from Agarose Gel

Double stranded DNA fragments were separated and purified from agarose gels using the QIAquick Agarose Gel Extraction Kit (QIAGEN) according to the manufacturers instruction. DNA was separated on the appropriate % of agarose gel as described in method 2.2.5. The gels were stained with ethidium bromide and viewed for the minimum time in UV light and the desired DNA fragment excised using a scalpel and the gel slice weighed. The weight of the gel was assumed to be equal to its volume. The gel slice was placed in a 1.5 ml Eppendorf and three volumes of buffer QX1 was added to 1 volume of gel. The agarose gel was dissolved by heating at 50°C for 10 minutes and the DNA was precipitated by the addition of 1 gel volume of isopropanol. The QIAquick spin column was centrifuged at 13000 rpm in a

microcentrifuge for 1 minute and washed with 0.5 ml of buffer QX1, centrifuging as before. The QIAquick column was washed with 0.75 ml of buffer PE and centrifuged as before. The column was centrifuged a second time to ensure complete removal of buffer PE. Elution of DNA was carried out by the addition of 20 µl of HPLC water to the column and centrifuging for 1 minute at 13000 rpm.

2.2.7 Restriction Enzyme Digests

All restriction enzymes were purchased from Promega and were supplied with an appropriate 10x buffer and 10x MULTI-CORE buffer. A typical reaction was for 3 hours at 37°C and comprised 500 ng of DNA, 1 µl of 10x buffer and 5-20 units of restriction endonuclease with the final volume being made up to 10 µl with Rathburn HPLC water.

2.2.8 DNA Polymerase I (Klenow) Fragment Reactions

The Klenow fragment of *E.coli* DNA polymerase I was used to fill in recessed 3' ends of double stranded DNA. The Klenow fragment was purchased from Promega and was supplied with an appropriate 10x Klenow buffer. A solution was made 1 mM with respect to each of the four deoxynucleotide triphosphates (dATP, dTTP, dGTP and dCTP) and stored at -20°C. A typical reaction was composed of 1 unit of Klenow polymerase per 2 µg of DNA, 2 µl of 10x Klenow buffer and 2 µl of dNTP's in a final volume of 20 µl. Incubations were for 30 minutes at room temperature and the reaction was stopped by the addition of 1 µl of 0.5M EDTA.

2.2.9 Phosphatase Treatment of DNA

Plasmid which had been linearised using one restriction endonuclease was treated with calf intestinal alkaline phosphatase (CIAP) to prevent the religation of the vector

by removing the phosphate group from both 5' termini. CIAP was obtained from Promega and was supplied with an appropriate 10x buffer. Typically 500 ng of linearised plasmid, 2 µl of 10x CIAP and 2 units of CIAP were added together in a final volume of 20 µl. The reaction was stopped by the addition of 1 µl of 0.5 M EDTA and heating for 20 minutes at 65°C.

2.2.10 Ligation Reaction

Insertion of genes into linearised plasmid DNA was carried out using T4 DNA ligase. The ligase was purchased from Promega and was supplied with a 10x ligase buffer. A typical ligation reaction comprised 2 µl linearised plasmid DNA and 14 µl of DNA insert which was incubated at 45°C for 5 minutes befor placing on ice. After 5 minutes 2 µl of T4 DNA ligase and 2 µl of 10x ligase buffer were added to the reaction mixture and incubated at room temperature for 4 hours for sticky end ligations and overnight at 16°C for mixed and blunt end ligations.

2.2.11 Mini Preparation of Plasmid DNA

Mini Preps were performed using a QIAprep Spin Miniprep kit supplied by Qiagen. Individual colonies were picked from L-agar/Amp plates, containing ampicillin at 50 µg/ml, using a sterile toothpick and grown overnight at 37°C in 5 ml of L-broth containing ampicillin at 50 µg/ml. Typically 1 ml from each overnight culture was centrifuged for 1 minute at 13,000 rpm in a microcentrifuge and the pellet resuspended in 0.3 ml of Qiagen buffer P1. An equal volume of Qiagen buffer P2 (lysis buffer) was added to the solution and incubated at room temperature. After 5 minutes 0.3 ml of pre-chilled Qiagen buffer P3 (neutralisation buffer) was added and left on ice for 10 minutes before being centrifuged for 15 minutes at 13000 rpm and the pellet discarded.

The supernatant was transferred to a clean Eppendorf and centrifuged at 13000 rpm for 5 minutes in order to pellet any remaining cell debris. The supernatant was transferred to a fresh Eppendorf and the DNA precipitated by the addition of 0.8 volumes of room temperature isopropanol. This was spun down for 15 minutes. The DNA pellet was washed twice in 70% (v/v) ethanol, air dried for 5 minutes and dissolved in 10 µl Rathburn HPLC water.

The DNA was analysed by restriction endonuclease digestion and agarose gel electrophoresis. Typically 7 µl of DNA was used for each restriction digest.

2.2.12 Plasmid Preparation

The preparation of plasmid DNA was performed using a Maxi Prep Kit (QIAGEN) according to manufacturers instructions. Typically the required plasmid was used to transform the slow growing *E.coli* strain XL1-Blue (Stratagene) cells for the plasmid preparation. A single colony was picked from an L-agar/Amp plate with a sterile toothpick and grown up in 2 ml of L-Broth containing ampicillin for 6 hours at 37°C. The 2 ml culture was used to inoculate 250 ml of L-Broth containing ampicillin and grown at 37°C overnight. The cells were harvested by centrifugation for 15 minutes at 6000 rpm in a Beckman JA-14 rotor, and the supernatant discarded. The pellet was resuspended in 10 ml of Qiagen buffer P1 (50 mM Tris-HCl pH 8, 10 mM EDTA with RNAase 100 µg/ml), and incubated at room temperature in the presence of 10 ml Qiagen buffer P2 (200 mM NaOH and 1% (w/v) SDS). After 5 minutes Qiagen buffer P3 (25% (v/v) potassium acetate, pH 4.8) was added to the solution, and incubated on ice for 20 minutes. The sample was centrifuged at 15,000 rpm in a Beckman JA-20 rotor for 30 minutes, and the supernatant saved. The supernatant was filtered using Whatman No.1 filter paper and loaded onto a Qiagen-tip 500 (Q-500) which had been equilibrated with 10 ml of buffer QBT. The Q-500 was washed twice with 30 ml of buffer QC and the DNA eluted with 15 ml of buffer QF. DNA

was precipitated with 0.7 volumes of room temperature isopropanol and centrifuged at 15000 rpm for 30 minutes in a Beckman JA-20 rotor. The pellet was washed twice with 70% (v/v) ethanol, air dried for 5 minutes and dissolved in 250 µl of HPLC water. Analysis of the DNA was carried by digestion with restriction endonucleases and agarose gel electrophoresis. The concentration of the plasmid was determined by measuring its absorbance at 260 nm.

2.2.13 DNA Concentration

The concentrations of DNA samples were determined by measuring the absorbance of the sample or an appropriate dilution, against a control of HPLC water, at 260 nm on a Cecil spectrophotometer and applying the formula:

(a) For double stranded DNA 1 absorbance unit indicates a concentration of 50 ng/µl.
(b) For oligonucleotides 1 absorbance unit indicates a concentration of 20 ng/µl.

The presence of any protein contaminants was determined by measuring the absorbance at 260 nm and 280 nm. The ratio between the readings at 260 nm and 280 nm should be between 1.8 and 2.0 for pure DNA solutions. Values less than 1.8 indicate that there are protein contaminants in the preparation and values greater than 2.0 indicate that there is RNA contamination of the sample.

2.2.14 DNA Sequencing

(a) Plasmid Preparation

Plasmids were prepared using a QIAGEN Maxi Prep Kit, see method 2.2.12 and the absorbance at 260 and 280 nm measured. If the ratio between the 260 nm and 280 nm readings was less than 1.8 the plasmid DNA was cleaned using a QIAGEN Mini Prep

Kit. The plasmid DNA was cleaned by adding 10 volumes of QIAGEN buffer PN to 20 μg of plasmid DNA. The solution was loaded onto a QIAquick column and centrifuged for one minute at 13000 rpm in a microfuge. Salt was washed off the column by adding 0.75 ml of QIAGEN buffer PE to the column and the column centrifuged twice as before. The DNA was eluted with 20 μl of HPLC water and the absorbance measured at 260 and 280 nm.

(b) PCR Product Preparation

PCR products were synthesised using method 2.2.4. A 5 μl sample was taken from the PCR reaction and analysed on an agarose gel, see method 2.2.5. If a single band of the correct length was visible after staining with ethidium bromide the PCR reaction was cleaned using a QIAquick column. The paraffin oil was removed from the PCR sample and 5 volumes of QIAGEN buffer PB added to the PCR reaction. The sample was loaded into a QIAquick column and centrifuged for 1 minute at 13,000 rpm in a microfuge. Salt was removed from the column by washing with 0.75 ml of QIAGEN buffer PE and centrifuged twice as before. The DNA was eluted by adding 20 μl of HPLC water to the column and centrifuging as before.

(c) Sequencing of DNA

DNA was sequenced on a Perkin Elmer ABI Prism 377 DNA Sequencer. Samples for sequencing were prepared in 12 μl total volume with 3.2 picomoles oligonucleotide and 500 ng plasmid DNA or 100 ng PCR product. Samples were prepared in duplicate and the DNA sequenced from both 5' and 3' ends.

2.3 Protein Purification Methods

2.3.1 Purification of Recombinant pVIII

The plasmid pSETVIII was transformed into the *E.coli* strain BL21(DE3). A single colony was picked from an L-agar/Amp plate and used to inoculate 1 ml of L-broth/Amp and grown overnight at 37°C. The overnight culture was used to inoculate 250 ml of L-broth/Amp and the absorbance of the culture at 600 nm was monitored. When the absorbance of the culture was between 0.5 and 0.6 units the expression of recombinant pVIII was induced by adding isopropyl-thiogalactoside (IPTG) to 0.4 mM. The culture was incubated at 37°C for 60 minutes and the cells harvested by centrifuging at 10,000 rpm for 5 minutes on a Beckman JA14 rotor. The pellet was resuspended in 5 ml of wash buffer (100 mM sodium bicarbonate pH 8, 0.5 M NaCl) and incubated with 100 μg/ml lysozyme for 15 minutes at 37°C. The lysed cells were freeze thawed three times in liquid nitrogen and were incubated for a further 15 minutes in the presence of 10 mM $MgSO_4$ and 100 μg/ml DNaseI. The sample was centrifuged at 3,000 rpm for 15 minutes and the pellet saved. The pellet was resuspended in 5 ml of wash buffer and 1 ml of 6 M guanidine chloride. The sample was centrifuged in a IEC Centra-3R centrifuge at 3,000 rpm for 15 minutes before loading on a ProBond nickel affinity column (supplied by Invitrogen), which had been equilibrated by washing in 4 volumes of wash buffer. Protein was washed off the column with 10 volumes of wash buffer and soluble protein eluted with 2.5 ml of elution buffer (250 mM imidazole, 100 mM sodium bicarbonate pH 8, 0.5 M NaCl). The column was washed with 10 ml wash buffer (containing 1 M guanidine chloride) and pVIII eluted with 5 volumes of elution buffer (with 1 M guanidine chloride) and collected in 0.5 ml fractions.

2.3.2 Purification of pVIII C-Terminal deletion mutant

Plasmid pSET110ΔC-T was transformed into the *E.coli* strain BL21(DE3). A single colony was picked from an L-agar/Amp plate and used to inoculate 1 ml of L-broth/Amp and grown overnight at 37°C. The overnight culture was used to inoculate 250 ml of L-broth/Amp and the absorbance of the culture at 600 nm was monitored. When the absorbance of the culture was between 0.6 and 0.7 units the expression of the pVIII C-Terminal deletion mutant was induced by adding isopropyl-thiogalactoside (IPTG) to 0.3 mM. The culture was incubated at 37°C for 60 minutes and the cells harvested by centrifuging at 10,000 rpm for 5 minutes on a Beckman JA14 rotor. The pellet was resuspended in 5 ml of wash buffer (100 mM sodium bicarbonate pH 8, 0.5 M NaCl, 10mM imidazole) and incubated with 100 μg/ml lysozyme for 15 minutes at 37°C. The lysed cells were freeze thawed three times in liquid nitrogen and were incubated for a further 15 minutes in the presence of 10 mM magnesium sulphate and 100 μg/ml DNaseI. The sample was centrifuged on a IEC Centra-3R centrifuge at 3,000 rpm for 15 minutes and the pellet saved. The pellet was discarded and the supernatant saved and loading on a ProBond nickel affinity column (supplied by Invitrogen), which had been equilibrated by washing in 4 volumes of wash buffer. Protein was washed off the column with 10 volumes of wash buffer and the pVIII C-Terminal deletion mutant eluted with 5 volumes of elution buffer (with 1 M guanidine chloride) and collected in 0.5 ml fractions.

2.3.3 Purification of Recombinant Protease

The plasmid containing the gene coding for the adenovirus type 2 protease was transformed into BL21(DE3) cells and a single colony used to inoculate 5 ml L-broth, containing ampicillin, and grown overnight at 37°C. The 5 ml starting culture was used to inoculate 250 ml of M9/Amp (19 mM NH_4Cl, 22 mM KH_2PO_4 and 42 mM Na_2HPO_4, 1 mM $MgSO_4$, 0.4 % (w/v) glucose) and the culture incubated at 37°C.

The absorbance of the culture was monitored at 600 nm and upon reaching 0.6 units the expression of the protease was induced by the addition of IPTG to 0.4 mM. After 5 hours the cells were harvested by centrifugation on a Beckman JA-14 rotor at 10000 rpm for 5 minutes and the pellet resuspended in 5 ml resuspension buffer (50 mM Tris-HCl, pH 8, 5 mM EDTA and 4% glycerol).

The resuspended cells were lysed with 100 µl of 10 mg/ml lysozyme for 15 minutes at 37°C and freeze/thawed three times in liquid nitrogen. The lysed cells were incubated for a further 15 minutes in the presence of 10 mM magnesium sulphate and 100 µg/ml DNaseI at 37°C. The sample was split into Eppendorfs and centrifuged at 13000 rpm in a microfuge for 5 minutes. The supernatant was saved and applied to a DE-Sepharose, which had been equilibrated with 50 mM Tris-HCl pH 8, on a Pharmacia GP-250 Plus FPLC. The flow rate was set at 1 ml/minute, the detection wavelength 280 nm and the full scale deflection was 2 absorbance units. The flow-through fractions that contained the protease were pooled and loaded onto a CM-Sepharose column which had been equilibrated with 50 mM Tris-HCl, pH 8. The flow rate was 1 ml/minute, the full scale deflection was set at 0.2 absorbance units and the detection wavelength 280 nm. After all the flow-through had passed through the column the protease was eluted with 50 mM Tris-HCl pH 8, 0.2M NaCl. The fractions collected were assayed for protease activity and run on a SDS PAGE gel to establish purity. Fractions which cleaved the synthetic peptide LSGGAFSW were aliquoted into 100 µl fractions and stored at -70°C.

2.3.4 Purification of MBP fusions expressed in Cytoplasm

A 1 ml overnight cultures (LB/Amp) of ER2508 cells containing a pMAL derived plasmid was used to inoculate 80 ml of rich broth (L-broth supplemented with 2 g/l glucose) which contained ampicillin at 100 µg/ml. The cells were grown at 37°C, with shaking, to an absorbance of approximately 0.6 units, and induced with 0.24 ml

of 0.1 M IPTG. After 1 hour, the cells were harvested by centrifugation. The supernatant was discarded and the cell pellet resuspended in 5 ml of column buffer (20 mM Tris-HCl pH 7.4, 20 mM NaCl, 1 mM EDTA, 10 mM ß-mercaptoethanol).

The harvested cells were frozen in liquid nitrogen and thawed three times before incubating in the presence of 100 µl lysozyme (1 mg/ml) for 15 minutes at 37°C. The viscosity of the sample was reduced by adding 100 µl DNAse (2 mg/ml) and 100 µl of 0.1 M MgSO4 were to the solution and incubating at 37°C for 15 minutes. After centrifugation at 10 000 rpm for 20 minutes on a JA-17 rotor the sample was separated into soluble and insoluble material. The supernatant, soluble material, was decanted and stored on ice before loading onto an amylose affinity column.

2.3.5 Purification of MBP fusions expressed in the cell periplasm

A 1 ml overnight cultures (LB/Amp) of ER2508 cells containing a pMAL derived plasmid was used to inoculate 80 ml of rich broth (L-broth supplemented with 2 g/l glucose) which contained ampicillin at 100 µg/ml. The cells were grown at 37°C, with shaking, to an absorbance of approximately 0.6 units, before expression of the MBP fusion was induced by the addition of 0.24 ml of 0.1 M IPTG. After 1 hour, the cells were harvested by centrifugation at 5000 rpm with a Beckman JA-14 rotor. The supernatant was discarded and the cell pellet saved.

The cell pellet was resuspended in 10 ml of 20%(w/v) sucrose, 30 mM Tris-HCl pH 8, 1 mM EDTA and incubated at room temperature with stirring. After 10 minutes the sample was centrifuged at 8000 rpm on a JA-17 Beckman rotor for 10 minutes and the supernatant discarded. The pellet was resuspended in 10 ml of ice cold 5 mM magnesium sulphate and incubated in an ice water bath, with stirring, for 10 minutes. In order to obtain the cold osmotic shock fluid the sample was centrifuged for 10 minutes at 8000 rpm on a JA-17 rotor and the supernatant retained. The supernatant

is the cold osmotic shock fluid which contains the proteins from the cell periplasm. The cold osmotic shock fluid was decanted and stored on ice before loading onto an amylose affinity column.

2.3.6 Purification of Maltose Binding Protein Fusions using an Amylose Column

An amylose affinity column was prepared by filling a column with 5 ml of amylose resin and the column equilibrated with eight volumes of column buffer (20mM Tris-HCl pH 7.4). The cold osmotic shock fluid was adjusted to 10 mM Tris-HCl pH 7.4 using 1 M Tris-HCl pH 7.4 whereas the crude cell extract was diluted 1 in 4 before loading onto the column at a flow rate of 1 ml/min. The column was washed with twelve volumes of column buffer. Elution of proteins bound to the column was carried out with elution buffer (20 mM Tris-HCl pH 7.4, 20 mM NaCl, 1 mM EDTA, 10 mM ß-mercaptoethanol, 10 mM maltose) and 1 ml fractions collected before analysing by SDS-PAGE.

2.3.7 Purification of Maltose Binding Protein Fusions using a DEAE column

Purification of MBP fusions was attempted using a Pharmacia GP-250 Plus FPLC with a DEAE Sepharose column. MBP does not bind to CM-Sepharose but, it binds to DEAE Sepharose at pH 8.0 and is eluted at 100-125 mM NaCl (New England Biolabs, 1993).

Competent ER2058 cells were transformed with a pMALc2 derived plasmid and plated out onto L-agar/Amp plates overnight. A single colony was picked and used to inoculate a 10 ml of rich broth (5% (w/v) glucose in L-broth)/Amp and incubated at 37°C overnight with shaking. The overnight culture was used to inoculate 250 ml of rich broth (RB)/Amp and induced with 1 ml of 0.25 M IPTG for one hour when the absorbance of the culture was between 0.6 and 0.7 units at 600 nm. The cells were

harvested by centrifugation and resuspended in resuspension buffer before freeze thawing three times in liquid nitrogen. After freeze thawing the cells were treated with 100 µl of 1 mg/ml lysozyme and incubated at 37°C in a waterbath. After fifteen minutes 100 µl of 1 M MgSO4 and 100 µl mg/ml DNAse I were added to the cell extract and incubated for fifteen minutes. The sample was centrifuged in order to separate the soluble proteins from the insoluble material. The soluble material (supernatant) was saved and diluted 1:1 with 50 mM Tris-HCl pH 8 before loading onto a DEAE column which had been equilibrated with 50 mM Tris-HCl pH 8. The FPLC was run with 90 mM NaCl until the absorbance at 280 nm had returned to the baseline, this took approximately 45 minutes at a flow rate of 1 ml/min. The protein was eluted from the column with 125 mM NaCl and 1 ml fractions collected which were analysed by SDS-PAGE.

2.3.8 Purification of MBP Fusions by Hydroxyapatite Chromatography

A hydroxyapatite column was prepared by swelling 2g of hydroxyapatite in column buffer overnight at 4°C, excess buffer was removed and fresh buffer added before resuspending the hydroxyapatite. The resuspended hydroxyapatite was poured into a 10 ml column and left to settle. Excess buffer was removed and 2 ml of cell extract loaded onto the column before washing the hydroxyapatite column with 50 ml of column buffer. Elution of the bound proteins was carried out by loading the column with 0.5 M Sodium phosphate, pH 7.2 and the elution fractions collected. Fractions collected were analysed by SDS-PAGE.

2.3.9 Column Purification of GST-fusion protein using glutathione Sepharose matrix

Competent BL21(DE3) cells were transformed with the vector pGEX227ΔN-T and plated onto restrictive media. A colony was picked used to inoculate 20 ml of LB/Amp which was incubated at 37°C overnight with shaking. The overnight culture

was used to inoculate 500 ml of LB/Amp which was incubated at 37°C until the absorbance was between 0.6 and 0.7 units at 600 nm. Expression of the pVIII N-terminal deletion mutant was induced by the addition of IPTG to 0.3 mM. After inducing for one hour the culture was centrifuged for 5 minutes at 10000 rpm on a Beckman JA-14 rotor and the pellet resuspend in 10 ml of ice-cold PBS. Cells were lysed by freeze thawing and incubating at 37°C in the presence of lysozyme and the DNA released by cell lysis cleaved by incubating with DNase I for 15 minutes at 37°C. The sample was split into ten Eppendorfs, each containing 1 ml, and centrifuged at 12,000 rpm in a microfuge for 10 minutes. The pellet was discarded and the supernatant in each Eppendorf pooled and stored on ice.

Approximately 10 ml of a 50% (v/v) glutathione Sepharose 4B slurry in PBS was poured into a column and allowed to settle. The storage buffer was drained from the column and the glutathione Sepharose washed with four volumes of PBS. The supernatant was loaded onto the column and washed with 10 volumes of PBS. Elution of the GST fusion protein was carried out by washing the column with 2 volumes of PBS containing 10 mM glutathione.

2.3.10 Batch Purification of GST-fusion protein using glutathione Sepharose matrix

Competent BL21(DE3) cells were transformed with the vector pGEX227ΔN-T and plated onto restrictive media. A colony was picked used to inoculate 20 ml of LB/Amp which was incubated at 37°C overnight with shaking. The overnight culture was used to inoculate 500 ml of LB/Amp which was incubated at 37°C until the absorbance was between 0.6 and 0.7 units at 600 nm. Expression of the pVIII N-terminal deletion mutant was induced by the addition of IPTG to 0.3 mM. After inducing for one hour the culture was centrifuged for 5 minutes at 10000 rpm on a Beckman JA-14 rotor and the pellet resuspend in 10 ml of ice-cold PBS.

Cells were lysed by freeze thawing and incubating at 37°C in the presence of lysozyme and the DNA released by cell lysis cleaved by incubating with DNase I for 15 minutes at 37°C. The sample was split into ten Eppendorfs, each containing 1 ml, and centrifuged at 12,000 rpm in a microfuge for 10 minutes. The pellet was discarded and the supernatant in each Eppendorf pooled and stored on ice. Approximately 2 ml of a 50% (v/v) PBS/glutathione Sepharose 4B slurry was added to supernatant and incubated at 4°C for 30 minutes on rolling mixer. The supernatant/resin mix was loaded into a column, saving the run through, and the column washed with 20 volumes of PBS. Elution of the GST fusion protein was carried out by washing the column with 2 volumes of PBS containing 10 mM glutathione. After elution the column was washed with 20 volumes of PBS and repeat the purification procedure using the column run through.

2.3.11 Purification EDS Protease by affinity chromatography

A cell extract was prepared by transforming competent BL21(DE3) cells with the plasmid pSETEDS. The transformed cells were incubated in 250 ml LB/Amp at 37°C until the culture reached an absorbance of 0.6 units at 600 nm, induction of the recombinant EDS protease was induced by the addition of 1 ml of 0.1M IPTG. After one hour the cells were harvested by centrifugation at 10 000 rpm on a Beckman JA-14 rotor for 5 minutes. The pellet resuspended in 5 ml of 50 mM Tris-HCl pH 8 and the cells were subjected to three cycles of freeze/thawing in liquid nitrogen. Lysozyme (0.1 mg/ml) was added to the solution to lyse the cells and the sample incubated at 37°C. After 15 minutes 100 µl DNase I (2 mg/ml) and 100 µl 100 mM magnesium sulphate were added to the sample in order to reduce the viscosity of the sample which had increased due to the release of genomic DNA and the cell extract was incubated for 30 minutes at 37°C. The soluble cell material was obtained by dividing the cell extract into Eppendorfs and centrifuging at 13 000 rpm in a

microcentrifuge for 15 minutes. The supernatant, soluble material, was pooled and saved.

A zinc affinity column was prepared using the chelating ligand iminodiacetic acid (IDA) as in methods 2.4.12. The zinc column was equilibrated with 5 volumes of 50 mM Tris-HCl pH 8 prior to the loading of the soluble cell extract material onto the column. The column was washed with 10 volumes of 50 mM Tris-HCl pH 8 and eluted with 3 volumes of 50 mM Tris-HCl pH 8, 1 M NaCl followed by 3 volumes of 50 mM Tris-HCl pH 8, 1 M NaCl, 50 mM EDTA.

2.4 Miscellaneous Methods

2.4.1 Peptide Synthesis

All peptides were synthesised by solid phase fluorenyl methoxycarbonyl (Fmoc) polyamide chemistry using a semi-automated Cambridge Research Biochemistry (UK) Pepsynthesiser II using CRB software on an Apple IIe computer essentially as Atherton *et al.*, (1988). The two peptide synthesised were :
NH_2-CPNFDAVKDSADGYD-COOH and NH_2-CGGIGTLQFIEEFVP-COOH.

The use of high grade reagents solvents free from aqueous or amine contaminants is essential for the success of this technique. The Pepsyn KA resin and the Fmoc pentafluorophenyl ester derivatives were obtained from Milligen except for the Fmoc phenylalanine pentafluorophenyl ester used in the synthesis of the second peptide which was purchased from Novabiochem. The o-rings and filters required for synthesis were obtained from CRB. Dimethylformamide (DMF), supplied by Rathburn (HPLC grade), was prepared for use by adding 50g of heat activated molecular sieves , supplied by Sigma, (sieves were activated by heating at 200°C for 2 hours) and leaving for 4 weeks routinely tested for the presence of dimethylamine

using difluoronitrobenzene thereafter. This is because dimethylamine can react with the Fmoc protecting groups and so when present, even at low concentrations, significantly reduce the final yield of peptide.

Dimethylamine was tested for by adding an equal volume of DMF to 5 mM difluoronitrobenzene in ethanol and leaving for 20 minutes. Absorbance at 391 nm of less than 0.15, when read against a blank of 2.5 mM difluoronitrobenzene in ethanol, indicates that the solvent was of a high enough grade to be used. Tests were carried out in duplicate. When the absorbance was greater than 0.15 the DMF was left at room temperature for another two weeks before it was retested. After storage on molecular sieves it is essential to filter the solvent through a 0.2 μm nylon filter (Ananchem) to prevent blockage of lines and valves in the Pepsynthesiser.

PepSyn KA resin, which had been pre-washed with three times with 5 ml DMF to remove fines was packed into a 5 x 1 cm column and the plunger adjusted to 5 mm above resin to remove air bubbles. All the lines were flushed out with DMF before priming the lines with DMF and 20% (v/v) piperidine (supplied by Applied Biosysems, peptide synthesis grade) in DMF. The main lines were given a final wash, which lasted 10 minutes, to remove all traces of piperidine and the flow rate adjusted to 3 ml/minute before the reaction column was attached.

Synthesis of the peptide was controlled in part by CRB software using an Apple IIe computer, see figure 19 for program used. Each amino acid residue was dissolved in 2 ml of quinoline yellow solution (1.3g hydroxybenzotriazole, 1.4 μl diisopropylethylamine, 0.5 mg quinoline yellow, 10 ml dimethylformamide (DMF), prepared every second day). The first residue was attached to the column (starting at step 4 in the programme) in the presence of the catalyst dimethyl amino pyridine, which was circulated in the column for 90 minutes. In order to ensure complete coupling of the first amino acid to the column this step was repeated. After the

binding of the first amino acid to the column, the column was washed for 15 minutes in DMF. The amino acid residue was deprotected by washing with 20% (v/v) piperidine in DMF for 10 minutes (this step removes the protecting Fmoc group), followed by washing with DMF for 15 minutes. The subsequent amino acid residues, which had been dissolved in quinoline yellow solution, were circulated consecutively in the column for 90 minutes and the washing and deprotection steps repeated. The synthesis of the peptide was monitored on a chart recorder at 320 nm. After deprotection of the last amino acid residue the column resin was transferred to a sinter filter and washed sequentially with, 25 ml DMF, 10 ml t-amyl alcohol (supplied by Aldrich Chemical Co.), 10 ml acetic acid (supplied by Fisons, HPLC grade), 10 ml t-amyl alcohol and twice with 25 ml of diethyl ether (supplied by Fisons, HPLC grade).

Program	Time (minutes)
1 FLOW A	5
2 FLOW B	10
3 FLOW A	15
4 CALL (load amino acid)	
5 RECIRCULATE	150
6 FLOW S	1
7 FLOW A	5
8 RECIRCULATE	0.5
9 FLOW A	5
10 CALL	
11 REPEAT	

Figure 19. CRB program used to control synthesis on an Apple IIe computer.

The peptide was cleaved from the resin by addition of 40 ml of Rathburn HPLC grade trifluoroacetic acid (TFA) which cleaves the acid sensitive 4-hydroxy-methyl-phenoxy-acetate linkage. The cations generated in the cleavage reaction may react with electron rich amino acid side chains. The possibility of this occurring is reduced by the addition of 1 ml of each of the scavengers ethanedithiol (supplied by Aldrich Chemical Co.) and anisole (supplied by Aldrich Chemical Co.). The resin containing solution was incubated at room temperature for 3 hours before being filtered, washed twice with 5 ml of TFA and the filtrate saved. The filtrate was reduced to approximately 1 ml by rotary evaporation and 20 ml of ether added to the filtrate producing a white precipitate of peptide. The ether was discarded and the precipitate washed five times in ether before drying. The peptide was dissolved in a minimum of water and freeze dried.

2.4.2 Antipeptide Antisera

Coupling of the synthetic peptide to human serum albumin (HSA) was performed as outlined by Scheidtmann (1989) by incubating HSA in a 30 molar excess of N-succinimidyl-3-2(2-pyridyldithio)propionate (SPDP) over amino groups at room temperature for 30 minutes. SPDP couples the peptide to carrier by linking the amino groups of the carrier to the cysteinyl residues of the peptide. HSA was dissolved in a solution of 0.1M sodium phosphate and 0.1M NaCl, pH 7.5 to a concentration of 10 mg/ml. SPDP was dissolved to 20 mM in ethanol and 225 µl was added dropwise to 1 ml of HSA. The solution was dialysed in PBS overnight at 4°C to remove excess SPDP. Coupling of the peptide to HSA was achieved by adding 1 ml of peptide (6.6 mg/ml in PBS) to 1.5 ml of activated HSA. The solution was left at room temperature for 1 hour, incubated at 4°C and dialysed in PBS overnight. The coupled peptide was aliquoted and stored at -20°C prior to immunisation of rabbits.

The synthetic peptide CGGIGTLQFIEEFVP,which was used to generate a sheep polyclonal antiserum, was coupled to bovine serum albumin, otherwise the procedure was identical to the above.

2.4.3 Immunisation Protocol

(a) Rabbits

An emulsion was prepared by mixing equal volumes of the peptide conjugate to Freund's complete adjuvant. Dutch rabbits were injected subcutaneously with 1 ml of the emulsion. The rabbits were subsequently given booster injections at 2-4 week intervals, of peptide conjugates mixed with an equal volume of Freund's incomplete adjuvant. Again the injections were subcutaneous. Samples of blood (5 ml) were taken from the rabbits' ears before the initial immunisation (pre-immune sera) and a week after each boost. Blood was collected into plastic tubes and allowed to clot by incubating it for 1 hour at 37°C and stored overnight at 4°C. The sera were collected by centrifugation for 10 minutes at 2000g and stored in 0.5 ml fractions at -20°C until required.

(b) Sheep

The pVIII deletion mutant 111ΔC-T was purified as in method 2.3.2, freeze dried and resuspended in 1 ml of 10 mM imidazole. This was repeated four times to produce four 1 ml samples which were given to SAPU to generate antiserum. After the initial immunisation further booster injection were given at four week intervals with samples of blood being taken a week after each booster injection.

The synthetic peptide CGGIGTLQFIEEFVP which forms part of the C-terminal sequence of pVIII was purified conjugated to BSA as in method 2.4.2, freeze dried

and resuspended in 1 ml of water. This was repeated four times to produce four 1 ml samples which were given to SAPU to generate antiserum. After the initial immunisation further booster injection were given at four week intervals with samples of blood being taken a week after each booster injection.

2.4.4 Screening of Colonies

Colonies were picked at random from a L-agar/Amp plate with a sterile toothpick. Each colony was grown up in 5 ml of L-broth/Amp until their absorbance at 600 nm was between 0.6 and 0.7 units. Expression of the recombinant protein was induced by the addition of IPTG to 0.4 mM. After 2 hours a 1 ml sample was taken from each culture and centrifuged at 13000 rpm for 1 minute in a microfuge. The pellet was resuspended in 100 µl PBS and 20 µl reducing solution before boiling for 2 minutes. The samples were allowed to cool before being sonicated for 1 minute on a Sanyo Soniprep 150 sonicator and analysed by SDS-PAGE.

2.4.5 SDS-polyacrylamide Gel Electrophoresis

Polyacrylamide gels are prepared by the copolymerisation of acrylamide with a cross linking agent, usually N, N'-methylenebisacrylamide in the presence of a chain initiator i.e. N, N , N', N' - tetramethylenediamine (TEMED) and the catalyst ammonium persulphate. Prior degassing of solutions is required as molecular oxygen inhibits chemical polymerisation and disrupts the gel matrix resulting in a decrease in the resolution of the gel. Sodium dodecylsulphate (SDS) is used in gel electrophoresis as it is an anionic detergent which binds strongly to proteins. In the presence of excess SDS the detergent binds to protein giving a constant negative charge per mass unit. Protein-SDS complexes move towards the anode during electrophoresis and because of the molecular-sieving properties of the gel their mobilities are usually inversely proportional to the $\log_{10}$ of their molecular weight.

Proteins were analysed using a Bio-Rad Mini Protean II gel apparatus which was assembled as manufacturers instructions and the gel electrophoresis performed as Laemmli (1970). The proteins were resolved in separating gels of between 10 and 20% acrylamide. The separating gels were prepared by mixing 40% (w/v) acrylamide (supplied by Scotlab), 2% (w/v) bisacrylamide (supplied by Scotlab), 2 M Tris pH 8.7, 10% (w/v) SDS and water according to table 3 and degassing for 15 minutes. Polymerisation was induced by addition of 10 µl TEMED and 50 ml of freshly prepared 10% (w/v) ammonium persulphate to the separating gel, which was immediately poured into the gel cassettes leaving a 2 cm gap at the top for the stacking gel. The running gel was overlaid with water saturated butanol, which was rinsed off with 70% (v/v) ethanol after the gel had set. The stacking gel was prepared by mixing 0.975 ml 40% (v/v) acrylamide, 0.7 ml 2% (v/v) bisacrylamide, 1.25 ml 1M Tris-HCl pH 6.9, 100 µl 10% (w/v) SDS, 6.75 ml water, 20 µl TEMED and 50 µl 10% (w/v) ammonium persulphate and poured on top of the running gel before insertion of the comb.

	10%	12.5%	15%	17.5%	20%
40% acrylamide	3.75 ml	4.69 ml	5.63 ml	6.56 ml	7.5 ml
2% bis-acrylamide	1 ml	0.75 ml	0.65 ml	0.55 ml	0.5 ml
2 M Tris-HCl pH 8.7	2.8 ml	2.8 ml	2.8 ml	2.8 ml	2.8 ml
10% SDS	150 µl	150 µl	150 µl	150 µl	150 µl
Water	7.45 ml	6.76 ml	5.92 ml	5.09 ml	4.2 ml

Table 3. Recipe for SDS-PAGE Separating Gels

(a) Sample Preparation and Separation of Proteins

Samples which were to be run on a polyacrylamide gel were dissolved in SDS reducing solution (7% (w/v) SDS, 0.18 M Tris pH 6.9, 18% (v/v) glycerol (supplied

by Fisons, analytical grade), bromophenol blue (0.38 mg/ml) and 25% (v/v) b-mercaptoethanol and heated at 100°C for 2 minutes. Electrophoresis was carried out for 1 hour at 40 mA (constant amperage) in Laemmli buffer.

(b) Coomassie Blue Staining of Gels

The gels were stained with 0.25% (w/v) Coomassie brilliant blue R250 (BDH) in 45% (v/v) methanol (supplied by Fisons, analytical grade) and 10% (v/v) acetic acid (supplied by Fisons, analytical grade) for 30 minutes. The gels were destained in 25% (v/v) methanol and 7.5% (v/v) acetic acid. During staining and destaining the gels were gently agitated on an orbital shaker. After destaining gels were stored in sealed plastic bags with the air removed.

2.4.6 Low Molecular Weight SDS-PAGE

(a) Gel Preparation

The protocol of Schägger and von Jagow (1987) was employed to analyse proteins with a molecular weight less than 14 kDa. Bio-Rad Mini Protean II gel rigs were assembled according to the manufacturers instructions. 16.5% separating gels were prepared by mixing 3.33 ml of acrylamide solution A (46.5% (w/v) acrylamide, 3% (w/v) bisacrylamide), 3.33 ml of gel buffer (3 M Tris-HCl, pH 8.45, 0.3% (w/v) SDS), 1.33 ml glycerol and 2 ml of water. After degassing for 15 minutes 5 µl TEMED and 50 µl 10% (w/v) ammonium persulphate were added to the solution. The separating gel was immediately poured into the gel cassette leaving a 3 cm gap at the top for the spacer gel and the stacking gel. The separating gel was overlaid with water saturated butanol, which was rinsed off with water and ethanol after the gel had set. The spacer gel was composed of 2 ml acrylamide solution B (48% (w/v) acrylamide and 1.5% (w/v) bisacrylamide), 3.33 ml gel buffer and 4.67 ml of water.

After degassing for 15 minutes 5 µl TEMED and 50 µl 10% (w/v) ammonium persulphate were added to the solution. The spacer gel was immediately poured on top of the separating gel leaving a 2 cm gap at the top for the stacking gel and overlaid with water saturated butanol. Once the spacer gel had set the water saturated butanol was washed off with water and ethanol. The separating gel was prepared by mixing 0.8 ml acrylamide solution B, 2.48 ml gel buffer, 6.72 ml water, 5 µl TEMED and 50 µl 10% ammonium persulphate and pouring on top of the spacer gel before inserting the comb.

(b) Sample Preparation and Running of Gels

Protein samples, typically 20 µl, were mixed with 1 volume of reducing buffer (20% (w/v) SDS, 50 mM Tris-HCl pH 6.8, 2% (v/v) ß-mercaptoethanol, 12% (v/v) glycerol and 0.0005% (w/v) bromophenol blue) and boiled for 2 minutes. The samples were loaded into the wells and proteins were separated by applying a constant current of 25 mA to each gel until the dye front was 0.5 cm from the bottom of the gel. The anode buffer used was 0.2M Tris-HCl, pH 8.9 and the cathode buffer used was 0.1M Tris-HCl pH 8.25, 0.1M Tricine and 0.3% SDS.

(c) Staining and Destaining

Gels were stained and destained using the same solutions and procedure as in method 2.4.5.

2.4.7 Western Blotting

Proteins were separated using the Bio-Rad Mini-Protean II Gel system as previously described. When the dye front was 0.5 cm from the bottom of the gel the power supply was switched off and the gel removed from its cassette for blotting in a semi-

dry blotter (LKB Multiphor II). Three pieces of 3MM Whatman filter paper were cut to 10 cm by 8 cm and soaked in transfer buffer (50 mM Tris, 40 mM glycine, 0.04% (w/v) SDS and 20% (v/v) methanol). The three pieces of filter paper were placed on the bottom graphite plate and hyperbond-PVDF (Amersham) which had been soaked with methanol, for 30 seconds and washed in transfer buffer for 5 minutes, placed on the pile of filter paper. The gel was rinsed with transfer buffer and placed on top of the membrane ensuring no air bubbles were trapped underneath the gel. The sandwich was completed with three pieces of filter paper which had been soaked in transfer buffer. The upper graphite plate was placed on top of the sandwich and the gel blotted for one hour at a constant current of 50 mA.

Blots were developed as in Amersham (1993) by blocking the blot for one hour minutes with blocking buffer (25 g of dried milk powder in 500 ml PBS with 500 ml Tween 20) with shaking. The first antisera were prepared in blocking buffer to at a dilution dependent upon their titre. The blot was incubated for 30 minutes on the shaker with the first antiserum and rinsed with blocking buffer. It was then washed with blocking buffer for 30 minutes prior to a 30 minute incubation with a 1 in 5000 dilution of the HRP conjugated second antibody (SAPU) raised against IgG from the species that the primary antibody was generated in. The blot was given a final 30 minute wash in blocking buffer and rinsed with TPBS (blocking buffer without milk powder).

Development of the blot was performed by incubating for 1 min in a solution containing 1 ml of blotting reagent A (2.5 mM Luminol, 0.1 M Tris-HCl, pH 8.5, and 0.4mM p-Coumaric acid) and 1 ml of blotting reagent B (0.02% (v/v) hydrogen peroxide, 0.1 M Tris-HCl, pH 8.5). The blot was drained, placed between two acetate sheets and exposed to Fuji X-ray film for between 1 and 15 minutes. The X-ray film was developed using a Kodak M35X-OMAT processor.

2.4.8 Stripping of Western Blots

Western blots which had been probed with antisera were stripped of primary and secondary antibodies with a buffer composed of 355 µl ß-mercaptoethanol, 10 ml of 10% (w/v) SDS, 3.125 ml of 1M Tris-HCl pH 6.9 and made up to a final volume of 50 ml. The stripping buffer was heated to 70°C and the Western blot placed in the buffer. After 30 minutes the blot was washed twice in 0.1% (v/v) Tween 20 in PBS and the blot developed as normal.

2.4.9 Adenovirus Protease Digestion of Recombinant pVIII.

Recombinant pVIII, obtained from nickel affinity purification, was precipitated with an equal volume of water, in order to remove the elution buffer which inhibited adenovirus protease activity. The sample was spun down in 1 ml aliquots at 10000 rpm for 5 minutes and each pellet was resuspended in 400 ml of resuspension buffer (50 mM sodium phosphate pH 6.5, 300 mM NaCl and 2 M urea). Digests were performed by incubating 10 ml Ad2 protease, 10 ml activating peptide (100 µg/ml), 0.01 ml b-mercaptoethanol and 5 ml of 5 mM EDTA at 37°C. After 10 minutes 25 ml of fusion protein and 10 ml resuspension buffer were added and the sample incubated at 37°C for 24 hours. The digest products were examined by SDS-PAGE and the cleavage products sequenced.

2.4.10 Preparation of Samples for Amino Acid Sequencing

(a) SDS-PAGE

Proteins were analysed using a Bio-Rad Mini Protean II gel apparatus which was assembled as per manufacturers instructions. The proteins were resolved in separating gels of between 8 and 17.5% acrylamide. The 30 (w/v)% acrylamide

solution was prepared by dissolving 7.5g of acrylamide (BDH) and 0.2g Piperazine diacrylamide (Bio-Rad) in water (final volume of 25 ml). The separating gels were prepared by mixing 30% (w/v) acrylamide 4x lower Tris buffer (1.5 M Tris-HCl pH 8.8, 0.004% (w/v) SDS) and water according to table 4. The gel solution was degassed for 15 minutes. Polymerisation was induced by addition of 2 µl TEMED and 15 µl of freshly prepared 10% (w/v) ammonium persulphate to the separating gel, which was immediately poured into the gel cassettes leaving a 2 cm gap at the top for the stacking gel. The sequencing gel was overlaid with iso-propanol, which was poured off and replaced with lower Tris buffer after the gel had set. The gel was left overnight at 4°C. The stacking gel was prepared by mixing 1 ml 30% (w/v) acrylamide, 1.5 ml 4x lower Tris buffer and 3.5 ml water. The solution was degassed for 15 minutes before being polymerised by the addition of 6µl TEMED and 45µl 10% (w/v) ammonium persulphate and poured on top of the running gel before insertion of the comb.

The gels were pre-run with upper Tris buffer (0.5 M Tris-HCl pH 6.8, 0.0004% (w/v) SDS) and 0.75 ml of 10 mM glutathione in the upper (inner) chamber. The lower (outer) chamber was filled with electrophoresis buffer (75 mM Tris, 0.575 M glycine (BDH) and 0.001% (w/v) SDS). In order to detect how far the gel had run an outer lane was filled with 10 µl of sample buffer (2.5 ml glycerol, 3 ml 10% (w/v) SDS, 1.25 ml upper Tris (x4), 2.75 ml water, 5 mg bromophenol blue and 50 µl/ml of ß-mercaptoethanol immediately prior to use). Gels were run at 15 mA per gel for 1 hour or until the dye front had reached the sequencing gel. The buffers were replaced with electrophoresis buffer and 150 µl 100 mM sodium thioglycolate in the upper chamber and electrophoresis buffer in the lower chamber. Samples were added to an equal volume of sample buffer and boiled for 2 minutes before loading. The gels were run at 20 mA per gel until dye front was 0.5 cm from the bottom of the gel.

	8%	10%	12.5%	15%	17.5%
30% Acrylamide	1.06 ml	1.33 ml	1.66ml	2 ml	2.33 ml
Lower Tris (x4)	1 ml	1 ml	1 ml	1 ml	1 ml
Water	1.94 ml	1.67 ml	1.33 ml	1 ml	0.66 ml

Table 4. Composition of acrylamide separating gels.

(b)Western Blotting

Protein samples were separated as above and the gel removed its cassette for blotting in a Bio-Rad Trans-Blot Electrophoretic Transfer Cell. The gel was washed in blotting buffer (10 mM CAPS pH 11, 20%(v/v) methanol) for five minutes to reduce the background levels of glycine. Pro-Bond PVDF (Applied Biosystems) was soaked in methanol for 1 minute before soaking in blotting buffer for 10 minutes. A fibre pad was soaked in blotting buffer and placed on the gel holder. A single piece of 3MM Whatman filter was soaked in blotting buffer and placed onto the fibre pad. The gel was placed on top of the filter paper and the membrane placed on top of the gel ensuring that no air bubbles were trapped between the gel and the membrane. A single sheet of 3MM Whatman filter which had been soaked in blotting buffer was placed on top of the membrane. The sandwich was completed with a fibre pad which had been soaked in blotting buffer. The gel holder was closed and placed into the blotting rig.

The buffer tank was filled with blotting buffer and a cooling trough. Blotting of the gel was carried out for 2 hours at 300 mA ensuring that the voltage did not exceed 100 V and the cooling trough was replaced after 1 hour. The blot was removed from the gel holder and stained with 0.1% amido black (40% methanol, 1% acetic acid) for 20 seconds. Destaining of the blot was carried out with 40% methanol and 1% acetic

acid and the blot dried before cutting the protein bands out with a scalpel. The excised bands were placed in Eppendorfs and stored at 4°C before sequencing.

(c) Amino Acid Sequencing

Amino acid sequencing was carried out by Paul Talbot on an Applied Biosystems Procise protein sequencer.

2.4.11 Capillary Electrophoresis

Capillary electrophoresis was carried out on a Bio-Rad Biofocus 3000 Capillary Electrophoresis System, see Biofocus 3000 instruction manual (version 3) for procedure.

Samples were prepared for analysis by adding 80 µl of Rathburn HPLC grade water to 10 µl TFA and 10 µl of sample and filtered using micopure filters (supplied by Amicon) before carrying out capillary electrophoresis.

2.4.12 Preparation of metal chelate affinity column

A metal chelate affinity column was prepared using the chelating ligand iminodiacetic acid (IDA). IDA immobilised on Sepharose 6B was purchased from Sigma and 5 ml of the resin poured into a column. The column was washed with 4 volumes of sterile water before charging, as in Hochuli et al. (1987) with 2 volumes of either 1% (w/v) nickle sulphate or 1% (w/v) zinc sulphate. The charged column was washed with 2 volumes of sterile water, 2 volumes of 0.2 M acetic acid (containing 0.2 M sodium chloride and 0.1% (v/v) Tween 20) and 2 volumes of water. The charged resin was stored in 20% (v/v) ethanol at 4°C until required for use.

2.4.13 Regeneration of Glutathione Sepharose column

A used glutathione Sepharose column was washed with three volumes of 0.1 M Tris, HCl, pH8, and 500 mM NaCl. It was subsequently washed with 0.1M Sodium acetate, pH 4.5 and 0.5 M sodium chloride. These two washes were repeated three times, at the end of washes all ionic material bound to the column was removed. The glutathione column was washed with five volumes of PBS prior to a wash with two volumes of 6 M guanidine hydrochloride. Next, the column was washed with guanidine chloride in order to remove precipitated, denatured, and non-specifically bound proteins. The column was immediately washed with five volumes of PBS. In order to remove proteins that are bound hydrophobically to the glutathione column, the colum was washed with four volumes of 70% (v/v) ethanol. The column was washed with five volumes of PBS and was stored in 20% (v/v) ethanol.

2.4.14 Dot Blots

A Hyperbond-PVDF membrane, supplied by Amersham, was cut to the desired size and washed with methanol. The membrane was placed in a square Petri dish and the protein to be bound to the membrane dissolved in a maximum volume of 3 ml, synthetic peptides were dissolved at 0.25 mg/ml. The solution was pipetted onto the membrane so that the entire membrane was covered and left to dry overnight. The membrane was blocked for a minimum of 1 hour with blocking buffer (5% (w/v) milk powder in PBS containing 0.1% (v/v) Tween 20). Two serial dilutions of each antiserum were prepared in 84 well plates with initial dilutions of 1 in 200 and 1 in 20 of the antiserum in 20 µl of blocking buffer, each dilution halved the concentration of the antiserum in the sample giving final dilutions of 1 in 400 000 and 1 in 40 000 respectively. The membrane was placed carefully on top of the 84 well plates containing the serial dilution and another 84 well plate placed on top of the membrane and the plates clamped together so that the membrane was held securely in place

before the assembly was inverted. The plate and membrane assembly was placed inside a humidity box and placed on an orbital shaker. After 15 minutes the membrane was removed from the plates and washed for 30 minutes in blocking buffer. Horse radish peroxidase conjugated secondary antibody, supplied by SAPU, was diluted in blocking buffer to 1 in 4000 and used to wash the membrane for 15 minutes. The membrane was washed for 30 minutes in blocking buffer and rinsed with PBS prior to developing with ECL reagents as in method 2.4.7.

2.4.15 pVIII Sepharose Column

pVIII was purified as in method 2.3.1 in 1 M guanidine chloride. The guanidine chloride solution used in the elution of pVIII from the nickel column, was buffered with 0.2 M sodium bicarbonate pH 8.3, and contained 0.5 M NaCl and 250 mM imidazole. In order to immobilise pVIII on a Sepharose column Sepharose CL was used as opposed to Sepharose 4B as guanidine chloride is a disruptive elutent. Tris buffered guanidine chloride could not be used in this procedure as the amino groups would couple with the resin preventing the binding of the ligand to the Sepharose.

Cyanogen Bromide activated Sepharose CL (supplied by Sigma) and 0.5g was swollen in 15 ml of 1 mM ice-cold HCl for one hour at 4°C, the resin was mixed constantly by rotation as magnetic stirrers can fragment the gel beads. The Sepharose was washed for 15 minutes with 200 ml of ice-cold 1 mM HCl as this preserves the activity of the reactive groups which hydrolyse in high pH conditions. The resin was filtered on a G3 sintered glass filter and as soon as the last aliquot of 1 mM HCl was drained from the Sepharose the resin was transferred into the solution containing pVIII as reactive groups on the Sepharose hydrolyse at the coupling pH of 8.

Approximately 1 ml of swollen Sepharose CL resin was added to 2 ml of pVIII as this ratio gives the optimum coupling solution. After four hours of mixing the

coupling solution by rotation at 37°C the unreacted groups on the resin was blocked by incubating in 2M Tris-HCl, pH 8 overnight at 4°C with constant rotation. In the morning the resin was washed with 1 M Tris-HCl, pH 4 and 1 M Tris-HCl, pH 8 in order to remove any proteins which may have bound ionically to the column. The column was stored in 0.2 M sodium bicarbonate pH 8, 0.5 M NaCl and 0.02% (w/v) sodium azide at 4°C for up to one month.

2.5 Tissue Culture Methods

2.5.1 Maintenance of HeLa Cells

(a) Spinner Cultures

Hela cells were grown in suspension in S-MEM (GIBCO-BRL) with 5% (v/v) newborn calf serum at 37°C to a density of 5 x 10^5 cells/ml. They were passaged every 3 to 4 days to give a cell concentration of 2 x 10^5 cells/ml. Cells were peroidically tested for the presence of mycoplasma with DAPI at 2 μg/ml.

(b) Monolayer Cultures

HeLa cultures were maintained in plastic 75cm^3 bottles (Sterilin) in 25 ml modified D-MEM (GIBCO-BRL) supplemented with 10% (v/v) foetal calf serum in an atmosphere of 5% carbon dioxide at 37°C in a Heraeus incubator. Cells were passaged as follows upon reaching 70-80% confluence. Spent media was poured off and the cells washed with 5 ml of Trypsin/EDTA. 2ml of fresh Trypsin/EDTA was left on the cells until cells started to come off the plastic. The cells were resuspended by adding D-MEM/10% foetal calf serum to 10 ml and a 2 ml aliquot of the cell suspension was transferred to a new 75cm^3 bottle with 25 ml of D-MEM/10% foetal calf serum. Cells were peroidically tested for the presence of mycoplasma with DAPI

at 2 μg/ml and in the event of a mycoplasma concentration cells were incubated for one week in the presence of 1% (v/v) mycoplasma removal agent (supplied by ICN).

2.5.2 Storage of HeLa Cells in Liquid Nitrogen

Cells were tested for the presence of mycoplasma with DAPI at 2 μg/ml immediately prior to storing the cells. In the event of a mycoplasma infection cells were incubated for one week in the presence of 1% (v/v) mycoplasma removal agent (supplied by ICN) and retested for the presence of mycoplasma. HeLa cultures were maintained in plastic 75cm^3 bottles (Sterilin) in 25 ml modified D-MEM (GIBCO-BRL) supplemented with 10% (v/v) foetal calf serum in an atmosphere of 5% carbon dioxide at 37°C.

Five flasks which were between 70 and 80% confluent were passaged as follows. Spent media was poured off and the cells washed with 5 ml of Trypsin/EDTA. 2ml of fresh Trypsin/EDTA was left on the cells until cells started to detach from the plastic and the Trypsin/EDTA gently poured off. The cells were resuspended, in each flask, by adding 2 ml of freezing mix (10% DMSO, 30% foetal calf serum, and 60% D-MEM) and 1 ml aliquots placed into sterile vials. The vials were placed on ice in a polystyrene container and stored at -70°C overnight before storing under liquid nitrogen.

2.5.3 Growing of HeLa cells taken from Liquid Nitrogen storage

A vial containing HeLa cells was taken out of liquid nirtogen storage and immediately placed in a 37°C waterbath to ensure rapid thawing. Upon thawing the cells were transferred into a 75 cm^3 flask and 20 ml of D-MEM plus 10% (v/v) FCS added to the flask. The HeLa culture was left overnight in an atmosphere of 5% carbon dioxide at 37°C in a Heraeus incubator before the spent media was carefully poured off and

replaced with 25 ml of fresh D-MEM/10% foetal calf serum. Cells were subsequently passaged as in method 2.5.1 on reaching 70-80% confluence.

2.5.4 Preparation of Adenovirus Type 2 Stock

HeLa cells were grown in 6 l of S-MEM, as in method 2.5.1, to a density of $3x10^5$ cells per ml. The cells were centrifuged for 15 minutes at 2500 rpm in a Mistral 6L centrifuge and resuspended in 250 ml of S-MEM prior to infection with 1 ml of Ad2 stock. Virus was allowed to absorb onto the cells in the absence of newborn calf serum for 2 hours at 37°C after which the cells were made up to 6 l with S-MEM and newborn calf serum was added to 2%. Cells were harvested at 72 hours post infection by centrifugation as before. The infected cell pellet was washed once with cold PBS and resuspended in 48 ml of 10 mM Tris-HCl, pH 7. The cell suspension was placed in 12 ml aliquots into sterile 50 ml tubes and 10 ml of trichloro-trifluoro-ethane (arcton) added to each tube prior to shaking for 30 minutes at 4°C on a mechanical shaker. Extracts were centrifuged at 2500 rpm for 15 minutes at 4°C using an IEC CENTRA-3R centrifuge. The top (aqueous) layer was removed and clarified by centrifugation at 10000 rpm for 30 minutes at 4°C using a JA17 rotor in a Beckman J2-21 centrifuge before being stored at -70°C. The titre of the virus was determined by carrying out plaque assays as described by Freshney (1987).

2.5.5 Preparation of Slides

Cells were grown to confluence in a 75 cm^3 flask and the media removed. The cells were washed with 5 ml of Trypsin-EDTA and incubated for one to two minutes in 2 ml of Trypsin-EDTA until the monolayer of cells started to detach from the plastic. After removing the Trypsin-EDTA from the flask 10 ml of D-MEM was added to the flask and the flask shaken to dislodge the monolayer.

Slides were prepared by soaking M-spot slides, supplied by Hendley-Essex, in ethanol and flaming prior to placing into sterile square Petri dishes. Four slides were placed in each dish and half of the freshly split cells added to the slides. The volume of media in the Petri dishes was made up to 20 ml by the addition of 10% (v/v) FCS enriched D-MEM. Cells were grown at 37°C, 5% CO_2 until they were confluent, the media was changed every three days, if required.

2.5.6 Adenovirus Infection of HeLa Cells

HeLa cells were grown to 80% confluence on multi-spot slides (supplied by Hendley-Essex) which were placed inside a 90 mm square Petri dish (Sterilin). Infection of the cells was carried by adding 10 p.f.u. (plaque forming units) per cell of arcton-extract prepared virus to 10 ml of D-MEM and incubating at 37°C, 5% carbon dioxide in a Heraeus incubator. After 30 minutes, the D-MEM was carefully removed and replaced with D-MEM plus 10% foetal calf serum. The slides were incubated at 37°C, 5% carbon dioxide for 12,16, 20, 24 and 28 hours before fixing.

2.5.7 Immunofluorescence

(a) Fixing Slides

The medium was carefully removed from the Petri dish with a pipette, taking care not to disturb the monolayer on the slides. After all the media was removed the slides were washed gently with 20 ml of PBS three times. The cells were fixed with 2% (w/v) paraformaldehyde in PBS. The paraformaldehyde was prepared in a fume cupboard by heating 80 ml of sterile water to 60°C and adding 4 g of paraformaldehyde. After the paraformaldehyde had dissolved 100 ml of sterile 2x PBS was added and the volume made to 200 ml with sterile water. Once the paraformaldehyde solution had cooled to room temperature 20 ml were added to each

Petri dish in order to fix the cells onto the slides. After 10 minutes the paraformaldehyde was removed and the slides washed gently with PBS. The slides were stored in PBS containing 0.1% (w/v) sodium azide at 4°C.

(b) Permeabilising Cells

The storage buffer was removed from the slides and the cells permeabilised with 0.2% (v/v) Triton X-100 for 5 minutes before washing with 20 ml of PBS. Blocking of the slides was performed by incubating 20 ml of 1% FCS in PBS for thirty minutes. After blocking the slides were wiped dry around the spots on the slide. Preprepared dilutions of preadsorbded antiserum were added carefully onto each spot. The antiserum was left for 1 hour at room temperature in humidity chamber. The slides were washed three times with 20 ml of PBS and permeablised as before. After blocking with 1% FCS in PBS the secondary antiserum was added and the slides stored in the dark for 1 hour at room temperature before washing as before. The edges of the slide were dried and drops of anti-quenching agent added onto the slide between the spots. The coverslip was lowered gently onto the slide and anchored into place using nail varnish. Slides were viewed using a Nikon Microphot-FXA microscope and photographs taken using an automatic exposure on Ilford HP5 Plus film.

3. Results

3.1. Constuction of Plasmids Containing pVIII and its Cleavage Products

Construction of plasmids containing pVIII and its cleavage products were created in order to over express the viral protein in *E.coli*. A variety of vectors were used in an attempt to produce soluble recombinant protein.

3.1.1 Construction of a Plasmid Containing pVIII

(a) Construction of a pVIII fusion protein in the vector Pin-Point Xa (pXa)

The construction of a pVIII fusion protein with a biotinylated tag was attempted using the vector Pin-Point Xa (Promega). Three different cloning routes were used in the effort to construct the plasmid pXaVIII. In each case the DNA insert coding for pVIII was generated from Ad2 DNA using PCR. The oligonucleotide primers used in the PCR were:

(1) 5'-GCGCCA**TCATGA**GCAAGG-3'
(2) 5'-GGTCG**AGATCT**GTCAGGC 3'
(3) 5'-GTCGTC**GGATCC**ATTATGAGCAAGG-3'
(4) 5'-CTCACC**GGATCC**GCGGGCAAAGCACT-3'

Each oligonucleotide primer was designed to have at least six bases at its 5' end followed by the desired restriction enzyme site, this being a Bsp HI, site in primer (1), a Bgl II site in primer (2) and a Bam HI site in primers (3) and (4). Primers (1) and (3) were taken from the + strand of Ad2 and primers (2) and (4) from the - strand. The inserts were copied from Ad2 DNA by PCR according to method 2.2.4 using the oligonucleotide primer pairings of (1) with (2), and (3) with (4). All restriction enzyme sites are highlighted and start codons are underlined.

The PCR products using primers (1) and (2) were purified from agarose gels and were either used directly in ligation reactions, after purification, or digested with Bsp HI and Bgl II, before being incubated with Klenow fragment. PCR products using oligonucleotides (3) and (4) were digested with Bam HI. The digestion products were purified from agarose gels and were in ligation reactions with pXa cut with Nru I. The ligation reaction products were transformed into competent XL1-Blue cells and plated onto LB/Amp plates. Mini preps (method 2.2.11) were carried out on each colony that had grown on the restrictive media.

(b) Construction of a pVIII fusion protein in the vector pRSETA

A pVIII fusion protein was constructed with a six histidine tag using the vector pRSETA (Invitrogen). The DNA insert coding for pVIII was generated from Ad2 DNA using PCR. The oligonucleotide primers used in the PCR were primers (3) and (4).

The pRSET vector was cleaved at the Bam HI cut-site and the 5' phosphate groups removed at each end of the cut plasmid by incubation in CIAP. This prevents self-religation of the vector, resulting in the vector only being able to religate by the incorporation of the insert. The PCR products were cut with Bam HI and added to cut vector with ligase and ligase buffer. The ligation reaction was carried out for four hours at 16°C. Competent XLI-Blues were transformed with DNA and grown overnight on a restrictive agar plate. Colonies that had grown on the restrictive media were grown in L-Broth and minipreps performed. The vector DNA was cut with Bam HI to detect the presence or absence of the insert. In a positive result two bands would appear at 2900 b.p. and 750 b.p. Two colonies (8A and 8E) were found to contain vector and insert DNA. The vector DNA from these colonies was cut with Eco RI to establish whether the insert had ligated in the correct orientation (see figure 20). A positive result would be the production of two bands at 3,000 b.p and 600 b.p.,

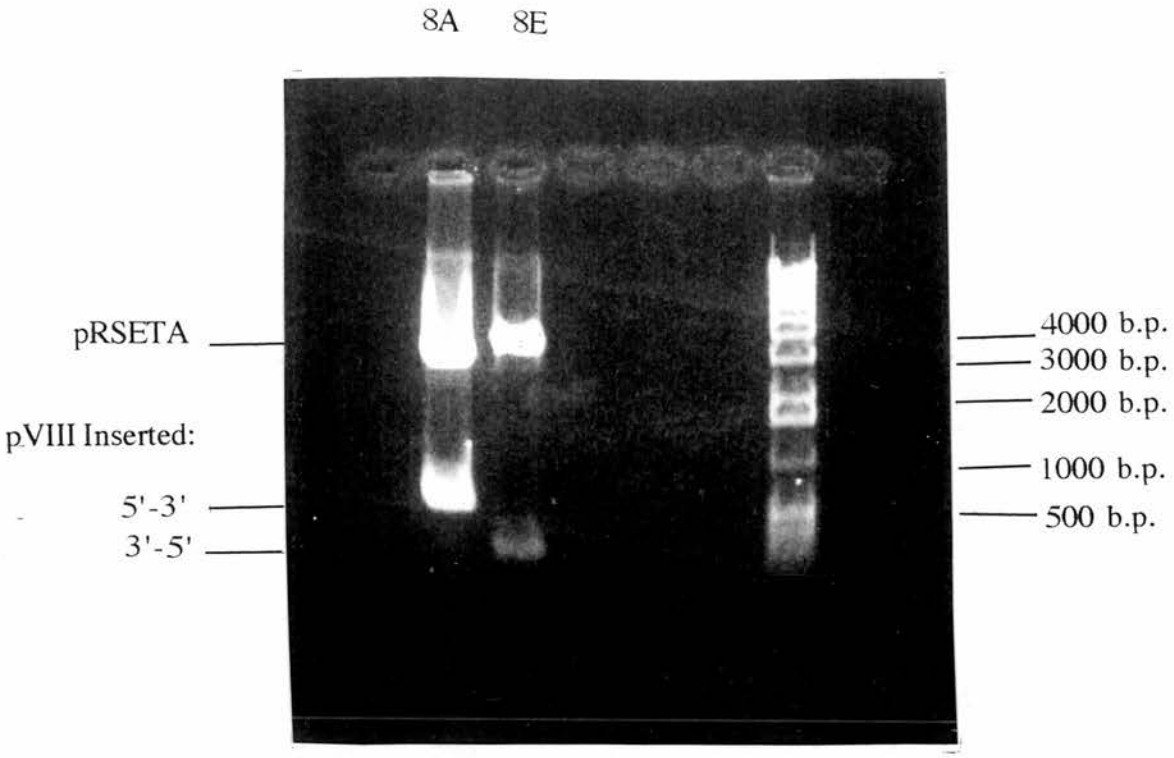


Figure 20: Analysis of DNA fragments produced vectors containing the pVIII gene. The vectors from colonies 8A and 8E were digested in the presence of Eco RI. The digestion products were resolved on a 1% (w/v) agarose gel and stained with ethidium bromide (0.5mg/ml)

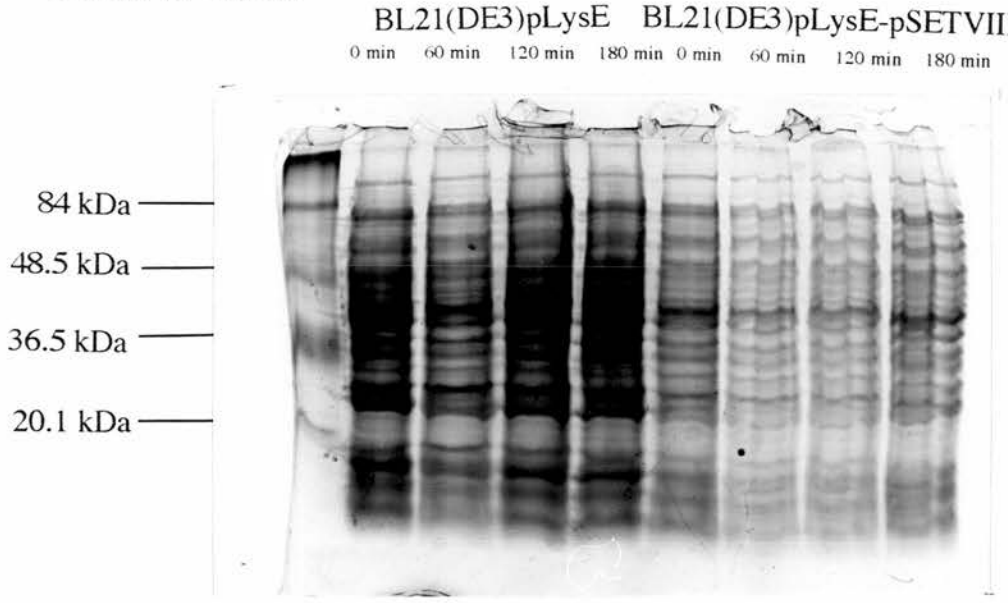


Figure 21: Analysis of induction products produced by BL21(DE3)pLysE and BL21(DE3)pLysE-pSETVIII cells by SDS-polyacrylamide gel electrophoresis. Production of recombinant pVIII was induced with 0.1M IPTG (4μl/ml) and the cell contents analysed at varying time intervals by loading onto a 20% polyacrylamide gel. The first lane was loaded with Prestained Molecular Weight Standards, supplied by Sigma, and the molecular weights indicated.

a negative result would be the production of two bands at 3,500 b.p. and 100 b.p. The insert DNA from colony 8A was found to have been inserted correctly and the vector was named pSETVIII.

(c) Screening of colonies for the expression of recombinant pVIII

BL21(DE3)pLysE cells were transformed with DNA from a maxi prep of the plasmid pSETVIII. Four colonies were picked at random and grown to an optical density of 0.6 at 600nm in 5 ml of LB/Amp, inducing expression with 20µl of IPTG at 37°C for one hour. Cell extracts were prepared from each colony and their expression examined by SDS-PAGE. It was found that recombinant pVIII was not expressed in sufficient quantities in BL21(DE3)pLysE for the protein to be visible by staining the SDS-PAGE gel with Coomassie blue, see figure 21. Consequently the vector pSETVIII was used to transform BL21(DE3) cells and the expression of recombinant pVIII examined by SDS-PAGE, see figure 22. Expression of pVIII in the four selected colonies was found to be sufficient to produce a visible pVIII band in the cell extract after staining with Coomassie blue. As the quantity of protein expressed by each colony was approximately the same colony number 1 was used to produce glycerol stocks of pSETVIII in BL21(DE3) cells.

3.1.2 Construction of N-terminal and C-Terminal Deletion Mutants.

(a) pSETVIII derived deletion mutants

(i) Construction of pSET55ΔC-T

Plasmid pSET55ΔC-T was constructed, encoding a six-histidine tag and the first 58 amino acid residues of pVIII. This construct was achieved by digesting the plasmid pSETVIII with Eco RI and purifying the 3450 b.p. fragment from an agarose gel. The

3.1 kbp fragment which had Eco RI sticky ends were ligated together and the ligation reaction products used to transform XLI-Blue cells and plated onto LB/Amp plates.

Three colonies that grew on the restrictive media were selected and named Eco A, Eco B and Eco C. Minipreps were carried out on three colonies and the DNA recovered digested with Xba I, see figure 23. It was found that colonies Eco A and Eco B were positive for the pVIII genetic material coding for amino acids 1-55 as a single 3100 bp band was detected. Colony Eco B was used to produce glycerol stocks and for a maxi prep of the plasmid.

(ii) Construction of pSET143ΔC-T

Plasmid pSET143ΔC-T was constructed, encoding a six-histidine tag and the first 143 amino acid residues of pVIII. This construct was achieved by digesting the plasmid pSETVIII with Sac I and purifying the 3.3 kbp fragment from an agarose gel. The ends of the 3.3 Kbp fragment were ligated together as they had compatible Sac I sticky ends. The reaction products used to transform XL1-Blue cells and plated onto LB/Amp plates.

Three colonies that grew on the restrictive media were selected and named Sac A, Sac B and Sac C. Minipreps were carried out on three colonies to purify the plasmid and the vector digested with Xba I in order to determine whether the deletion mutant had been created. It was found that colonies Sac A and Sac B were positive for the pVIII genetic material coding for amino acids 1-143, see figure 23, as a single 3300 bp band was detected. It was not possible to determine if colony Sac C contained a positive plasmid as the miniprep was contaminated with bacterial DNA. Colony Sac A was used to produce glycerol stocks and for a maxi prep of the plasmid.

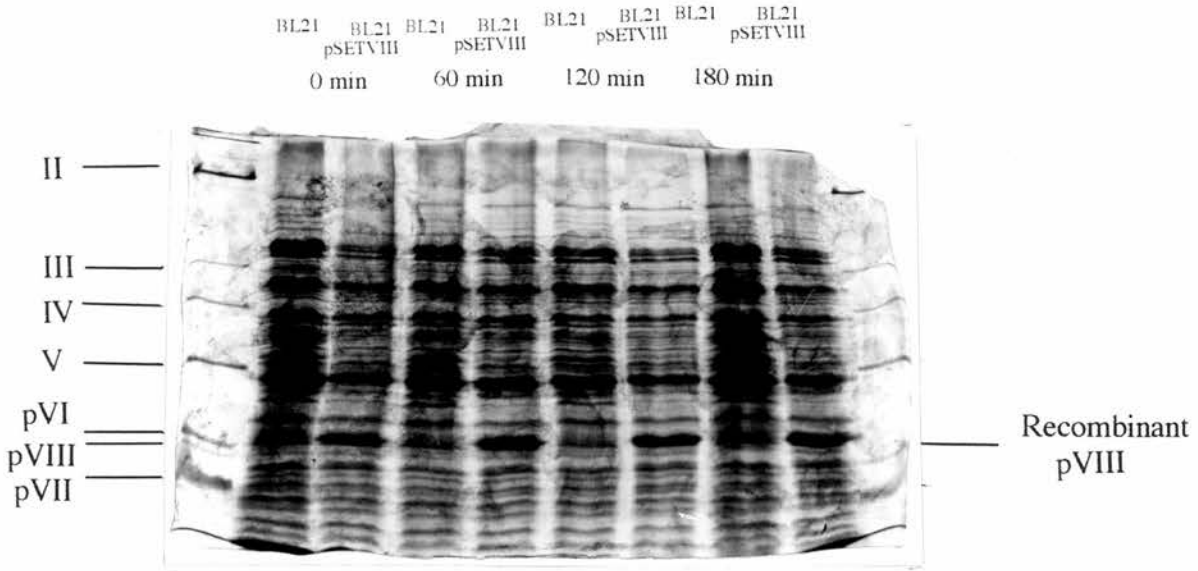


Figure 22: Analysis of induction products produced by BL21(DE3) and BL21(DE3)-pSETVIII cells by SDS-polyacrylamide gel electrophoresis. Production of recombinant pVIII was induced with 0.1M IPTG (4μl/ml) and the cell contents analysed at varying time intervals by loading onto a 20% polyacrylamide gel. Adenovirus serotypes ts1 and 2 were ran in parallel lane as standards and the locations of pVI (27,000 Da) and pVIII (26,000 Da) indicated.

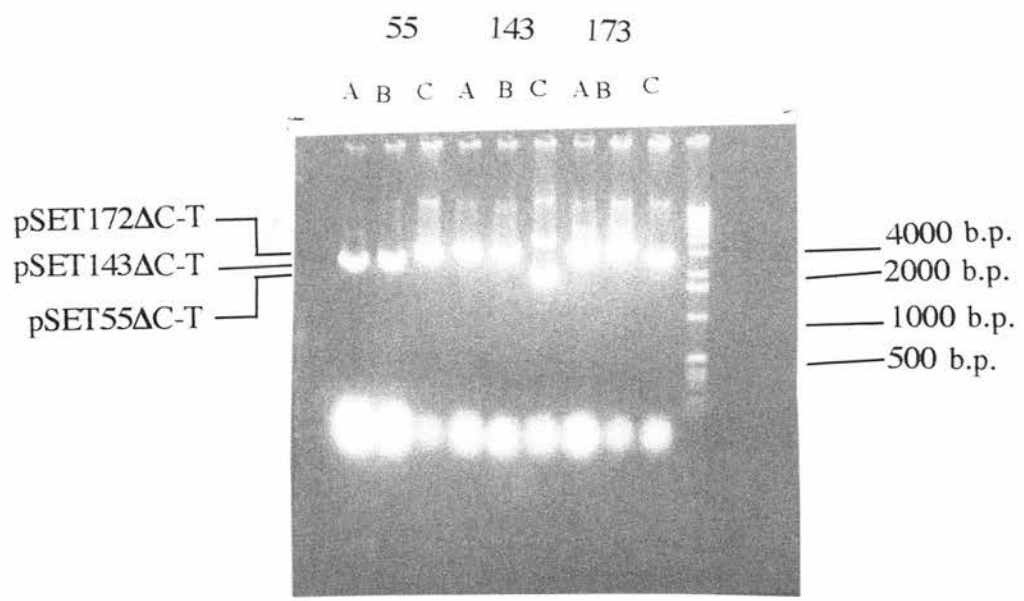


Figure 23: Analysis of DNA fragments produced by an Xba I / Hind III digest of pVIIIΔC-T mutants. The vectors from colonies 55 A-C, 143 A-C, and 172 A-C were digested in the presence of Eco RI, Sac I and Pst I respectively. The digestion products were resolved on a 1% (w/v) agarose gel and stained with ethidium bromide (0.5 mg/ml)

(iii) Construction of pSET172ΔC-T

Plasmid pSET172ΔC-T ,which encoded for the first 172 amino acid residues of pVIII and a six-histidine N-terminal tag, was constructed. This construct was achieved by digesting the plasmid pSETVIII with Pst I and purifying the 3.4 kbp fragment from an agarose gel. Both ends of the 3.4 kbp fragment were ligated together using their compatible Pst I sticky ends. The ligation reaction products used to transform XL1-Blue cells and plated onto LB/Amp plates.

The three colonies that grew on the restrictive media were selected and named Pst A, Pst B and Pst C. Minipreps were carried out on three colonies to purify the plasmid and the vector digested with Xba I in order to determine whether the deletion mutant had been created. It was found that colony Pst B was positive for the pVIII genetic material coding for amino acids 1-172, see figure 23, as a single 3400 bp band was detected. Colony Pst B was used to produce glycerol stocks and for a maxi prep of the plasmid.

(iv) Expression of recombinant proteins by plasmids pSET55ΔC-T, pSET143ΔC-T and pSET172ΔC-T

Each plasmid was used to transform BL21(DE3) cells. Three colonies were picked at random from each plasmid and grown to an optical density of 0.6 at 600nm in 5 ml of LB/Amp, inducing expression with 20 μl of IPTG at 37°C for one hour. Cell extracts were prepared from each colony and their expression examined by low molecular weight SDS-PAGE. It was found, after staining with Coomassie blue, that the plasmids pSET143ΔC-T and pSET172ΔC-T expressed a 20 kDa and a 24 kDa band respectively which was visible in the cell extract and was not present in BL21(DE3) cells. As expression between the colonies of pSET143ΔC-T and pSET172ΔC-T were approximately identical the first colony of each plasmid was used to create glycerol

stocks in BL21(DE3) cells. However, the plasmid pSET55ΔC-T did not express a visible 10 kDa band that was not present in BL21(DE3) cells and it was therefore concluded that either the vector was unable to express the protein in BL21(DE3) cells or unable to express the protein in sufficient quantities to enable the purification of the protein from the cellular proteins.

(b) Constructs using pUC18

(i) Construction of pUCVIII

Plasmid pUCVIII which encoded for entire pVIII protein was constructed using PCR. This was achieved by copying the DNA from the linearised plasmid pSETVIII (cut with Nde I) using the oligonucleotide primers:

(5) 5'-TGGGGA**GGTACC**ATGAGCAAGG-3'
(6) 5'-CGCTCTGCC**TCTAGA**CTGGTC-3'

Primer (5) matches with +strand over the start codon (underlined) of pVIII in the plasmid pSETVIII. The Bam HI cleavage site before the initiation codon has been removed and a Kpn I cleavage site (highlighted) inserted. Oligonucleotide primer (6) matches with 3' strand after the pVIII stop codon and contains an Xba I restriction enzyme cleavage site (bold). The PCR product produced by primers (5) and (6) was digested with Kpn I and Xba I and incubated, in the presence of DNA ligase, with pUC18 that had been cleaved with Kpn I and Xba I. The ligation reaction products used to transform XL1-Blue cells and plated onto LB/Amp plates.

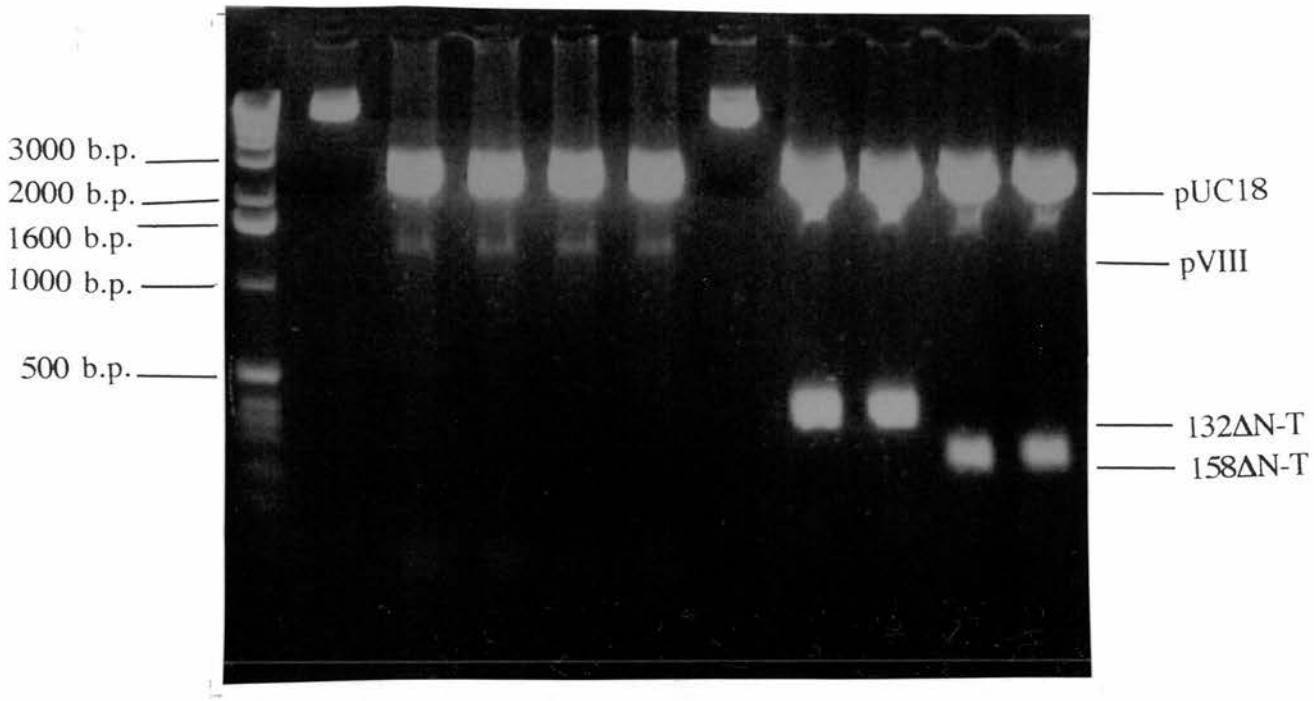


Figure 24: Analysis of DNA fragments produced by restriction enzyme digest of pUCVIII, pUC132ΔN-T and pUC158ΔN-T ligation products. The vectors from colonies VIII A-D were digested with Kpn I and Xba I. Plasmids from colonies132 A-B and 158 A-B were digested in the presence of Eco RI and Xba I. The digestion products were resolved on a 2% (w/v) agarose gel and stained with ethidium bromide (0.5mg/ml)

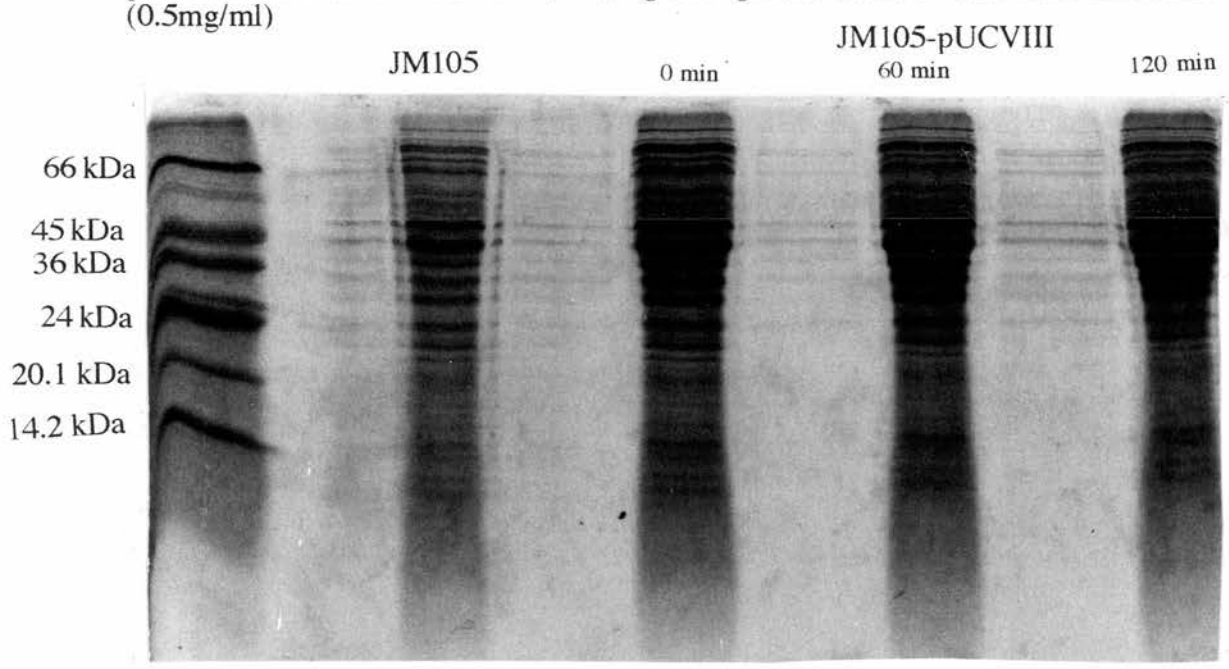


Figure 25: Analysis of induction products produced by JM105 and JM105-pUCVIII cells by SDS-polyacrylamide gel electrophoresis. Production of recombinant pVIII was induced with 0.1M IPTG (4μl/ml) and the cell contents analysed at varying time intervals by loading onto a 20% polyacrylamide gel. Molecular Weight markers were loaded in the first lane and the molecular weights are as indicated.

Four colonies that grew on the restrictive media were selected and named VIII A-D. Minipreps were carried out on four colonies to purify the plasmid and the vector digested with Kpn I and Xba I in order to determine whether the gene coding for pVIII had been inserted into the vector. It was found that all the colonies were positive for the pVIII insert, see figure 24, as a 750 bp band was detected. Colony VIII B was used to produce glycerol stocks and for a maxi prep of the plasmid.

(ii) Expression of recombinant pVIII by JM105 cells transformed with pUCVIII

The plasmid pUCVIII was used to transform the *E.coli* strain JM105 and plated out on LB/Amp agar plates. Three colonies which grew on the restrictive media were selected at random and grown to an optical density of 0.6 at 600nm in 5 ml of LB/Amp, inducing expression with 20µl of IPTG at 37°C for one hour. Cell extracts were prepared from each colony and their expression examined by SDS-PAGE, see figure 25. After staining with Coomassie blue it was observed that the cells did not produce a protein band that was not present in untransformed JM105 cells. The plasmid was subsequently used to transform BL21(DE3) cells but, as with the JM105 cells, it was found that recombinant pVIII was not expressed.

(iii) Construction of pUC111ΔC-T

Plasmid pUC111ΔC-T which encoded for the first 111 amino acid residues of pVIII was constructed. This was achieved by copying the DNA from the linearised plasmid pSETVIII (cut with Nde I) using the oligonucleotide primers:

(5) 5'-TGGGGA**GGTACC**ATGAGCAAGG-3'

(7) 5'-CCGCAC**TCTAGA**ACGTCAGCCGCC-3'

Oligonucleotide (5) was used as the forward primer, see 3.1.2b (i) pUCVIII for details. Primer (7) matches with opposite strand at site corresponding to the pVIII cleavage site closest to the N-terminus of the protein. The Xba I cleavage site is highlighted and the DNA bases coding for Phe 112 are replaced with a stop codon (underlined). The PCR product produced by primers (5) and (7) was digested with Kpn I and Xba I and incubated, in the presence of DNA ligase, with pUC18 that had been cleaved with Kpn I and Xba I. The ligation reaction products used to transform XL1-Blue cells and plated onto LB/Amp plates.

The eight colonies that grew on the restrictive media were selected and named 111 A-H. Minipreps were carried out on four colonies to purify the plasmid and the vector digested with Eco RI and Hind III in order to determine whether the gene coding for pVIII had been inserted into the vector. It was found that all the colonies apart from 111 B were positive for the DNA insert coding for the first 111 amino acids of pVIII, see figure 26, as two bands of size 200 and 150 bp band were detected. The double band was caused by the presence of the Eco RI restriction enzyme site near the start of the pVIII gene. Colony 111 D was used to produce glycerol stocks and for a maxi prep of the plasmid.

(iv) Expression of protein by JM105 cells transformed with pUC111ΔC-T

The plasmid pUC111ΔC-T was used to transform the *E.coli* strain JM105 and plated out on LB/Amp agar plates. Three colonies which grew on the restrictive media were selected at random and grown to an optical density of 0.6 at 600nm in LB/Amp, inducing expression with IPTG. After 1 hour post induction cell extracts were prepared from each colony and their expression examined by SDS-PAGE. After staining with Coomassie blue it was observed that the cells did not produce a protein band that was not present in untransformed JM105 cells. The plasmid was

subsequently used to transform BL21(DE3) cells but, as with the JM105 cells, it was found that recombinant pVIII was not expressed.

(v) Construction of pUC132ΔN-T

The construction of plasmid pUC132ΔN-T, which encoded for the 96 amino acid residues of pVIII immediately after the second protease cleavage site was made using the primers below. The PCR was carried out using linearised plasmid pSETVIII (cut with Nde I) as the template.

(8) 5'-GAAAATCAGA**GAATTC**GGTATT-3'
(6) 5'-CGCTCTGCC**TCTAGA**CTGGTC-3'

Primer (8) is the oligonucleotide which matches with the 5' strand over the sequence coding for the 2nd (from N-terminus) adenovirus 23 kDa protease cleavage site. The oligonucleotide contains an Eco RI cleavage site (bold) just before the adenovirus 23K protease cleavage site. Oligonucleotide (6) was used as the reverse primer, see 3.1.2b (i) pUCVIII for details. The PCR product produced by primers (8) and (6) was digested with Eco RI and Xba I and incubated, in the presence of DNA ligase, with pUC18 that had been cleaved with Eco RI and Xba I. The ligation reaction products used to transform XL1-Blue cells and plated onto LB/Amp plates.

The two colonies that grew on the restrictive media were selected and named 132 A and 132 B. Minipreps were carried out on the two colonies to purify the plasmid and the vector digested with Eco RI and Xba I in order to determine whether the DNA coding for the last 96 amino acids of pVIII had been inserted into the vector. It was found that both colonies were positive for the insert, see figure 24, as a 275 bp band was detected. Colony 132 B was used to produce glycerol stocks and for a maxi prep of the plasmid.

(vi) Expression of protein by JM105 cells transformed with pUC132ΔC-T

The *E.coli* strain JM105 was transformed with the plasmid pUC132ΔC-T and plated out on LB/Amp agar plates. Three colonies which grew on the restrictive media were selected at random and grown to an optical density of 0.6 at 600nm in LB/Amp, inducing expression with IPTG. At 1 hour post induction cell extracts were prepared from each colony and their expression examined by SDS-PAGE. After staining with Coomassie blue it was observed that the cells did not produce a protein band that was not present in untransformed JM105 cells. The plasmid was subsequently used to transform BL21(DE3) cells but, as with the JM105 cells, it was found that recombinant pVIII was not expressed.

(vii) Construction of pUC158ΔN-T

Plasmid pUC158ΔN-T, which was designed to express the last 70 C-terminal amino acids residues of pVIII was produced using the oligonucleotides primers below. Linearised plasmid pSETVIII (cut with Nde I) was used as the template for the PCR.

(9) 5'-GAAAATCAGA**GAATTC**GGTATT-3'
(6) 5'-CGCTCTGCC**TCTAGA**CTGGTC-3'

Oligonucleotide primer (9) matches with the 5' strand over the sequence coding for the third (from N-terminus) adenovirus 23K protease cleavage site. The primer contains an Eco RI cleavage site (bold) immediately before the adenovirus 23K protease cleavage site. Oligonucleotide (6) was used as the reverse primer, see 3.1.2b (i) pUCVIII for details. The PCR product produced by primers (9) and (6) was digested with Eco RI and Xba I and incubated, in the presence of DNA ligase, with

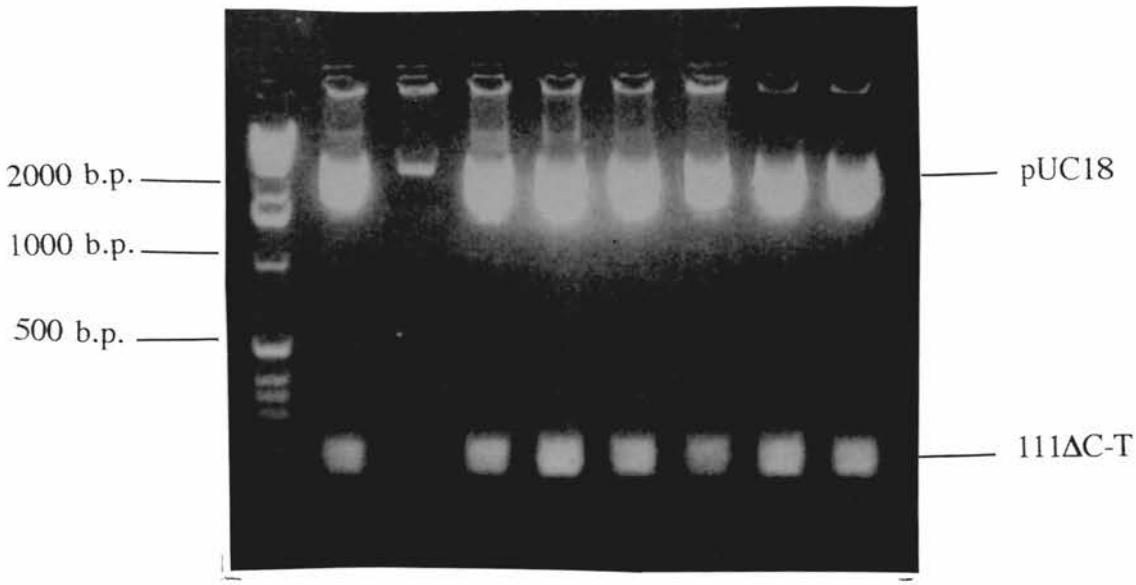


Figure 26: Analysis of DNA fragments produced by an Eco RI and Xba I digest of pUC111ΔC-T ligation products. The vectors from colonies 111 A-H were digested in the presence of Eco RI and Hind III. The digestion products were resolved on a 2% (w/v) agarose gel and stained with ethidium bromide (0.5mg/ml)

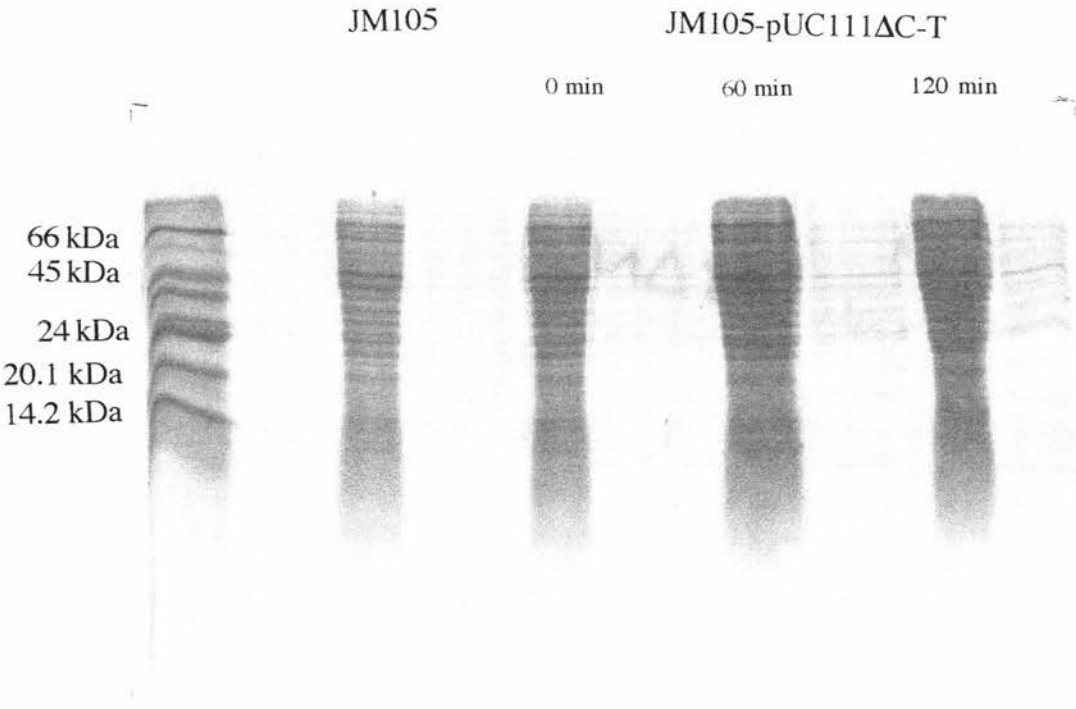


Figure 27: Analysis of induction products produced by JM105 and JM105-pUC158ΔN-T cells by SDS-polyacrylamide gel electrophoresis. Production of recombinant pVIII was induced with 0.1M IPTG (4μl/ml) and the cell contents analysed at varying time intervals by loading onto a 20% polyacrylamide gel. Molecular Weight markers were loaded in the first lane and the molecular weights are as indicated.

pUC18 that had been cleaved with Eco RI and Xba I. The ligation reaction products used to transform XL1-Blue cells and plated onto LB/Amp plates.

The two colonies that grew on the restrictive media were selected and named 158 A and 158 B. Minipreps were carried out on both colonies to purify the plasmid and the vector digested with Eco RI and Xba I in order to determine whether the DNA coding for the last 70 amino acids of pVIII had been inserted into the vector. It was found that both colonies were positive for the insert, see figure 24, as a 200 bp band was detected. Colony 158 A was used to produce glycerol stocks and for a maxi prep of the plasmid.

(viii) Expression of protein by JM105 cells transformed with pUC158ΔN-T

The plasmid pUC158ΔN-T was used to transform the *E.coli* strain JM105 and plated out on LB/Amp agar plates. Three colonies which, grew on the restrictive media were selected at random and grown to an optical density of 0.6 at 600nm in LB/Amp. Expression was induced for 1 hour by the addition of IPTG. Cell extracts were prepared from each colony and their expression examined by low molecular weight SDS-PAGE. After staining with Coomassie blue it was observed that the cells did not produce a protein band that was not present in untransformed JM105 cells, see figure 27. The plasmid was subsequently used to transform BL21(DE3) cells but, as with the JM105 cells, it was found that recombinant pVIII was not expressed.

(c) Deletions mutants in the vector pRSETA

Plasmid was used to transform competent XL1-Blue cells. The transformed cells grown to an optical density of greater than 1.0 at 600nm and a Maxi Prep (see method 2.2.12) performed. The pRSETA obtained had a 260/280 ratio of 1.8 and concentration of 500 ng/μl.

(i) Construction of pSET111ΔC-T

Plasmid pSET111ΔC-T was constructed, encoding a six-histidine tag and the first 111 amino acid residues of pVIII. This plasmid was constructed using the two oligonucleotides primers below and the plasmid pSETVIII, which had been linearised with Nde I, as a template for the PCR.

(10) 5'-CGATGG**GGATCC**ATTATGAGCAAGG-3'

(11) 5'-GGGCGACC**GGATCC**TGTGACGTTAGCC-3'

(12) 5'-CGACCGCACC**CTGAG**CTCTCAGCCGCCCG-3'

(13) 5'-GGTTTCCC**TCTAGA**ATAATTT-3'

(14) 5'-CCGGCT**GGTACC**ACGTCAGCCGCC-3'

(A) Primer (10) matches with the positive strand over the start codon (underlined) of pVIII, in pSETVIII, and contains a Bam HI cleavage site before the start codon. Oligonucleotide primer (11) matches with the opposite strand at the first adenovirus 23 kDa protease cleavage site in pVIII from the N-terminal. The Bam HI cleavage site is highlighted and the DNA bases coding for Phe 112 are replaced with a TTA stop codon (underlined). The plasmid pRSETA was cleaved with Bam HI and treated with Calf Intestinal Alkaline Phosphatase in order to prevent the ligation of the vector without insert.

A maximum of twenty four colonies were selected at random from each ligation reaction and mini preparations of the plasmid DNA performed. The purified DNA was digested with the restriction enzymes used to insert the DNA into the plasmid to check for the presence of, and the length of insert in the plasmid. Several attempts were made using the primers (10) and (11) and after finding no positive colonies it was abandoned and the 3' primer redesigned.

(B) Oligonucleotide (10), see above for details, was used as the 5' primer. Oligonucleotide primer (12) matches with the 3' strand between 335 and 353 base pairs from the pVIII start codon. The Xho I cleavage site is highlighted and the AAA codon which codes for Phe 112, in pVIII, is replace with the stop codon TCA (underlined). The vector was cleaved with Bam HI-Xho I and, when this failed to produce a successful clone, the vector was cleaved with Bam HI and Sal I. Sal I was used as an alternative to Xho I as it produced a compatible 5' overhang and was a unique cleavage site and was present in the multiple cloning site of pRSETA . The PCR product was always digested with Bam HI and Xho I.

A maximum of twenty four colonies were selected at random from each ligation reaction and mini preparations of the plasmid DNA performed. The purified DNA was digested with the restriction enzymes used to insert the DNA into the plasmid to check for the presence of, and the length of insert in the plasmid. After several attempts using the primers (10) and (12) and finding no positive colonies this cloning route abandoned. Both the 5' and 3' primers were redesigned.

(C) Oligonucleotide primer (13) was the 5' primer designed to bind to the pSETVIII template, linearised with Hind III, around the Xba I cleavage site at -39 bp from the start codon of the histidine tagged pVIII. The Xba I cleavage site is highlighted. Oligonucleotide primer (14) was designed against the 3' strand with a stop codon (underlined) to replace the codon coding for Phe 112 in pVIII. The Kpn I cleavage site is located six base pairs after the stop codon and is highlighted. The PCR product produced by primers (13) and (14) was digested with Kpn I and Xba I and incubated, in the presence of DNA ligase, with pRSETA that had been cleaved with Kpn I and Xba I. The ligation reaction products used to transform XL1-Blue cells and plated onto LB/Amp plates.

The ten colonies that grew on the restrictive media were selected and named 111 A-I. Minipreps were carried out on each colony to purify the plasmid and the vector digested with Kpn I and Xba I in order to determine whether the DNA coding for the first 111 amino acids of pVIII had been inserted into the vector. It was found that all the colonies were positive for the insert, see figure 28, as a 500 bp band was detected. The insert size was 500 bp as it included the histidine tag of the pRSET vector. Colony 111 A was used to produce glycerol stocks and for a maxi prep of the plasmid.

(ii) Screening of colonies for the expression of pSET111ΔC-T in BL21(DE3) cells

BL21(DE3) cells were transformed with DNA from a maxi prep of the plasmid pSET110ΔN-T. Six colonies were picked at random and grown to an optical density of 0.6 at 600nm in 5 ml of LB/Amp, inducing expression with 20μl of IPTG at 37°C for one hour. Cell extracts were prepared from each colony and their expression examined by SDS-PAGE, see figure 29. It was found that after staining with Coomassie blue that a 16 kDa protein band was detected in the cell extract of BL21(DE3) cells that was not present in untransformed BL21(DE3) cells. As the quantity of protein expressed by each colony was approximately the same colony number 1 was used to produce glycerol stocks of pSET111ΔC-T in BL21(DE3) cells.

(iii) Construction of pSET227ΔN-T

Plasmid pSET227ΔN-T was constructed, encoding a fusion protein with a six-histidine tag and the last 70 amino acid residues of pVIII. The DNA to be inserted into the vector was generated using the two oligonucleotides primers below and the plasmid pSETVIII, which had been linearised with Nde I, as a template for the PCR.

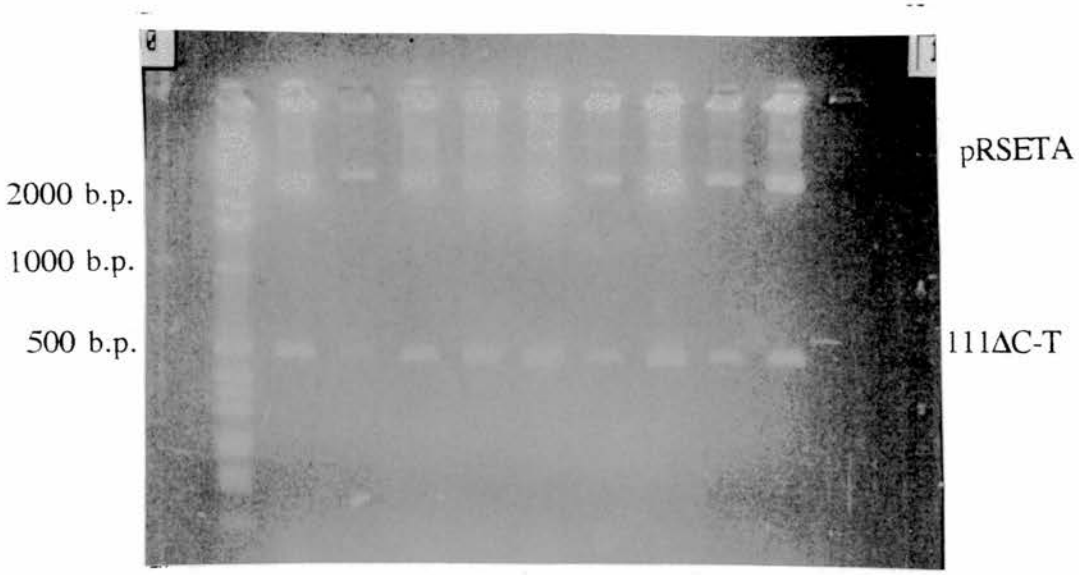


Figure 28: Analysis of DNA fragments produced by restriction enzyme digest of pSET111ΔC-T ligation products. The vectors from colonies 111 A-I were digested in the presence of Bam HI and Kpn I. The digestion products were resolved on a 2% (w/v) agarose gel and stained with ethidium bromide (0.5mg/ml)

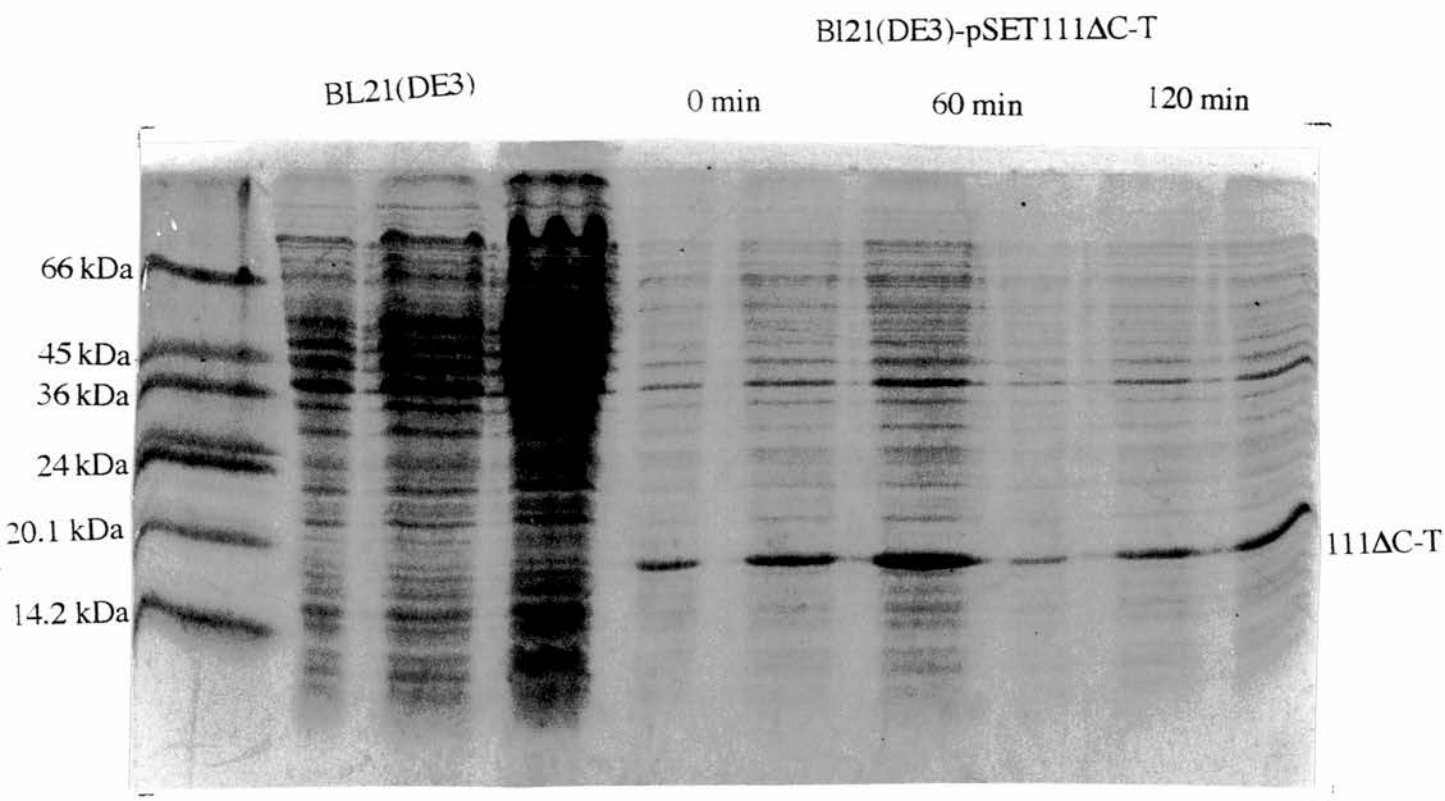


Figure 29: Analysis of induction products produced by BL21(DE3) and BL21(DE3)-pSET111ΔC-T cells by SDS-polyacrylamide gel electrophoresis. Production of recombinant pVIII was induced with 0.1M IPTG (4μl/ml) and the cell contents analysed at varying time intervals by loading onto a 20% polyacrylamide gel. Molecular Weight markers were loaded in the first lane and the molecular weights are as indicated.

(15) 5'-GGCGGC**GGATCC**CGCTCTTCATTTAC-3'

(16) 5'-TTATGCTAGTTATTGCT-3'

(17) 5'-CAGTCGCTCTGC**CTCGAG**ACTGGT-3'

(18) 5'-GCGGCAGTG**GAATTC**GTGTGTCAG-3'

(A) Oligonucleotide primer (15) was designed to match the 5' strand and with a Bam HI cleavage site (highlighted) replacing the two codons coding for Gly 156 and Arg 157. Primer (16) was designed to match the 3' strand of pSETVIII DNA at a site downstream of the pVIII stop codon, the Bam HI cleavage site incorporated into the primer highlighted. The plasmid pRSETA was cleaved with Bam HI and treated with CIAP in order to prevent the ligation of the vector without insert. The Bam HI digested PCR product and digested vector were incubated in the presence of DNA ligase for four hours at room temperature. The ligation reaction products were used to transform competent XL1-Blue cells which were plated out onto LB/Amp agar plates.

A maximum of ten colonies were selected at random from each ligation reaction and mini preparations of the plasmid DNA performed. The purified DNA was digested with Bam HI in order to determine whether or not the DNA coding for the last 70 amino acids of pVIII had been inserted into the vector. After several attempts using the primers (15) and (16) and finding no positive colonies this cloning route abandoned and 3' primer (16) redesigned.

(B) Oligonucleotide (15), see above for details, was used as the primer for the positive strand. Oligonucleotide primer (17) matches with the negative strand after the pVIII stop codon, the Xho I cleavage site is highlighted. The PCR product and vector were digested with Bam HI and Xho I before being incubated together in the presence of DNA ligase for four hours at room temperature. The ligation reaction products were used to transform competent XL1-Blue cells which were plated out onto LB/Amp agar plates.

A maximum of ten colonies were selected at random from each ligation reaction and mini preparations of the plasmid DNA performed. The purified DNA was digested with Bam HI and Xho I in order to determine whether or not the DNA coding for the last 70 amino acids of pVIII had been inserted into the vector. After several attempts using the primers (15) and (17) and finding no positive colonies this cloning route abandoned and 3' primer (17) redesigned.

(C) Primer (15) was designed as the 5' strand primer, see above for details. Oligonucleotide primer (18) was designed with an Eco RI cleavage site and matches against Adenovirus 2 DNA included in the vector pSETVIII after the pVIII stop codon. The PCR product and vector were digested with Bam HI and Eco RI before being incubated together in the presence of DNA ligase for four hours at room temperature. The ligation reaction products were used to transform competent XL1-Blue cells which were plated out onto LB/Amp agar plates.

The fourteen colonies than grew on restrictive media were named 227 A-N. A miniprep was performed on each colony in order to purify the plasmid DNA. The purified DNA was digested with Bam HI and Eco RI in order to determine whether or not the DNA coding for the last 70 amino acids of pVIII had been inserted into the vector. It was found, see figure 30, that colonies 227 B, D, F-H, J, L, and N were all positive colonies producing a 200 bp band. Colony 227 D was used to produce glycerol stocks and for a maxi prep of the plasmid.

(iv) Expression of protein by BL21(DE3) cells transformed with pSET227ΔN-T

The plasmid pSET227ΔN-T was used to transform the *E.coli* strain BL21(DE3) and plated out on LB/Amp agar plates. Six colonies which grew on the restrictive media were selected at random and grown to an optical density of 0.6 at 600nm in LB/Amp,

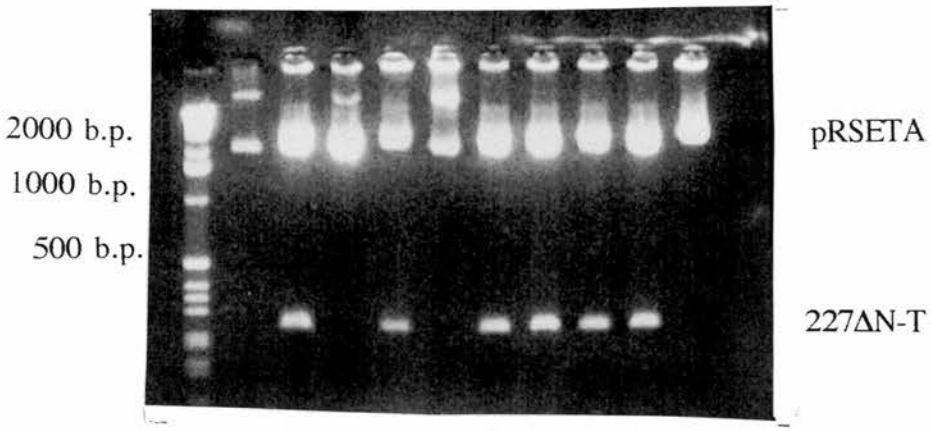


Figure 30: Analysis of DNA fragments produced by a restriction enzyme digest of the vector pSET227ΔN-T. The vectors from colonies 227 A-N were digested in the presence of Bam HI and Eco RI. The digestion products were resolved on a 2% (w/v) agarose gel and stained with ethidium bromide (0.5mg/ml).

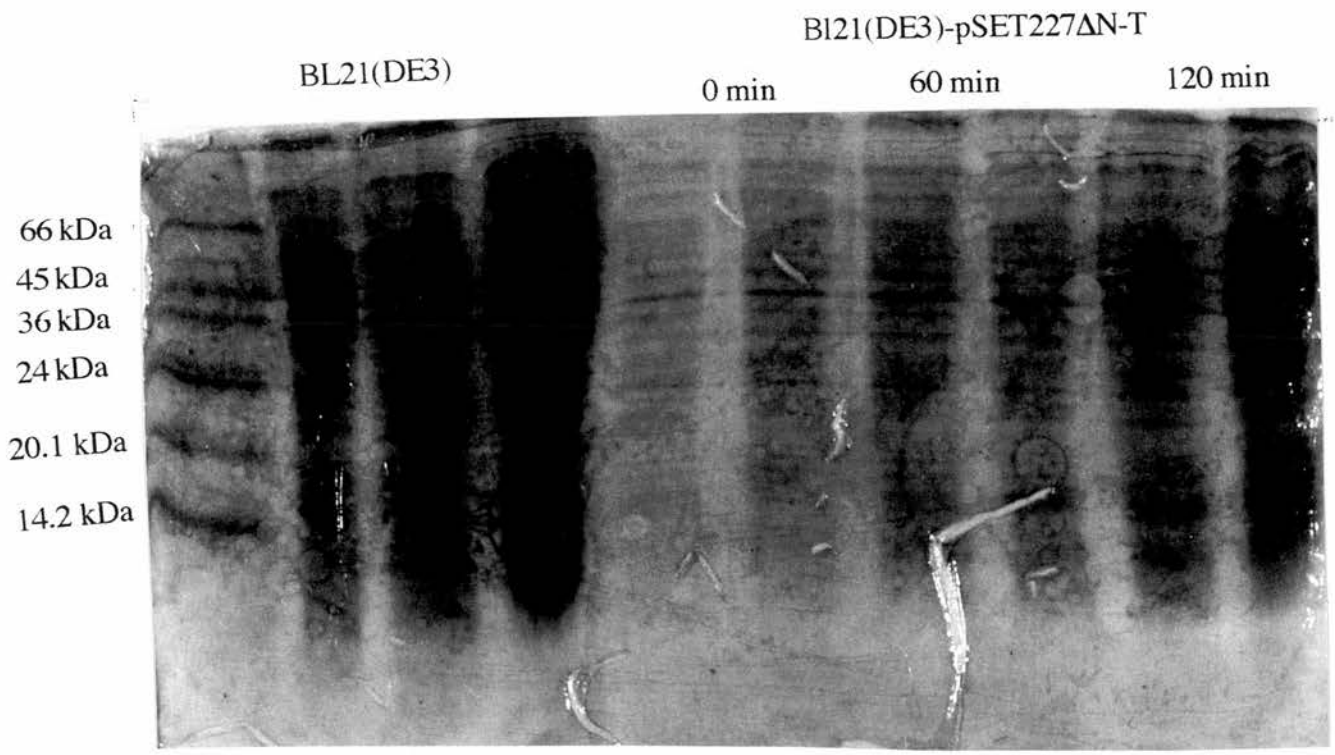


Figure 31: Analysis of induction products produced by BL21(DE3) and BL21(DE3)-pSET227ΔN-T cells by SDS-polyacrylamide gel electrophoresis. Production of recombinant pVIII was induced with 0.1M IPTG (4μl/ml) and the cell contents analysed at varying time intervals by loading onto a 20% polyacrylamide gel. Molecular Weight markers were loaded in the first lane and the molecular weights are as indicated.

expression was induced for 1 hour by the addition of IPTG. Cell extracts were prepared from each colony and their expression examined by SDS-PAGE, see figure 31. After staining with Coomassie blue it was observed that the cells did not produce a protein band that was not present in untransformed BL21(DE3) cells. As the expected molecular weight of the protein was 10.5 kDa it was possible that the protein had not been retained within the gel and therefore a low molecular weight gel was used to analyse the cell extracts. However, it was found that the cells did not express any protein band that was not detected in the untransformed BL21(DE3) cells.

(d) Constructs using the plasmid pGEX 4T-1

(i) Contruction of pGEX55ΔC-T

The plasmid was constructed by cleaving pGEX 4T-1 with Bam HI and Eco RI, the insert was obtained by cleaving pSETVIII with Bam HI and Eco RI and isolating the 165 b.p. fragment produced from digestion. The two digestion products were incubated together in a ligation reaction and the reaction products used to transform competent XL1-Blue cells.

The ten colonies than grew on restrictive media were named 55 A-J. A miniprep was performed on each colony in order to purify the plasmid DNA. The purified DNA was digested with Eco RV in order to determine whether or not the DNA coding for the first 55 amino acids of pVIII had been inserted into the vector. It was found, see figure 32, that colonies 55 A-E were all positive colonies producing a 1900 bp band. This was due to the unique Eco RV cleavage site in the vector pGEX4T1 and the Eco RV restriction site near the start of the pVIII gene. Colony 55 E was used to produce glycerol stocks and for a maxi prep of the plasmid.

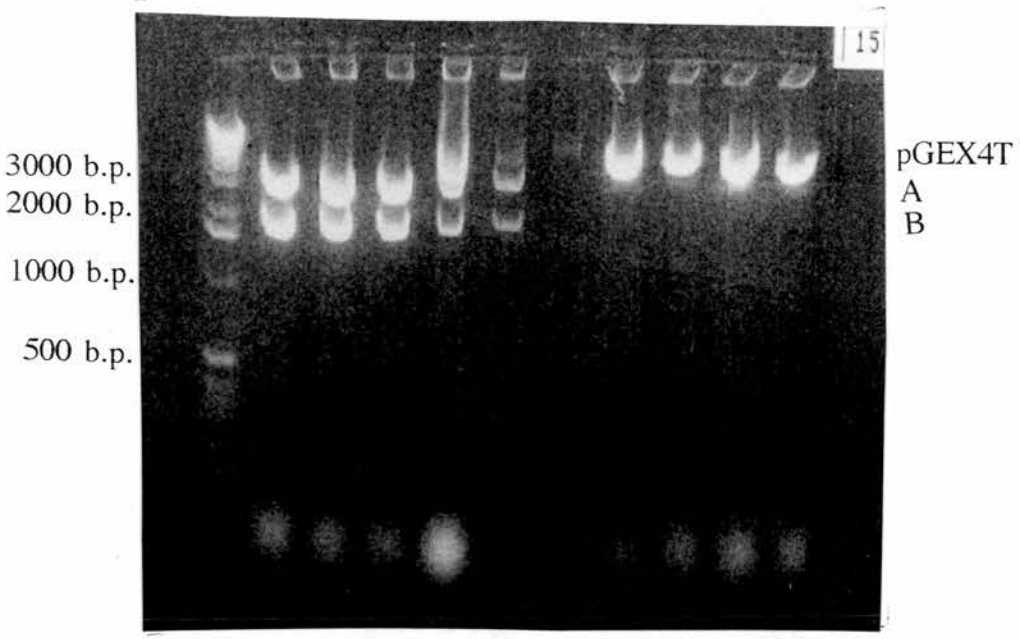


Figure 32: Analysis of DNA fragments produced by a restriction enzyme digest of the pGEX55ΔC-T ligation products. The vectors from colonies 55 A-J were digested in the presence of Eco RV. The digestion products were resolved on a 2% (w/v) agarose gel and stained with ethidium bromide (0.5mg/ml). A and B are th large and small fragments of pGEX55ΔC-T respectively.

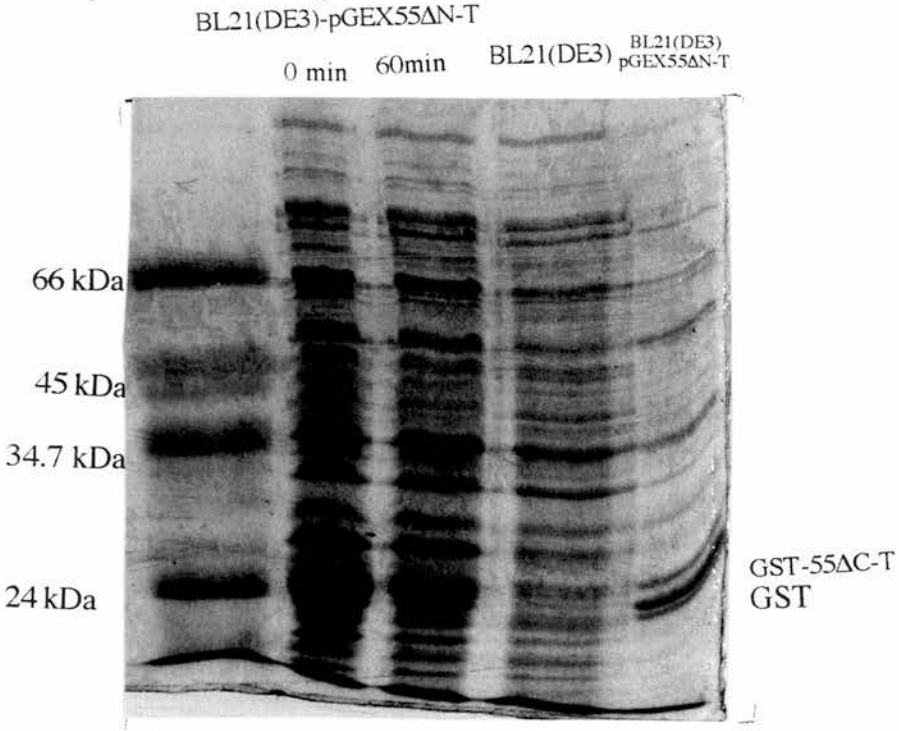


Figure 33: Analysis of induction products produced by BL21(DE3) and BL21(DE3)-pGEX55ΔC-T cells by SDS-polyacrylamide gel electrophoresis. Production of recombinant pVIII was induced with 0.1M IPTG (4μl/ml) and the cell contents analysed at varying time intervals by loading onto a 20% polyacrylamide gel. Molecular Weight markers were loaded in the first lane and the molecular weights are as indicated.

(ii) Screening of colonies for the expression of pGEX55ΔC-T in BL21(DE3) cells

BL21(DE3) cells were transformed with DNA from a maxi prep of the plasmid pGEX55ΔC-T. Two colonies were picked at random and grown to an optical density of 0.6 at 600nm in LB/Amp, and expression induced for 1 hour by the addition of IPTG. Cell extracts were prepared from each colony and their expression examined by SDS-PAGE, see figure 33. It was found that after staining with Coomassie blue that two protein bands were detected in the cell extract of BL21(DE3) cells that was not present in untransformed BL21(DE3) cells at 25 kDa and 30 kDa. The 25 kDa band corresponds to the molecular weight of the GST tag and the 30 kDa band corresponds to the GST-pVIIIΔC-T fusion protein. As the quantity of protein expressed by both colonies were approximately the same colony number 1 was used to produce glycerol stocks of pGEX55ΔC-T in BL21(DE3) cells.

(iii) Construction of pGEX227ΔN-T

The plasmid pGEX227ΔN-T was designed to express the last seventy amino acids of pVIII fused to GST in order to increase the solubility of the protein and to aid in the purification of the protein. The insert was obtained by digesting pSET227ΔN-T with Bam HI and Eco RI and purifying the 210 b.p fragment from agarose. The vector was digested with Bam HI and Eco RI and incubated, with the insert, in a ligation reaction and the reaction products used to transform competent XL1-Blue cells which were plated onto LB/Amp agar plates.

The seventeen colonies than grew on restrictive media were named 227 A-Q. A miniprep was performed on each colony in order to purify the plasmid DNA. The purified DNA was digested with Pst I in order to determine whether or not the DNA coding for the last 70 amino acids of pVIII had been inserted into the vector. It was found, see figure 34, that colonies 227 A-F, and 227 H-O were all positive colonies

producing a 1100 bp band. This was due to the unique Pst I cleavage site in the vector pGEX4T1 and the Pst I restriction site near the end of the pVIII gene. Colony 227 D was used to produce glycerol stocks and for a maxi prep of the plasmid.

(iv) Screening of colonies for the expression of pGEX227ΔN-T in BL21(DE3) cells

A maxiprep of the plasmid pGEX227ΔN-T was used transform competent BL21(DE3) cells. Six colonies were picked at random and grown to an optical density of 0.6 at 600nm in LB/Amp, and expression induced for 1 hour by the addition of IPTG. Cell extracts were prepared from each colony and their expression examined by SDS-PAGE. It was found that after staining with Coomassie blue that three protein bands were detected in the cell extract of BL21(DE3) cells that was not present in untransformed BL21(DE3) cells at 25 kDa, 29 kDa and 33 kDa. The 25 kDa band corresponds to the molecular weight of the GST tag, the 30 kDa band corresponds to a truncated GST-pVIIIΔC-T fusion protein and the 33 kDa band corresponds to the full length GST-pVIIIΔC-T fusion protein. As the quantity of protein expressed by both colonies were approximately the same colony number 1 was used to produce glycerol stocks of pGEX227ΔC-T in BL21(DE3) cells.

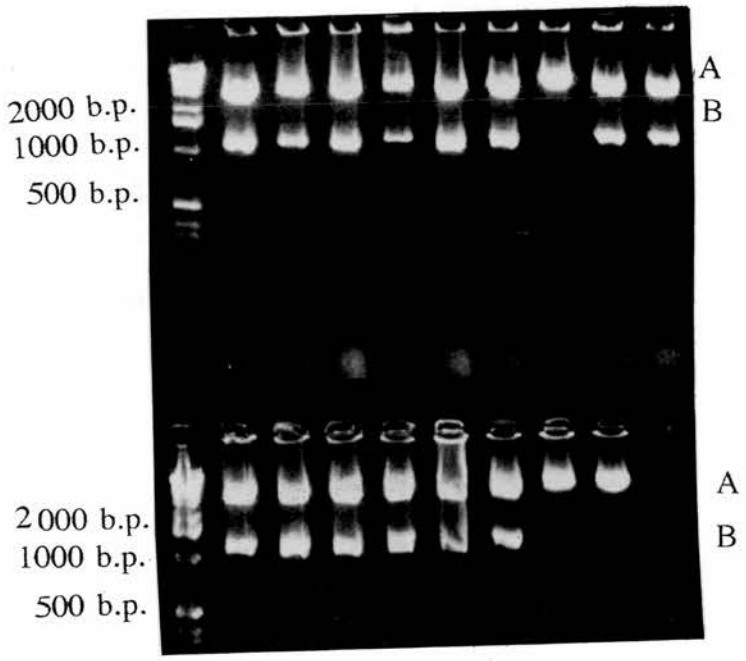


Figure 34: Analysis of DNA fragments produced by restriction enzyme digest of pGEX227ΔN-T ligation products. The vectors from colonies 227 A-Q were digested in the presence of Kpn I and Xba I. The digestion products were resolved on a 2% (w/v) agarose gel and stained with ethidium bromide (0.5mg/ml)

3.1.3. Construction of pGSV and pBSV vectors

(a) Creation of plasmid pGSV

The vector pGSV, see figure 35, was designed to increase the choice of restriction enzymes that could be used with the plasmid pMAL-p2. The pMAL-p2 vector contains the normal *malE* signal sequence, which directs the fusion protein through the cytoplasmic membrane. pMAL-p2 fusion proteins are purified from the cell periplasm where there is less contaminating protein than in the cytoplasm.

The pUC18 multiple cloning site was chosen to replace the pMAL2-p2 MCS as it increases the number of single restriction enzymes sites to twelve. Vector pUC18 was cleaved with Eco RI and Hind III and the 54 bp product ligated into pMAL2-p2. Sequencing of the new vector at the multiple cloning site was performed using the oligonucleotides below.

(19) 5'-AGACGCGCAGACTAATTC-3' (247-5')
(20) 5'-CTGCAAGGCGATTAAGTTGG-3' (248-3')

(b) Creation of plasmid pBSV

The vector pBSV, see figure 36, was designed on the same principle as pGSV but, the vector used was pMAL-c2. The pMAL-c2 vector has a specific 10 amino acid deletion of the malE gene which prevents the secretion of the maltose binding protein fusion.
The pGSV multiple cloning site was chosen to replace the pMAL-c2 MCS (identical to that of pMAL-p2). However, due to the method devised the Sph I restriction enzyme site was not included in pBSV. The omission of Sph I in pBSV restored the Blue/White selection which had been lost by the creation of pGSV.

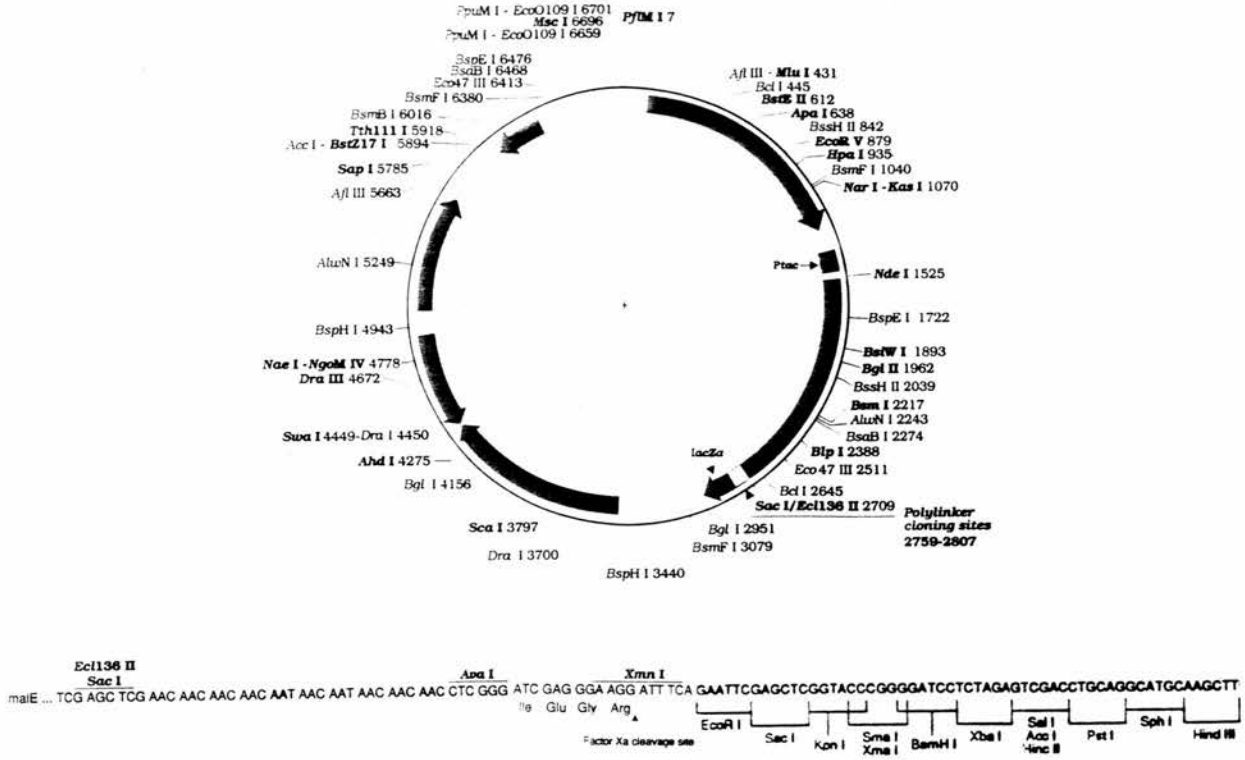


Figure 35: Map of the plasmid pGSV. The map shows the restriction sites of those enzymes that cut the plasmid once or twice; the unique sites are shown in bold type. The multiple cloning site is shown below the map.

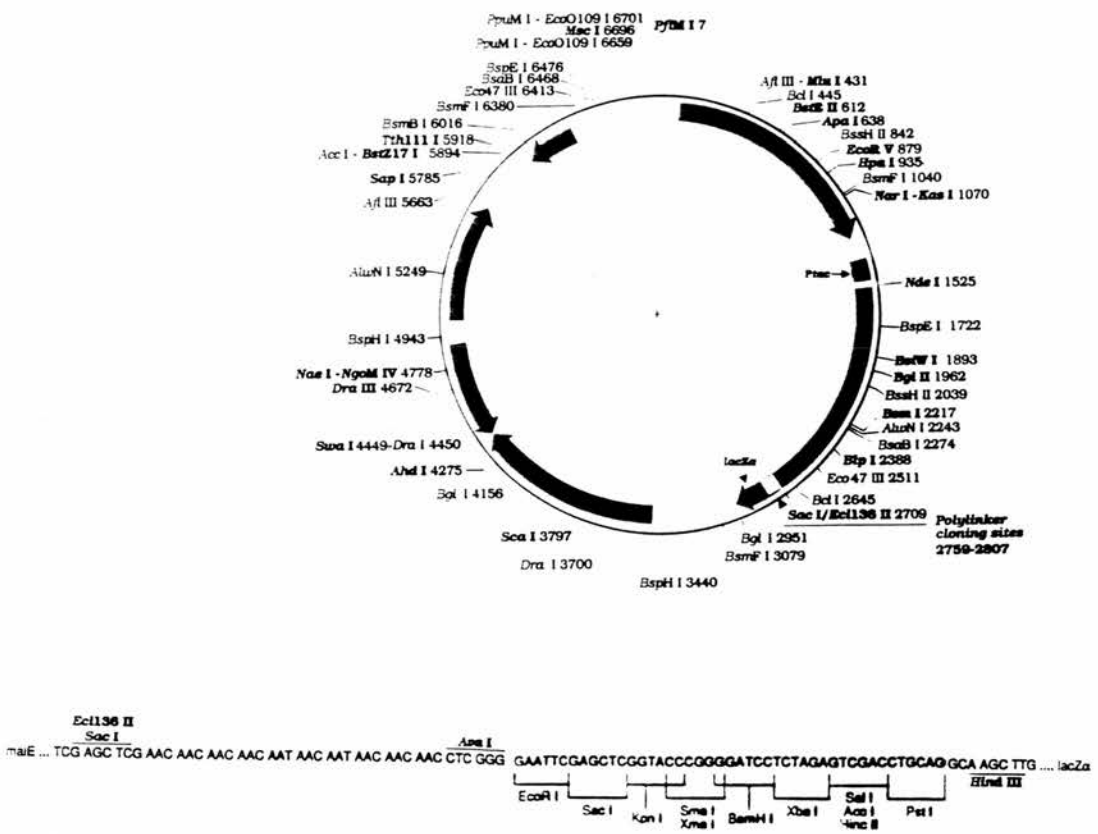


Figure 36: Map of the plasmid pBSV. The map shows the restriction sites of those enzymes that cut the plasmid once or twice; the unique sites are shown in bold type. The multiple cloning site is shown below the map.protein. The pMAL-c2 vector is capable of producing fusion protein than the pMAL-p2 vector.

Vector pMAL-c2 was cleaved with Bgl II and Pst I. Plasmid pGSV was cleaved with Bgl II and Pst I, and the 1000 bp fragment ligated into pMAL-c2. The new vector was named pBSV and contained eleven unique restriction enzyme sites in the MCS and could be used for blue/white selection. Sequencing of the new vector at the multiple cloning site was performed using the oligonucleotides (19) and (20).

3.1.4 Constructs with Maltose Binding Protein Fusions Exported to the Periplasm

(a) Construction of pGSVpVIII

Vector pGSVpVIII which encoded the adenovirus protein pVIII, fused to the maltose binding protein, was constructed by subcloning from the vector pUCVIII. The gene coding for pVIII was cleaved from the vector pUCVIII using Kpn I and Xba I and the 750 bp fragment purified from agarose. The insert was incubated with pGSV, which had been cleaved with Kpn I and Xba I in the presence of T4 DNA ligase. The ligation reaction products were used to transform competent XL1-Blue cells and plated onto LB/Amp agar plates.

The four colonies which grew on the restrictive media were named 1-4 and transferred onto two LB/Amp agar plates, one agar plate contained 80 μg/ml X-gal and 0.3 mM IPTG. These colonies were examined by miniprep, in order to purify the plasmid, and digested with Eco RI and Xba I to determine whether or not the plasmid contained the pVIII gene. It was found that all the colonies were positive for the pVIII gene, see figure 37, as bands at 575 bp and 175 bp were detected. The double band was a caused by the presence of an Eco RI restriction enzyme site near the start of the pVIII gene. Colony 1 was selected for use and glycerol stocks and a maxi prep were made using the corresponding positive colony that had not been grown on agar plates containing X-Gal.

(b) Expression of recombinant proteins by plasmids pGSVpVIII in ER2508 cells

Plasmid pGSVpVIII was used to transform ER2508 cells. Two colonies were picked at random and grown to an optical density of 0.6 at 600nm in 5 ml of LB/Amp, inducing expression with 20µl of IPTG at 37°C for one hour. Cell extracts were prepared from each colony and their expression examined by SDS-PAGE. It was found, after staining with Coomassie blue, that the plasmids pGSVpVIII did not express a visible protein band that was not present in the ER2508 cells, see figure 38. Similar results were also obtained when XL1-Blue and BL21(DE3) cells were transformed with the plasmid. Therefore it was concluded that either the vector was unable to express the protein in the three *E.coli* strains or unable to express the protein in sufficient quantities to enable the purification of the protein from the cellular proteins.

(c) Constructs of pMAL158ΔN-T

The plasmid pMAL158ΔN-T was constructed by subcloning the genetic material coding for the C-terminal of pVIII from pUC227ΔN-T. This was done by cleaving pUC227ΔN-T with Eco RI and Xba I and purifying the 210 bp fragment from agarose. The insert was incubated with pMALp2, which had been cleaved with Eco RI and Xba I and the contents of the ligation reaction used to transform competent XL1-Blue cells and plated onto LB/Amp agar plates.

The two colonies which grew on the restrictive media were named 158A and 158B and were transferred onto two LB/Amp agar plates, one agar plate contained 80 µg/ml X-gal and 0.3 mM IPTG. These colonies were examined by miniprep, in order to purify the plasmid, and digested with Eco RI and Xba I to determine whether or not the plasmid contained the pVIII gene. It was found that both colonies were positive for the

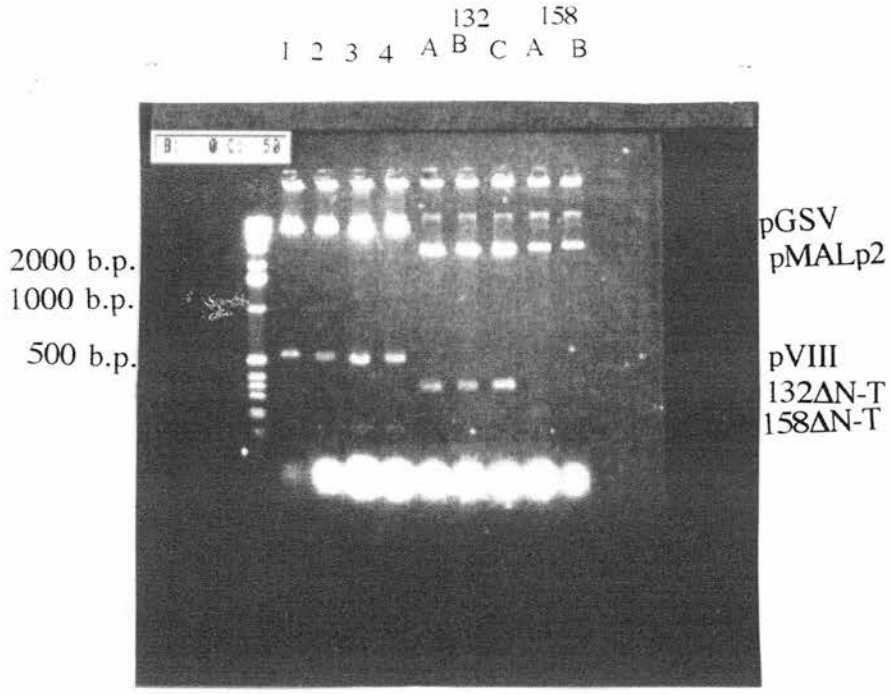


Figure 37: Analysis of DNA fragments produced by restriction enzyme digest of pGSVpVIII, pMAL132ΔN-T and pMAL158ΔN-T ligation products. The vectors from colonies 1-4, 132 A-C and 158 A-B were digested in the presence of Eco RI and Xba I. The digestion products were resolved on a 2% (w/v) agarose gel and stained with ethidium bromide (0.5mg/ml)

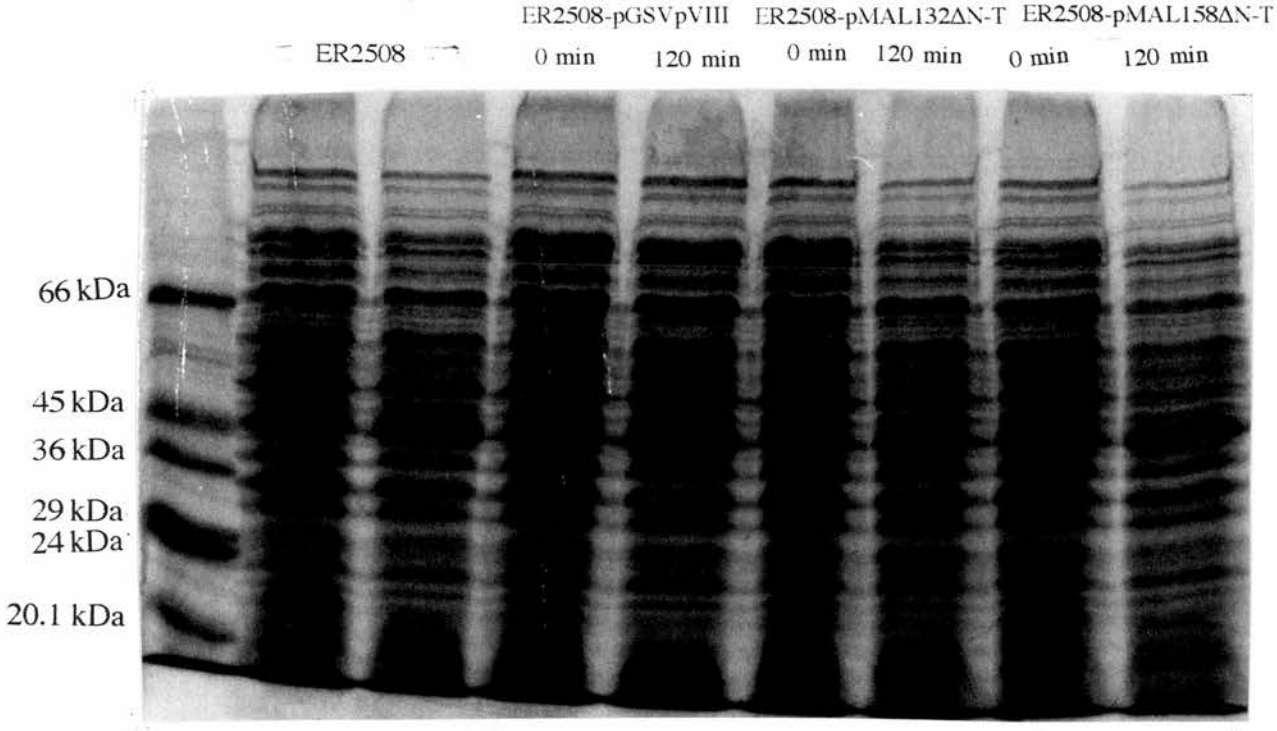


Figure 38: Analysis of induction products produced by ER2508 and ER2508-pGSVpVIII, ER2508-pMAL132ΔN-T and ER2508-pMAL158ΔN-T cells by SDS-polyacrylamide gel electrophoresis. Production of recombinant pVIII was induced with 0.1M IPTG (4μl/ml) and the cell contents analysed at 2 hours post induction by loading onto a 20% polyacrylamide gel. Molecular Weight markers were loaded in the first lane and the molecular weights are as indicated.

pVIII genetic material coding for amino acids 158-227, see figure 37, as a single 200 bp band was detected. Colony 158 B was selected for use and glycerol stocks and a maxi prep were made using the corresponding positive colony that had not been grown on agar plates containing X-Gal.

(d) Expression of recombinant proteins by plasmids pMAL158ΔN-T in ER2508 cells

A maxi prep of plasmid pMAL158ΔN-T was used to transform competent ER2508 cells. Two colonies were picked at random and grown to an optical density of 0.6 at 600nm in LB/Amp, expression was induced for one hour by the addition of IPTG. Cell extracts were prepared from each colony and their expression examined by SDS-PAGE. It was found, after staining with Coomassie blue, that the plasmids pMAL158ΔN-T did not express a visible protein band that was not present in the ER2508 cells, see figure 38. Similar results were also obtained when XL1-Blue and BL21(DE3) cells were transformed with the plasmid. Therefore it was concluded that either the vector was unable to express the protein in the three *E.coli* strains or unable to express the protein in sufficient quantities to enable the purification of the protein from the cellular proteins.

(e) Construction of pMAL132ΔN-T

The subcloning of the genetic material encoding for the last 96 amino acid residues of pVIII was subcloned into the pMALp2 vector from the plasmid pUC132ΔN-T. Vector pUC132ΔN-T with digested with Eco RI and Xba I and the resulting 300 bp fragment purified from 2% (w/v) agarose. The fragment was used in a ligation reaction with purified pGSV, which had been previously digested with Eco RI and Xba I. The contents of the ligation reaction were used to transform competent XL1-Blue cells and plated onto restrictive agar plates.

The three colonies which grew on the restrictive media were named 132 A, 132 B and 132 C. The colonies were transferred onto two LB/Amp agar plates, one agar plate contained 80 μg/ml X-gal and 0.3 mM IPTG. These three colonies were examined by miniprep, in order to purify the plasmid, and digested with Eco RI and Xba I to determine whether or not the plasmid contained the insert. It was found that all three colonies were positive for the pVIII genetic material coding for amino acids 132-227, see figure 37, as a single 300 bp band was detected. Colony 132 A was selected for use and glycerol stocks and a maxi prep were made using the corresponding positive colony that had not been grown on agar plates containing X-Gal.

(f) Expression of recombinant proteins by plasmids pMAL132ΔN-T in ER2508 cells

A maxi prep of plasmid pMAL132ΔN-T was used to transform competent ER2508 cells. Four colonies were picked at random and grown to an optical density of 0.6 at 600nm in LB/Amp, expression was induced for one hour by the addition of IPTG. Cell extracts were prepared from each colony and their expression examined by SDS-PAGE It was found, after staining with Coomassie blue, that the plasmids pMAL132ΔN-T did not express a visible protein band that was not present in the ER2508 cells, see figure 38. Similar results were also obtained when XL1-Blue and BL21(DE3) cells were transformed with the plasmid. Therefore it was concluded that either the vector was unable to express the protein in the three *E.coli* strains or unable to express the protein in sufficient quantities to enable the purification of the protein from the cellular proteins.

3.1.4 Constructs with Maltose Binding Protein Fusions Retained in the Cytoplasm

(a) Construction of pBSVpVIII

Vector pBSVpVIII was created by subcloning the gene coding for pVIII from pGSVpVIII and inserting it into pBSV. The gene coding for pVIII was isolated from pGSVpVIII by incubating the plasmid with Kpn I and Xba I for three hours at 37°C and purifying the 750 bp fragment from agarose. The insert was incubated with pBSV, which had been cleaved with Kpn I and Xba I, at room temperature for 4 hours. The contents of the ligation reaction was used to transform competent XL1-Blue cells and plated onto LB/Amp agar plates.

Colonies which grew on restrictive media were labelled and transferred onto two LB/Amp agar plates, one agar plate contained 80 μg/ml X-gal and 0.3 mM IPTG. The screening of colonies was enhanced by using the blue/white selection capability of the malE-lacZα fusion. Colonies which failed to turn blue, when grown in an α complementing host, i.e. XL1-Blue, indicated an inactivation of the ß-galactosidase α fragment activity and therefore either contained the pVIII gene or a mutation. The six colonies that did not turn blue were named 8A-F and the plasmid purified from each colony using the miniprep procedure. In order to determine if the vector contained the pVIII gene the plasmids were digested with Kpn I and Hind III, see figure 39. It was found that colonies 8 A, 8 B, 8 D and 8F were positive as they produced a 750 bp band. The minipreps of colonies 8 C and 8 E were contaminated with bacterial DNA so it was not possible to determine whether or not the plasmids contained pVIII. Colony 8 A was selected for use and glycerol stocks and a maxi prep were made using the corresponding positive colony that had not been grown on agar plates containing X-Gal.

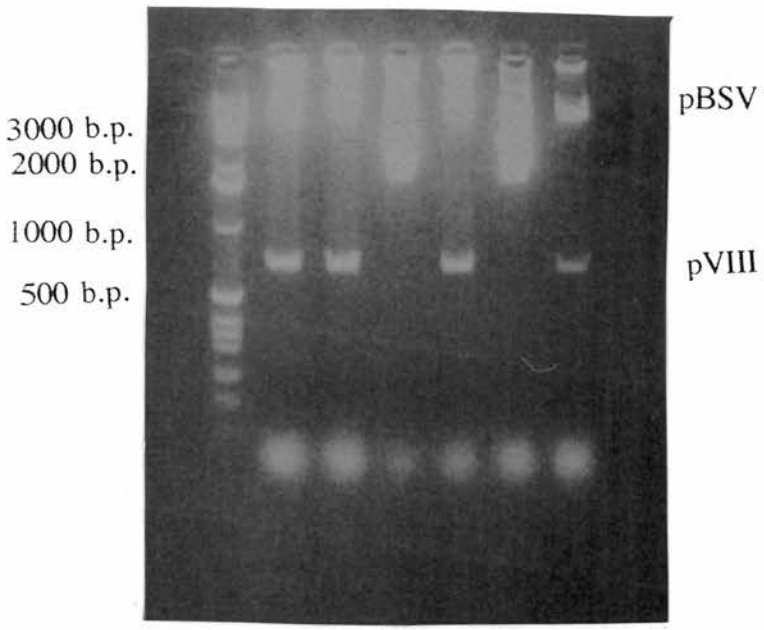


Figure 39: Analysis of DNA fragments produced by restriction enzyme digest of pBSVpVIII ligation products. The vectors from colonies 1-6 were digested in the presence of Kpn I and Hind III. The digestion products were resolved on a 2% (w/v) agarose gel and stained with ethidium bromide (0.5mg/ml)

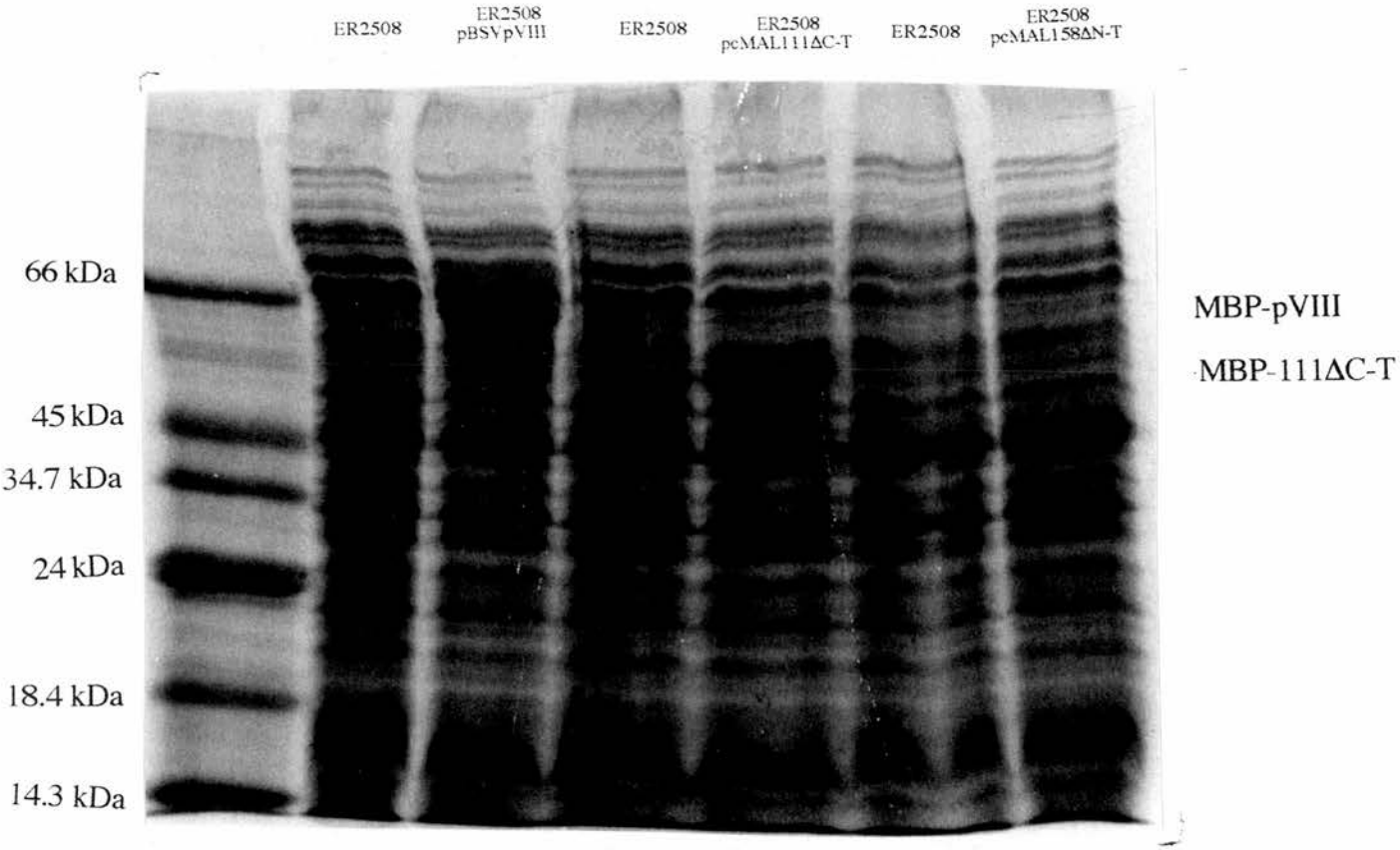


Figure 40: Analysis of induction products produced by ER2508 and ER2508-pBSVpVIII, ER2508-pcMAL111ΔC-T and ER2508-pcMAL158ΔN-T cells by SDS-polyacrylamide gel electrophoresis. Production of recombinant pVIII was induced with 0.1M IPTG (4μl/ml) and the cell contents analysed at 2 hours post induction by loading onto a 20% polyacrylamide gel. Molecular Weight markers were loaded in the first lane and the molecular weights are as indicated.

(b) Expression of recombinant proteins by plasmids pBSVpVIII in ER2508 cells

A maxi prep of plasmid pBSVpVIII was used to transform competent ER2508 cells. A colony was picked at random and grown to an optical density of 0.6 at 600nm in LB/Amp, expression was induced for one hour by the addition of IPTG. A cell extract was prepared from the colony and the expression examined by SDS-PAGE, see figure 40. It was found, after staining with Coomassie blue, that the plasmid pBSVpVIII expressed a visible protein band at 70 kDa that was not present in the ER2508 cells, this band corresponds to the full length MBP-pVIII fusion protein.

(c) Construction of pBSV111ΔC-T

Plamid pBSV111ΔC-T was constructed by subcloning the gene fragment coding for the N-terminal of pVIII from pUC110ΔC-T. Vector pUC110ΔC-T was digested with Kpn I and Xba I and the resulting 350 bp fragment purified from 2% (w/v) agarose. Plamid pBSV was also cleaved with Kpn I and Xba I and purified from agarose. The insert and the digested vector were incubated with T4 DNA Ligase at room temperature. After four hours the contents of the ligation reaction were used to transform competent XL1-Blue cells which were subsequently plated onto LB/Amp agar plates.

Colonies which grew on restrictive media were labelled and transferred onto two LB/Amp agar plates, one agar plate contained X-gal and IPTG. The screening of colonies was enhanced by using the blue/white selection capability of the malE-lacZα fusion. The six colonies that did not turn blue were named 158 A-F and the plasmid purified from each colony using the miniprep procedure. In order to determine if the vector contained the DNA coding amino acid 1-111 of pVIII the plasmids were digested with Eco RV, see figure 41. It was found that colony 158 F was positive as it

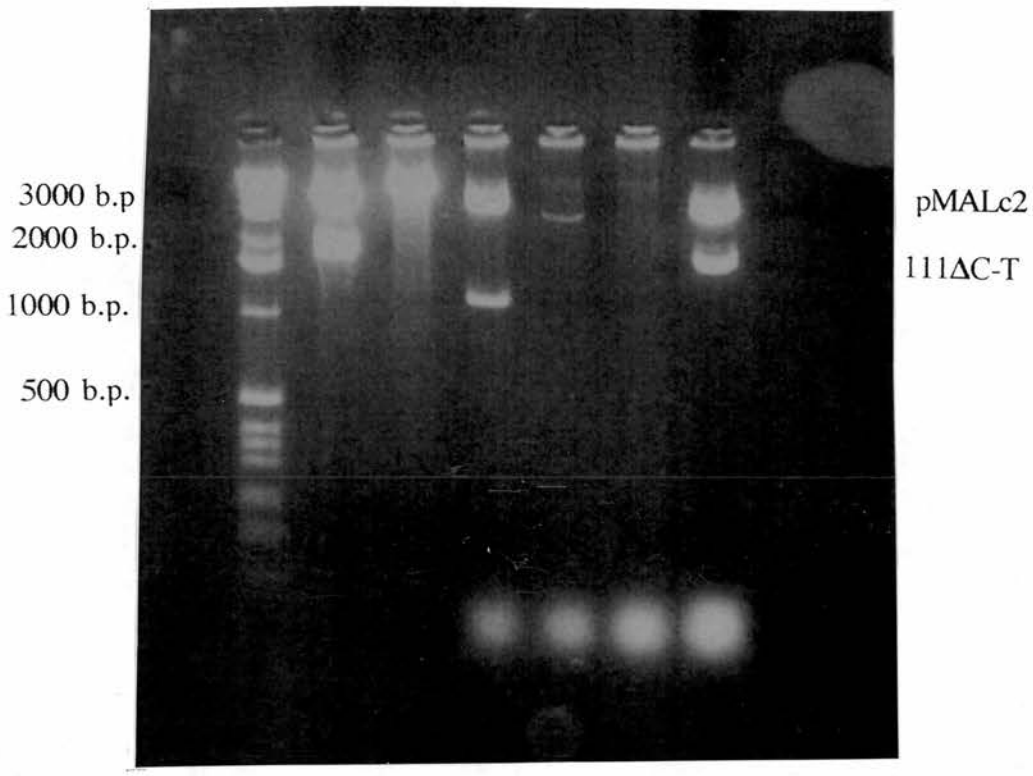


Figure 41: Analysis of DNA fragments produced by restriction enzyme digest of pBSV111ΔC-T ligation products. The vectors from colonies 111 A-F were digested in the presence of Eco RV. The digestion products were resolved on a 2% (w/v) agarose gel and stained with ethidium bromide (0.5mg/ml)

produced a 2000 bp band due to the unique Eco RV site in the vector pBSV and the internal Eco RV cleavage site present in the DNA coding for the pVIII C-Terminal deletion mutant. Colony 158 F was selected for use and glycerol stocks and a maxi prep were made using the corresponding positive colony that had not been grown on agar plates containing X-Gal.

(d) Expression of recombinant proteins by plasmids pBSV111ΔC-T in ER2508 cells

A maxi prep of plasmid pBSV111ΔC-T was used to transform competent ER2508 cells. A colony was picked at random and grown to an optical density of 0.6 at 600nm in LB/Amp, expression was induced for one hour by the addition of IPTG. A cell extract was prepared from the colony and the expression examined by SDS-PAGE, see figure
40. It was found, after staining with Coomassie blue, that the plasmids pBSVpVIII expressed a visible protein band at 58 kDa that was not present in the ER2508 cells, this band corresponds to the full length MBP-pVIIIΔC-T fusion protein.

(e) Construction of pBSV227ΔN-T

The gene fragment encoding for the last 70 amino acid residues of pVIII was subcloned into the pBSV vector from the plasmid pUC227ΔN-T. Vector pUC227ΔN-T was digested with Eco RI and Xba I, the resulting 200 bp fragment was purified from 2% (w/v) agarose. The vector pBSV, which had been cleaved with Eco RI and Xba I for 3 hours at 37°C was purified from 1% (w/v) agarose. The insert and the vector were heat shocked prior to the addition of the DNA ligase and ligase buffer. After four hours at room temperature the contents of the ligation reaction were used to transform competent XL1-Blue cells which were subsequently plated onto LB/Amp agar plates.

Colonies which grew on restrictive media were labelled and transferred onto two LB/Amp agar plates, one agar plate contained X-gal and IPTG. The screening of colonies was enhanced by using the blue/white selection capability of the malE-lacZα fusion. The eight colonies that did not turn blue were named 227 A-H and the plasmid purified from each colony using the miniprep procedure. In order to determine if the vector contained the pVIII gene the plasmids were digested with Eco RI and Xba I, results not shown. It was found that colonies 227 H was positive as it produced a 300 bp band. Colony 227 H was selected for use and glycerol stocks and a maxi prep were made using the corresponding positive colony that had not been grown on agar plates containing X-Gal.

(f) Expression of recombinant proteins by plasmids pBSV158ΔN-T in ER2508 cells

A maxi prep of plasmid pBSV158ΔN-T was used to transform competent ER2508 cells. A colony was picked at random and grown to an optical density of 0.6 at 600nm in LB/Amp, expression was induced for one hour by the addition of IPTG. A cell extract was prepared from the colony and the expression examined by SDS-PAGE, see figure 40. It was found, after staining with Coomassie blue, that the plasmids pBSV158ΔN-T did not express a visible protein band that was not present in the ER2508 cells. Similar results were also obtained when XL1-Blue and BL21(DE3) cells were transformed with the plasmid. Therefore it was concluded that either the vector was unable to express the protein in the three *E.coli* strains or unable to express the protein in sufficient quantities to enable the purification of the protein from the cellular proteins.

3.2 Construction of a Plasmid which Expresses the EDS L3 23K Protease

A genomic fragment of the EDS DNA coding for the protease was identified by Dr D. Todd and a copy supplied in the vector BlueScript with a partial sequence of the

fragment. The protease gene was cloned into the vector pET11c at Nde I and Bam HI restriction enzyme sites by Lewis Murray creating the vector pETEDS. Attempts to purify the EDS protease from BL21(DE3) cells which had been transformed with pETEDS failed as the cells did not express the EDS protease.

Subcloning of the gene was performed by cleaving the pETEDS vector with Xba I and Bam HI. Xba I cleaves pETEDS upstream of the protease start (ATG) codon and upstream of the Shine-Dalgarno site. BamHI cleaves pETEDS downstream of the protease stop (TAA) codon. Plasmid pRSETA was cleaved with Xba I, which cleaves upstream of the Shine-Dalgarno site and Bam HI which cleaves downstream of the histidine tag. The gene coding for the EDS protease was inserted into pRSETA replacing the Shine-Dalgarno site with that of pET11c and replacing the histidine tag of pRSETA with the protease gene.

3.2.1 Screening of Colonies

BL21(DE3) cells were transformed with DNA from a maxi prep of the plasmid pSETEDS. Six colonies were picked at random and grown overnight in 5 ml of LB/Amp at 37°C. A 0.5 ml aliquot of the overnight culture was used to inoculate 5 ml of LB/Amp which was grown at 37°C with shaking until the optical density at 600 nm reached 0.6. Each 5 ml culture was induced for 1 hour by the addition of 20 µl of 0.1M IPTG. The cultures were harvested by centrifugation for 1 minute in a microcentrifuge and the pellets resuspended in 100 µl of PBS. The samples were sonicated three times for 30 seconds on ice and 20 µl were analysed by SDS-PAGE, see figure 42. It was found, after performing a Western Blot and probing with anti-protease antiserum that a single band was detected in the lanes that contained cells transformed with the plasmid pSETEDS, see figure 43. Glycerol stocks were made using the colony which expressed the greatest quantity of fusion protein and stored at -70°C.

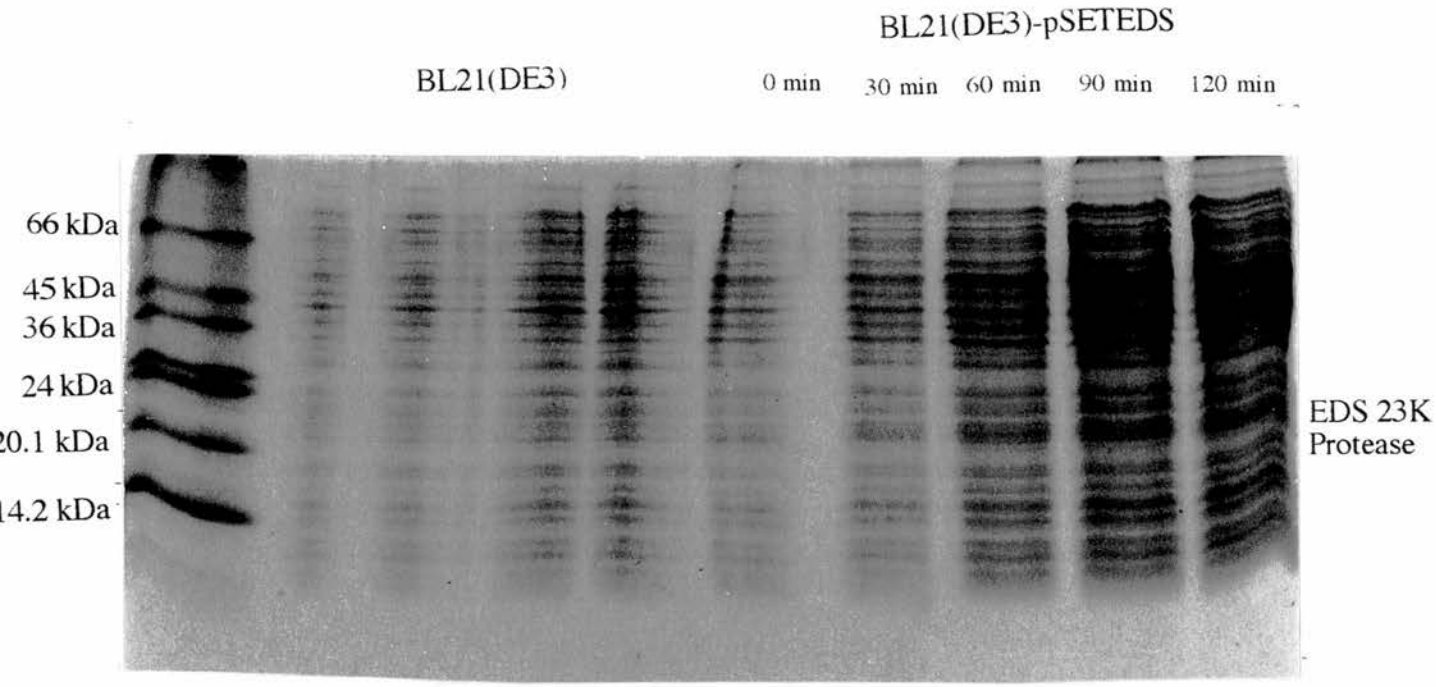


Figure 42: Analysis of induction products produced by BL21(DE3) and BL21(DE3)-pSETEDS cells by SDS-polyacrylamide gel electrophoresis. Production of recombinant pVIII was induced with 0.1M IPTG (4μl/ml) and the cell contents analysed at varying time intervals by loading onto a 20% polyacrylamide gel. Molecular Weight markers were loaded in the first lane and the molecular weights are as indicated.

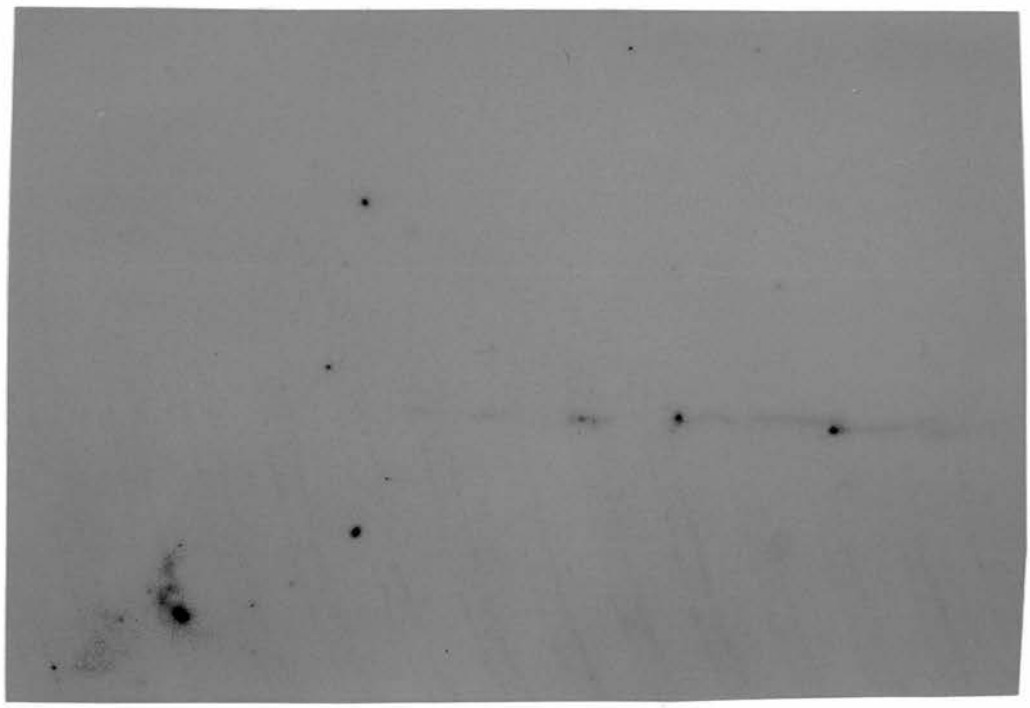


Figure 43: Western blot analysis of induction products from BL21(DE3) and BL21(DE3)-pSETEDS cells. Production of recombinant protease was induced with 0.1M IPTG (4μl/ml). The contents of BL21(DE3) and BL21(DE3)-pSETEDS cells were sampled at two hours post induction and electrophoresed, blotted onto Hyperbond-PVDF and probed with anti-protease N-Terminal antisera. Molecular weights indicated are taken from position of Molecular Weight Standards on blot after staining with amido black.

3.2.2 DNA Sequencing of the EDS 1976 virus L3 23 kDa Protease

The EDS 1976 virus L3 23 kDa protease had previously been partially sequenced by Dr D. Todd see figure 44. However, there remained three base pairs which had not been called and thus three amino acids in the c-terminal half of the protease were unidentified. In order to establish the identity of the three unknown bases the six sequencing primers below were designed, using the program Amplify, under the principle of using primer walking to sequence the DNA fragment, see figure 45.

(1) 5'-GGTTTCCCTCTAGAATAATTT-3'
(2) 5'-GTTACACCATTTACATGTTTG-3'
(3) 5'-GAAGCTTCTATATTTCAAGCT-3'
(4) 5'-TTATGCTAGTTATTGCT-3'
(5) 5'-CATAGCTTGAAATATAGAAGC-3'
(6) 5'-CAAACATGTAAATGGTGTAAC-3'

Sequencing primer (1) is designed against the 5' strand of the vector pRSETA and binds upstream of the start codon and Shine-Dalgarno site between bases -29 to -49. The oligonucleotide also contains an Xba I restriction enzyme site which is underlined. The oligonucleotide (2) was designed to prime against the 5' strand of the EDS DNA between 194 and 214 as it was assumed that the sequencing would produce an accurate read length of approximately 300 bases. The last 5' primer was designed to bind between bases 424 and 444, assuming the accurate read length to be 300 the primer product would extend beyond the EDS protease stop codon at bases 607 to 609.

In order to verify the results obtained from the forward reading primers the DNA sequence was sequenced in the opposite direction. Oligonucleotide (4) was designed to prime against the 3' sequence of pRSETA over the multiple frame stop codon

```
1                                                                   50
ATGAGCGGCA CATCCGAATC AGAGTTGAAA GCATTGATGA AATCCTTAGG
M  S  G  T   S  E  S   E  L  K   A  L  M  K   S  L  G

51                                                                 100
CATAGCCGGT AATTTTCTCG GGACTTTCGA CTGTACATTT CCTGGTTTTA
 I  A  G   N  F  L  G   T  F  D   C  T  F   P  G  F  I

101                                                                150
TTAACAAACA TAAACGCCAA ACGGCAATCA TTAATACCGG CTCTCGGGCA
  N  K  H   K  R  Q   T  A  I  I   N  T  G   S  R  A

151                                                                200
AGCGGCGGTT TACATTGGCT TGCGTTTGCG TGGGACCCGC TTCGTTACAC
S  G  G  L   H  W  L   A  F  A   W  D  P  L   R  Y  T

201                                                                250
CATTTACATG TTTGATCCTT TGGGATGGAA AGAAAAGGAT CTTTTCAAAC
 I  Y  M   F  D  P  L   G  W  K   E  K  D   L  F  K  L

251                                                                300
TTTATGGGTT TTCCTATAAG ACTATGATTA AGCGGTCAGC TTTACAGAGT
  Y  G  F   S  Y  K   T  M  I  K   R  S  A   L  Q  S

301                                                                350
GACAACAGAT GTGTAAAACT TGTAAAAAAT ACTGAAGCTG TACAGTGTAC
D  N  R  C   V  K  L   V  K  N   T  E  A  V   Q  C  T

351                                                                400
TTGCGCTGGT AGTTGTGGTT TATTTTGTGT ATTTTTTCTG TATTGTTTTA
 C  A  G   S  C  G  L   F  C  V   F  F  L   Y  C  F  N

401                                                                450
ACTTGTGTCA CATTAATCCC TTTGAAGCTT CTATATTTCA AGCTATGCAT
  L  C  H   I  N  P   F  E  A  S   I  F  Q   A  M  H

451                                                                500
GGTACCTCCC CGGCTTTGTA TCCTTCCAAA CCTCATCTGT TGSATGCTAA
G  T  S  P   A  L  Y   P  S  K   P  H  L  L   ?  A  N

501                                                                550
TCAGCAAATG TTATACGATT TTCTTCGCTC GCACAGKTCC TATTTTGKAA
 Q  Q  M   L  Y  D  F   L  R  S   H  ?  S   Y  F  ?  N

551                                                                600
ATAATGAACG CACACTGGTT TGCAATACTA AACTCAATTT AATAAACATT
  N  E  R   T  L  V   C  N  T  K   L  N  L   I  N  I

601   609
CATCAATAA
H  Q  *
```

Figure 44. Partial nucleotide sequence of EDS L3 23 kDa protease with translated amino acid sequence. The start codon (ATG) and stop codon (TAA) are underlined.

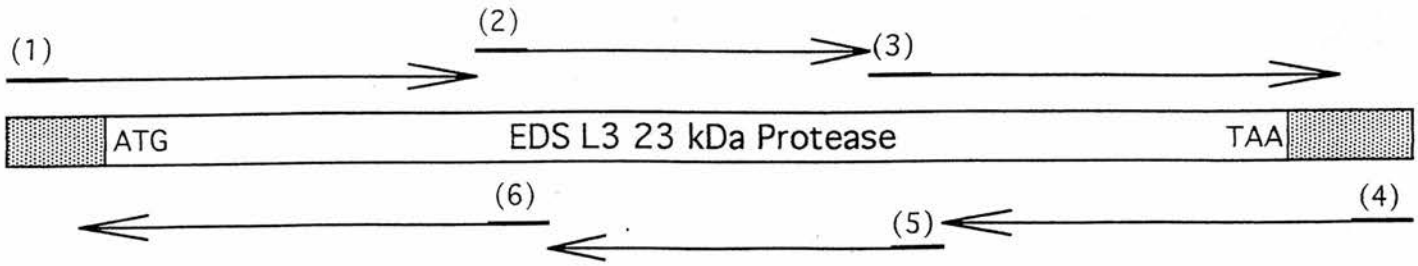


Figure 46. Sequencing by primer walking along the EDS protease gene. The numbers of the primers correspond to the primers used to sequence the gene.

region. The two stop codons included in the primer are underlined and the final thymine base of the primer is part of the third stop codon. Sequencing primer (5) binds against the 3' strand between 427 and 447, approximately 300 bases from the priming site of primer (4). Oligonucleotide (6) is designed to prime against bases 194 to 214 of the 3' strand and is the opposite of primer (2). Assuming a reliable read length of 300 bases the product of primer (6) would extend beyond the start codon of the EDS protease.

3.2.3 Sequencing Data for the EDS L3 23K Protease

The reliability of the primers varied from an accurate read length of 248 bases for primer (6) to 598 bases for primer (1). The accurate read length of a primer was taken to the point where the third base was either uncalled or miscalled. Using this definition the average read length was approximately 400 bases and all primers, with the exception of primer (2), produced a sequence result. All bases were called at least once in both forward and reverse directions.

The nucleotide sequence obtained, see figure 46 is identical to the sequence of the EDS protease submitted by Hess *et al.,* 1997 and is in agreement with the partial sequence.

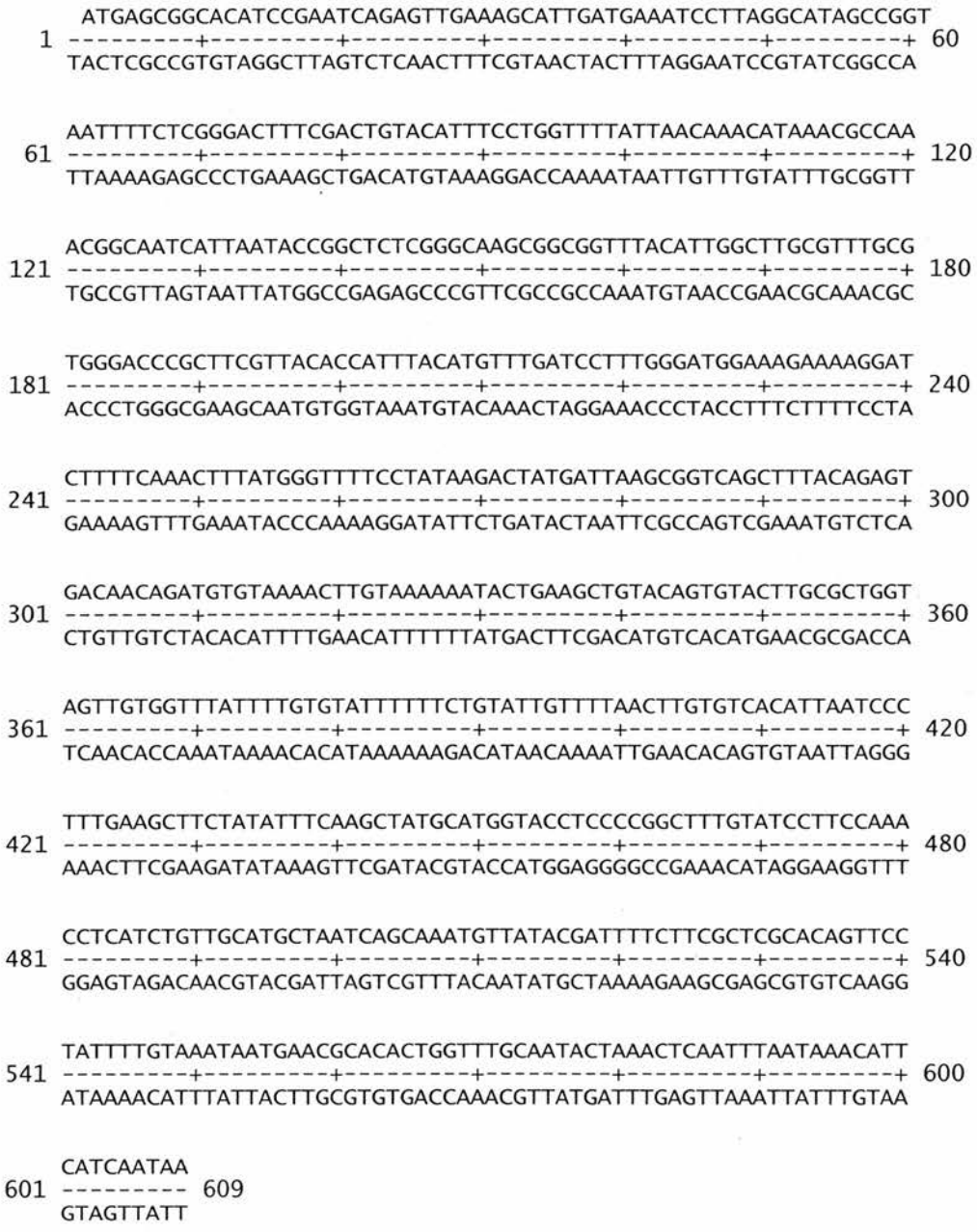


Figure 46. Complete nucleotide sequence of the EDS L3 23 kDa protease.

3.2.4 Computer Software Analysis of the EDS L3 23K Protease Gene

(a) Analysis of EDS Protease nucleotide sequence using the program Translate.

The GCG program Translate was used to translate the EDS L3 23 kDa protease sequence, see figure 47. The translated polypeptide is 202 a.a. long which falls within the known size range of 201-214 a.a. for the adenovirus protease. The catalytic triad of His, Cys and Asp are conserved and are located at residues 55, 122 and 72 respectively. Binding of the activating peptide will be to the cysteine residue located at position 104. The previously unidentified amino acids are a histidine at position 165, a serine residue at position 179 and a valine residue at position 183.

MSGTSESELK ALMKSLGIAG NFLGTFDCTF PGFINKHKRQ TAIINTGSRA
SGGLHWLAFA WDPLRYTIYM FDPLGWKEKD LFKLYGFSYK TMIKRSALQS
DNRCVKLVKN TEAVQCTCAG SCGLFCVFFL YCFNLCHINP FEASIFQAMH
GTSPALYPSK PHLLHANQQM LYDFLRSHSS YFVNNERTLV CNTKLNLINI
HQ

Figure 47. Translation of EDS L3 23 kDa nucleotide sequence. The catalytic triad of H55, C122 and D72 are highlighted as is the activating binding C104.

(b) Analysis of EDS Protease predicted amino acid sequence using the program Fasta.

The GCG Fasta program conducts a Pearson and Lipman search for similarity between the EDS protease amino acid sequence and all other known adenovirus protease sequences. Results obtained are displayed in table 5, variable factors were all used at their default settings. The data for bovine 4 is not included in the table below as only a partial sequence was available. However, there was a 68.3% similarity in the 63 a.a. overlap but, as the N-terminal region of the protease is

generally conserved to a greater extent than the C-terminal this value cannot be regarded as representative for the entire protease.

Adenovirus Serotype	% Similarity to EDS	Length of overlap (a.a.)
bovine 7	57.7	201
ovine 287	56.7	201
murine 1	44.0	184
human 41	43.5	184
human 40	42.4	184
bovine 2	42.1	197
human 12	40.6	187
bovine 10	40.5	190
bovine 3	40.4	198
human 3	40.2	204
canine u	40.2	199
human 5	39.6	197
human 2	39.6	197
human 4	38.8	188
human 17	37.5	200

Table 5. Similarity between the EDS L3 23 kDa Protease and all known adenovirus protease sequences.

(c) Pileup Analysis of EDS Protease and all known adenovirus protease sequences

The GCG program Pileup creates a multiple sequence alignment using the progressive pairwise alignment method of Feng and Doolittle. All variable parameters are used at their default settings. The results obtained are shown in figure 48. The alignment of the sequences show that catalytic residues His 55 and Cys 122 are conserved in all serotypes. The third catalytic residue Asp 72 is only replaced by glutamic acid in two serotypes, the Ad2 protease being one of these. Also conserved in all serotypes is the activating peptide binding residue Cys 104.

```
                1                                                   50
 vprt_adeeds  .....MSGTS ESELKALMKS LGIAGNFLGT FDCTFPGFIN KHKRQTAIIN
  vprt_ade02  ......MGSS EQELKAIVKD LGCGPYFLGT YDKRFPGFVS PHKLACAIVN
  vprt_ade03  MTCGSGNGSS EQELKAIVRD LGCGPYFLGT FDKRFPGFMA PDKLACAIVN
  vprt_ade04  ....MAAGSG EQELRAIIRD LGCGPYFLGT FDKRFPGFMA PHKVACAIVN
  vprt_ade05  ......MGSS EQELKAIVKD LGCGPYFLGT YDKRFPGFVS PHKLACAIVN
  vprt_ade12  ......MGSS EQELTAIVRD LGCGPYFLGT FDKRFPGFVS RDRLSCAIVN
  vprt_ade17  .....MSGSS ERELASIVRD LGCGPTFWAP TTQRFPGFLA GDKLACAIVN
  vprt_ade40  ......MGSS EQELVAIVRE LGCGPYFLGT FDKRFPGFMA PHKLACAIVN
  vprt_ade41  ......MGSS EQELVAIARD LGCGSYFLGT FDKRFPGFMA PNKLACAIVN
  vprt_adeb2  ......MGSR EEELRAIVRD LGISPYFLGT FDKRFPGFLH KDKLSCAIVN
  vprt_adeb3  ......MGSR EEELRFILHD LGVGPYFLGT FDKHFPGFIS KDRMSCAIVN
  vprt_adeb4  .....MSGTS ESELKHLLSS LHLTYGFLGT FDCRFPGFLQ KNKVQTAIVN
  vprt_adeb7  .....MSGLS EKEVFLLLSS LQCTHGFLGT FDCRFPGFIN KVKVQTAIIN
 vprt_adeb10  ......MGTS EEELKHIVID LGCGPFFLGI NDKHFPGFLN KQSNACAIVN
  vprt_adem1  ......MGSS ETELRQLVAD LGIGS.FLGI FDKHFPGFIS VNKPACAIVN
  vprt_adecu  ...MAEGGSS EEELRAIVRD LAVTPFFLGT FDKRFPGFIS SQRITCAVVN
vprt_adeo287  .....MSGTS ESELKNLISS LHLNNGFLGI FDCRFPGFLQ KSKIQTAIIN

                51      *                   *                   100
 vprt_adeeds  TGSRASGGLH WLAFAWDPLR YTIYMFDPLG WKEKDLFKLY GFSYKTMIKR
  vprt_ade02  TAGRETGGVH WMAFAWNPRS KTCYLFEPFG FSDQRLKQVY QFEYESLLRR
  vprt_ade03  TAGRETGGEH WLAFGWNPRY NTCYLFDPFG FSDERLKQIY QFEYEGLLRR
  vprt_ade04  TAGRETGGEH WLAFAWNPRS NTCYLFDPFG FSDQRLKQIY QFEYEGLLRR
  vprt_ade05  TAGRETGGVH WMAFAWNPHS KTCYLFEPFG FSDQRLKQVY QFEYESLLRR
  vprt_ade12  TAGRETGGVH WLAFGWNPKS HTCYLFDPFG FSDQRLKQIY QFEYESLLRR
  vprt_ade17  TAGRETGGVH WLAFGWNPRS RTCYMFDPFG FSDRRLKQIY SFEYEAMLRR
  vprt_ade40  TAGRETGGVH WLALAWNPKN RTCYLFDPFG FSDERLKQIY QFEYEGLLKR
  vprt_ade41  TAGRETGGVH WLALAWNPKS HTCYLFDPFG FSDERLKQIY QFEYEGLLKR
  vprt_adeb2  TAARETGGAH WLALAWFPNA KNFYFFDPFG FSDHKLKQIY QFEYEGLLRR
  vprt_adeb3  TAGRETGGVH WLAMAWHPAS QTFYMFDPFG FSDQKLKQIY NFEYQGLLKR
  vprt_adeb4  TGPREKGGVH WVAMAWDP.. .......... .......... ..........
  vprt_adeb7  TGPREQGGIH WIALAWDPKS YQMFIFDPLG WKNDQLMKYY KFSYSNLIKR
 vprt_adeb10  TASRETGGVH WIAMGWHPPS N.FYLFDPFG FSDKKLLQIY QFEYNALLKR
  vprt_adem1  TASRETGGVH WLAMAWYPTS STFYLFDPFG FSDRKLQQVY KFEYERLLKR
  vprt_adecu  TAGRETGGVH WLAMAWNPRS KTFYMFDPFG FSDSKLKQVY SFEYEGLLRR
vprt_adeo287  TGPREQGGIH WITLALEPIS YKLFIFDPLG WKDTQLIKFY NFSLNSLIKR

               101      x                          *                150
 vprt_adeeds  SALQS.DNRC VKLVKNTEAV .....QCTCA GSCGLFCVFF LYCFNLCHIN
  vprt_ade02  SAIASSPDRC ITLEKSTQSV .....QGPNS AACGLFCCMF LHAFANWPQT
  vprt_ade03  SALA.TKDRC ITLEKSTQSV .....QGPRS AACGLFCCMF LHAFVHWPDR
  vprt_ade04  SALA.TKDRC VTW.KSHQTC RVRVGRCGFS AACSTACA.. ......WP.T
  vprt_ade05  SAIASSPDRC ITLEKSTQSV .....QGPNS AACGLFCCMF LHAFANWPQT
  vprt_ade12  SALAATKDRC VTLEKSTQTV .....QGPFS AACGLFCCMF LHAFTHWPDH
  vprt_ade17  SAVASSPDRC LSLEQSTQTV .....QGPDS AACGLFCCMF LHAFVHWPDR
  vprt_ade40  SALASTPDHC ITLIKSTQTV .....QGPFS AACGLFCCMF LHAFVNWPTS
  vprt_ade41  SALASTPDHC ITLVKSTQTV .....QGPFS AACGLFCCMF LHAFIHWPSN
  vprt_adeb2  SALAG..DGC VNLVKSTETV .....QGPNS AACGLFCCMF LHAFVNWPDR
  vprt_adeb3  SALTSTADRC LTLIQSTQSV .....QGPNS AACGLFCCMF LHAFVRWPLR
  vprt_adeb4  .......... .......... .......... .......... ..........
  vprt_adeb7  SALSS.PDKC VKVIKNSQSV .....QCTCA GSCGLFCVFF LYCFYKYKSN
 vprt_adeb10  SAITSSPDRC VQLFQNNESV .....QSPHS AACGLYCCMF LHAFANWPAH
  vprt_adem1  SAVSSSSSKC VTLVKSHQTV .....QGPHS AACGLFCVLF LAAFGKYPQN
  vprt_adecu  SAIASTPDRC VTLAKSNETI .....QGPNS AACGLFCCMF LHAFVNWPDN
vprt_adeo287  SALNN.SDRC ITVERNTQSV .....QCTCA GSCGLFCIFF LYCFHFYKQN
```

```
            151                                                   200
 vprt_adeeds  PFEASIFQAM HGTSPA...L YPSKPHLLHA NQQMLYDFLR SHSSYFVNNE
  vprt_ade02  PMDHNPTMNL ITGVPNSMLN SPQVQPTLRR NQEQLYSFLE RHSPYFRSHS
  vprt_ade03  PMDGNPTMKL VTGVSNSMLQ SPQVQPTLRR NQEVLYRFLN THSSYFRSHR
  vprt_ade04  PMDKNPTMNL LTGVPNGMLQ SPQVEPTLRR NQEALYRFLN SHSAYFRSHR
  vprt_ade05  PMDHNPTMNL ITGVPNSMLN SPQVQPTLRR NQEQLYSFLE RHSPYFRSHS
  vprt_ade12  PMDKNPTMDL LTGVPNCMLQ SPQVVGTLQR NQNELYKFLN NLSPYFRHNR
  vprt_ade17  PMDGNPTMNL LTGVPNGMLQ SPQVLPTLRR NQEELYRFLA RHSPYFRSHR
  vprt_ade40  PMERNPTMDL LTGVPNSMLQ SPQVVPTLRH NQERLYRFLA QRSPYFQRHC
  vprt_ade41  PMEQNPTMDL LTGVPNSMLQ SPQVEPTLRR NQERLYRFLT QHSPYFRRHR
  vprt_adeb2  PMTRNPTMDL LTGVPNADMM KPSSLAILRE NQNQLYKFLS THSQYFRTHR
  vprt_adeb3  AMDNNPTMNL IHGVPNNMLE SPSSQNVFLR NQQNLYRFLR RHSPHFVKHA
  vprt_adeb4  .......... .......... .......... .......... ..........
  vprt_adeb7  AFKNCLFQSL YGSIPS...L TPPNPTNLHK NQDFLYKFFK EKSLYFRQNE
 vprt_adeb10  PFD.NPTMDQ LVGVPNNMLE APRAQSIFKQ NQETLYSFLH YNSSFFRRYE
  vprt_adem1  PMNNNPIMGP IEGVPNDQMF NPCYTKTLYR NQQWVYSYLN KNSLYFRLHV
  vprt_adecu  PFNHNPTMGP LKSVPNYKLY DPTVQHVLWE NQEKLYKFLE KNSAYFRAHA
vprt_adeo287  VFKSWLFQKL NGSTPS...L IPCEPHLLHE NQTFLYDFLN AKSVYFRKNY

            201                       225
 vprt_adeeds  RTLVCNTKLN LINIHQ.... .....
  vprt_ade02  AQIRSATSFC HLKNM..... .....
  vprt_ade03  ARIERATAFD RMDMQ..... .....
  vprt_ade04  ARIEKATAFD RMNQDM.... .....
  vprt_ade05  AQIRSATSFC HLKNM..... .....
  vprt_ade12  ERIEKATSFT KMQNGLK... .....
  vprt_ade17  AAIEHATAFD KMKQLRVSQ. .....
  vprt_ade40  ERIKKATAFD QMKNNM.... .....
  vprt_ade41  ERIEKATAFD QMKNAQVLFH NKIFY
  vprt_adeb2  PQIERDTSFN KLLELKNQ.. .....
  vprt_adeb3  AQIEADTAFD KMLTN..... .....
  vprt_adeb4  .......... .......... .....
  vprt_adeb7  EYIVSNTKIG LIKSHI.... .....
 vprt_adeb10  NKLRKQTDP. .......... .....
  vprt_adem1  ELIKKNTAFD KLLVRK.... .....
  vprt_adecu  AAIKTRTAFN KLKQ...... .....
vprt_adeo287  RTFIENTKTG LIKTH..... .....
```

Figure 48. Pileup GCG analysis of all known adenovirus protease sequences. The catalytic triad of His 54, Asp/Glu 71 and Cys 122 are asterisked. Cys 104, the residue which interacts with the pVIct is crossed.

(d) Isoelectric point determination of the EDS L3 23 kDa Protease

The ExPASY program pI/Mw Tool was used to determine the theoretical isoelectric point of the EDS protease, human adenovirus serotype 2, see table 6. The computer program makes a plot of the positive and negative charges and the net charge of the protein as a function of pH. The isoelectric point, pI, of a protein is the pH at which the net charge of the polypeptide is zero and the predicted results for EDS and Ad2 are shown in the table below.

Adenovirus Serotype	Isoelectric Point
Ad2	8.95
EDS	9.06

Table 6. Predicted isoelectric points of the EDS and human Ad2 proteases.

(e) Motifs of Adenovirus proteases

The GCG program Motifs was used to search all the known adenovirus proteases for known motifs. Adenovirus human serotypes 12, 40 and 41 contained a prokaryotic membrane lipoprotein lipid attachment site. This result probably has little significance other than the fact that in the light of this motif Ad12, Ad40 and Ad41 recombinant protease may prove problematical to purify from prokaryotic cells.

(f) Predicted Secondary structure of EDS protease

The GCG program PeptideStructure was use to predict the secondary structure of the EDS L3 23 kDa protease according to Chou-Fasman. The structure of the human adenovirus serotype 2 was also analysed as the crystal structure of the Ad2 protease is known. The actual structure of the Ad2 protease was determined using a stereo view of all the main chain atoms and a MOLSCRIPT generated figure of the adenovirus protease (Ding *et al.*, 1996). See figure 49 for details.

The predicted structure of EDS protease is similar either to the predicted structure of the Ad2 protease or the actual structure of the protease until the final catalytic residue. From Cys 122 onwards the C-terminal of the EDS is dominated by ß-sheets unlike the predicted and crystal structure of the protease which are both dominated by helices.

```
      1                                                  50
EDS   .ttttHHHHH HHHHHH.... .bbbbbbbbb TThhhhhh.B BBBBBBtttt
Ad2p  .tttHHHHHH HHHHHtt..B BBBBB....T T...TT.BBB BBBBBB...T
Ad2c  ........HH HHHHH..... BBBBBB.... .........B BBBBB.....

      51                                                 100
EDS   TTtHHHHHHH HH.BBBBBBB B.TTtHHHHH HHHH.....h hhhhhhhhhT
Ad2p  T.HHHHHHHH .TTt.BBBBB .TT..tt..B BBBBBBhhhh hhhhhhhhhh
Ad2c  ....BBBBBB B....BBBBB ......HHHH HHHH....HH HHHHHHHHH.

      101                                                150
EDS   TTTBBBBB.. ..BBBBBBTT TTtBBBBBBB BBBBBBBB.T THHHHHHHHH
Ad2p  TTBBBBB... BBBBB.TThh hhhhhhhhhh hhhh...... tttt.BBBBB
Ad2c  ...BBBBBB. BBB....... .HHHHHHHHH HHHH...... ..........

      151                                                204
EDS   ttttt..TTh hhhhhhhBBB BBBBB.ttTT BBBBBttBBB BBttBBBBBB B.
Ad2p  BBTT...... ........tt ..BBBBBB.. .tTT..tt.h hhhhhhhh.. ....
Ad2c  .BBBB..... ....HHHHHH HHHHHHHHHH H.HHHHH.HH HHHHH..... ....
```

Figure 49. Predicted Secondary structure of the EDS L3 23 kDa protease. The human adenovirus serotype 2 protease predicted structure is indicated by Ad2p and the structure as determined by crystallography as Ad2c. H denotes an alpha helix, B denotes a ß-sheet.

3.3 Purification of histidine tagged fusion proteins

3.3.1 Purification of histidine tagged pVIII fusion protein

Colony 8A was grown to an Optical density at 600 nm of 1.0 in 250 ml of L-Broth. The cells were centrifuged, lysed and the pSETVIII DNA extracted using a Qiagen 500 filter column. The purified vector was used to transform competent BL21(DE3)-pLysE cells. The BL21(DE3)-pLysE cells were selected for the expression of the fusion protein as it was thought that the failure of the pin-point Xa cloning route was due to the fusion protein being a toxic protein. The BL21(DE3)-pLysE cells are resistant to low quantities of toxic proteins owing to a higher than normal rate of lysozyme production. This allows the cell to grow normally as the over expression of the T7 lysozyme gene suppresses the production of the fusion protein. However, this control mechanism ceases to function after induction of the fusion protein and expression of the protein kills the cell. The disadvantage of this system is that only small quantities of the fusion protein are produced as three vectors are present in the cell. Expression of the fusion protein in BL21(DE3)-pLysE cells, with pSETVIII,

was carried out by growing the cells in 250 ml of L-Broth containing carbenicillin and chloramphenicol to an optical density at 600 nm of 0.6 units. Production of the fusion protein was induced by the addition of 1 ml 0.1 M IPTG per 250 ml of cells.

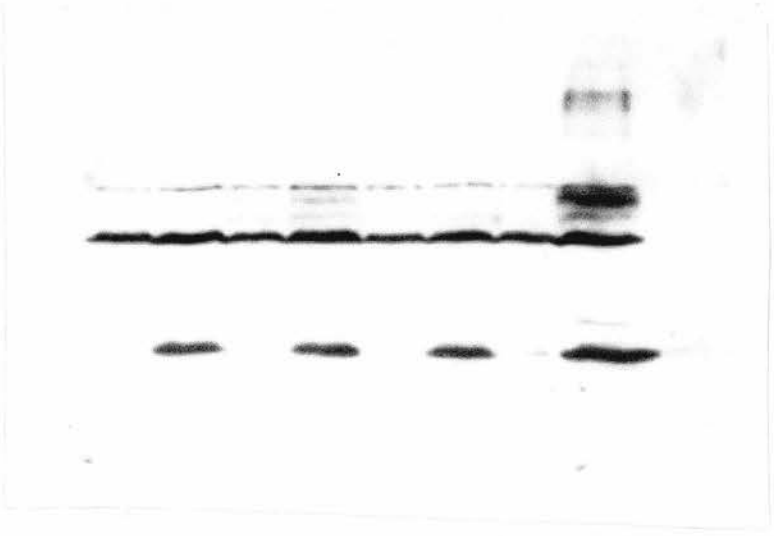
The induction products of BL21(DE3)-pLysE with pSETVIII were examined on a 15% SDS-PAGE gel . The theoretical weight of the fusion protein is 30,000 Da as the pVIII has a calculated weight of 26,000 Da and the histidine tag attached to the protein had a calculated weight of 4,000 Da. After staining the gel with Coomassie blue and then destaining, no visible band was detected around the 30,000 Da region which was not present also in BL21(DE3)-pLysE cells. A Western blot was made from a SDS-PAGE gel and probed with N-terminal and C-Terminal antisera that were generated from synthesised peptides (N-T and C-T sequences of pVIII) coupled to HSA. After 45 minutes exposure the photographs produced by each antisera were examined and a faint band was detected by the C-terminal and N-terminal antisera at the 30,000 Da region which was slightly above the band produced by pVIII from Ad2ts1. The blots were overlaid and it was found that the antisera had picked out the same bands. It was concluded that this band was the fusion protein.

Production of the recombinant pVIII was minimal in BL21(DE3)pLysE cells and thus, not present in sufficient quantities to be purified from the cell extract. To overcome the lack of recombinant pVIII production the plasmid pSETVIII was transformed into BL21(DE3) cells. Selection of BL21(DE3) for the expression of pVIII was over BL21(DE3)-pLysS cells. BL21(DE3)-pLysS cells have a minimal resistance to toxic proteins and express the fusion protein in greater quantities than BL21(DE3)-pLysE cells. BL21(DE3) cells produce large quantities of induced non-toxic proteins but have no resistance to toxic proteins, it was for the former reason that BL21(DE3) cells were selected as there was no direct evidence of recombinant pVIII being a toxic protein. Expression of pVIII in BL21(DE3)-pSETVIII was induced by adding 20 µl of 0.1 M IPTG to 5 ml of cells, which had been grown from a single colony in L-

Broth, at 37°C for 4 hours and incubated for 2 hours at 37°C, sampling 1 ml every 30 minutes. Each 1 ml sample was centrifuged, and the pellet resuspended in 40 µl reducing solution. The solution was heated at 100°C for 2 minutes before loading onto a 20% SDS-polyacrylamide gel. The samples were run next to a control of untransformed BL21(DE3) cells, which had been grown and the sample prepared identically to that of BL21(DE3)-pSETVIII. After development of the gel with Coomassie blue a large band was visible, only in the samples from BL21(DE3)-pSETVIII, and located slightly above the pVIII band from ts1 (see figure 22). The location of the band above the ts1 pVIII band was expected as recombinant pVIII has a calculated weight of 30,000 Da, and pVIII a weight of 26,000 Da (Edvardsson *et al.*, 1976). In order to confirm that the 30 kDa band produced by BL21(DE3)-pSETVIII was pVIII a Western blot was made from a 20% SDS-PAGE gel. Both C-terminal and N-terminal antisera detected a 30 kDa band and pVIII from ts1 (see figure 50). The Western blots were overlaid with each other and the gel. It was found that the blots had detected the same 30 kDa band, which also corresponded to the same band detected on the gel in the BL21(DE3)-pSETVIII samples. It was concluded that the 30 kDa band was recombinant pVIII.

The fusion protein pVIII was purified from BL21(DE3) cellular proteins using Pro-Bond Resin (a nickel affinity resin) supplied by Invitrogen. A sample of recombinant pVIII was induced in BL21(DE3)-pVIII cells, which had been grown up in 250 ml L-broth at 37°C for 4 hours, by the addition of 1 ml of 0.1M IPTG, and incubated for a further 45 minutes at 37°C. The sample was centrifuged and the pellet resuspended in 5 ml of wash buffer. Lysozyme was added to the solution to lyse the BL21(DE3)-pVIII cells, releasing the cell contents. After 5 minutes 1 ml of Triton X-100 was added to the sample which was sonicated for 30 seconds before centrifuging at 3,000 rpm for 15 minutes. The pellet was saved and the supernatant centrifuged at 10,000 rpm for 10 minutes and both supernatant and pellet saved. The pellets were

(a)



(b)

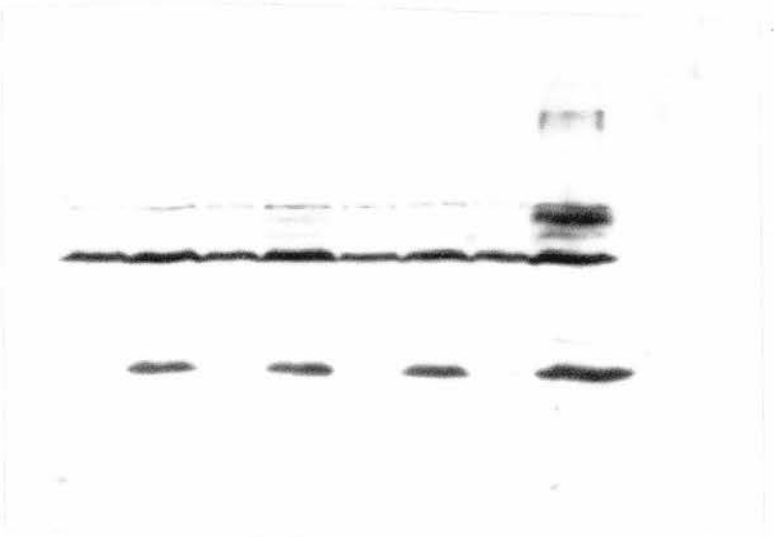


Figure 50: Western blot analysis of induction products from DE3 and DE3-pSETVIII cells. The contents of DE3 and DE3-pSETVIII cells were sampled at varying time intervals and electrophoresed, blotted onto nitrocellulose and probed with (**a**) C-Terminal antisera and (**b**) N-Terminal antisera. Adenovirus serotype ts1 was ran in parallel lane as a standard (pVIII) for the expected location of the fusion protein.

resuspended in wash buffer and ran with the supernatant on a non-denaturing SDS-PAGE gel, with Ad2ts1 as a standard. After staining of the gel, it was found that most of pVIII was located in the original pellet. Guanidine chloride was added to the pellet to a concentration of 1 M in an attempt to dissolve the recombinant pVIII. The solution was centrifuged at 3,000 rpm for 15 minutes and a non-reducing SDS-PAGE gel showed that pVIII was present in the supernatant, only trace amounts were present in the pellet. The nickel affinity column was prepared by suspending 1 ml of resin in 20 ml of wash buffer and centrifuging for 15 minutes at 3,000 rpm. The nickel resin was resuspended in 0.5 ml of wash buffer and loaded onto a 2.5 ml column. The sample containing pVIII was loaded onto the column, and the resin washed with 30 ml of wash buffer. Elution of the proteins bound to the nickel column was carried out by adding 2.5 ml of elution buffer and collecting five 0.5 ml fractions. Analysis by SDS-PAGE gel showed that pVIII was not present in any of the eluted fractions. The nickel column was washed with 10 ml of wash buffer containing 1 M guanidine chloride and the elution of proteins bound to nickel column was performed by adding 2.5 ml of elution buffer (containing 1 M guanidine chloride) and collecting eight 0.25 ml fractions. Analysis on a SDS-PAGE gel showed the elution buffer washings contained a large quantity of the fusion protein, and a amaximum of three contaminating proteins, which were only detected after silver staining of the SDS-PAGE gel, see figure 51.

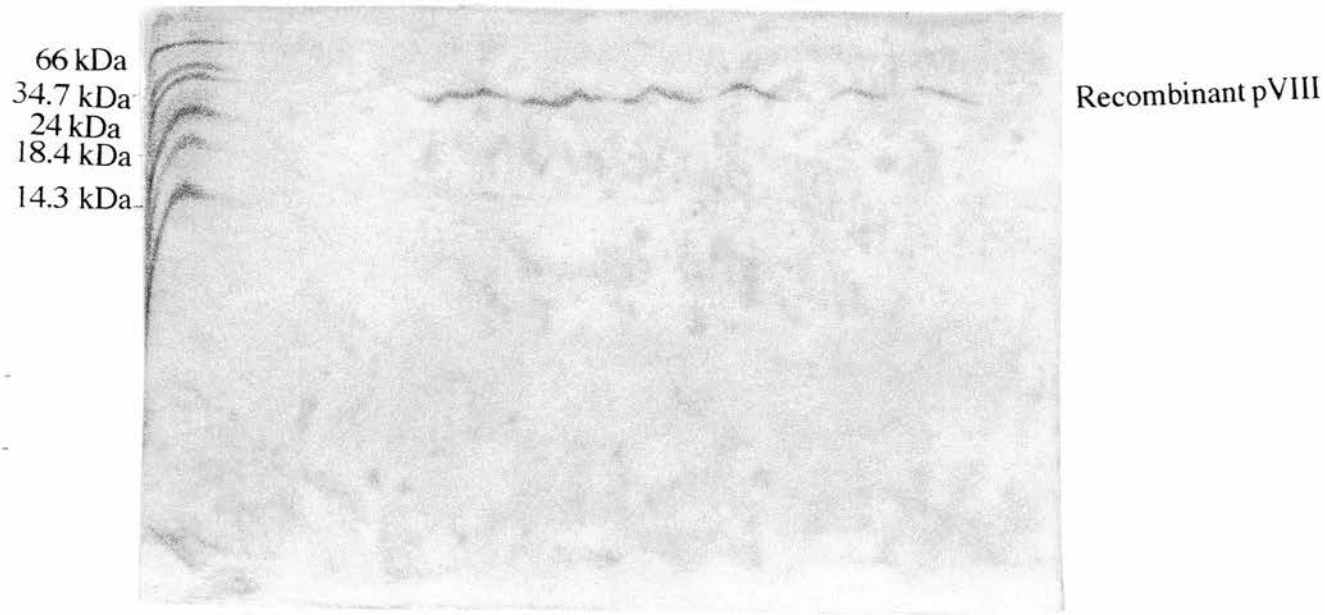
Figure 51: SDS-PAGE analysis of elution fractions obtained from a nickel affinity column. Recombinant pVIII was eluted with 250 mM imidazole and 1 M guanidine chloride and elution fractions resolved on a 15% polyacrylamide gel. Molecular weight standards and dirupted Ad2ts1 virus were ran in parallel lanes.

3.3.2 Purification of recombinant pVIII using ProBond and Nickel IDA Columns

As an alternative to using the Invirogen Probond nickel affinity column a nickle affinity column was prepared as in method 2.4.12 using IDA-Sepharose. Cell extracts were prepared as in method 2.3.1 and half of the solubilised cell material loaded onto a ProBond column while the rest was loaded onto a Ni-IDA column. The purification procedure 2.3.1 was followed for both columns and the recombinant pVIII eluted with 250 mM imidazole, 1 M guanidine chloride, 0.1 M sodium bicarbonate pH 8 and the elution fractions analysed by SDS-PAGE. Staining the polyacrylamide gels with Coomassie blue showed that both columns purified pVIII to a single band, see figure 52. However, it was found that the procedure using the ProBond column had resulted in the purification of the greater quantity of pVIII. Therefore all further purifications were carried out using the ProBond nickel affinity column.

As both samples were purified from the same cell culture it is extremely unlikely that the difference between the two purifications can be accounted for by differing quantities of recombinant protein being loaded onto each column. It is probable that the difference between the two purification is a result of the differing properties of the columns. When bound to the chelating ligand IDA nickel ions have three co-ordination sites free to interact with biopolymers but, are weakly bound to the ligand and can be released during chromatography. However, when nickel ions are bound to a nitriiotriacetic acid derived ligand, as with the ProBond resin, the divalent cation is bound strongly to the column and two co-ordination sites are free to interact with proteins (Hochuli et al, 1987). Therefore it is probable that the difference between the two purifications can be accounted for by the leaching of the nickel cations from the IDA column during chromatography as the column is subjected to treatment with 1 M guanidine chloride prior to elution.

(a)



(b)

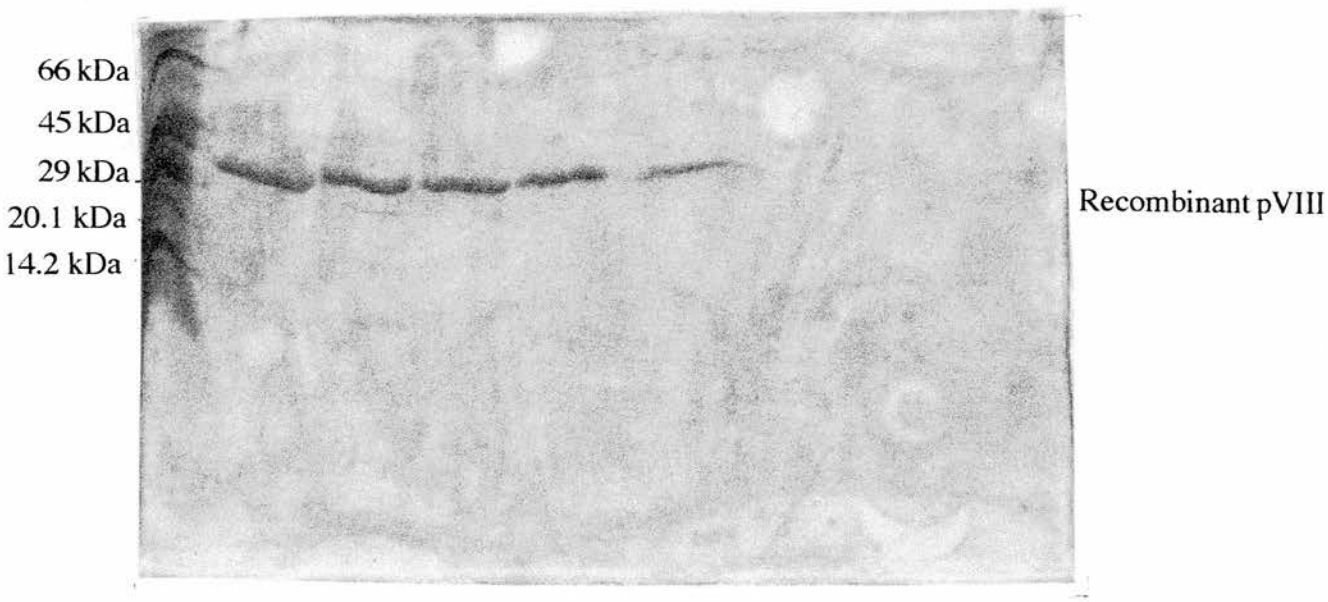


Figure 52: SDS-PAGE analysis of elution fractions obtained from two different nickel affinity column. Recombinant pVIII was eluted with 250 mM imidazole and 1 M guanidine chloride from (a) IDA-nickel affinity column and (b) ProBond nickel affinity column. The elutions fractions were resolved on a 20% polyacrylamide gel and stained with Coomassie blue. Molecular weight standards were resolved in a parallel lane.

3.3.3 Purification of His tagged pVIII C-Terminal deletion mutant.

Competent BL21(DE3) cells were transformed with pSET110ΔC-T and plated out onto LB Agar/Amp and incubated at 37°C. BL21(DE3) cells were selected as they contained the necessary BL21(DE3) gene for the expression of proteins with a T7 promoter. After incubating overnight a single colony was picked from the plate and used to inoculate 5 ml of LB/Amp. The culture was incubated at 37°C, with shaking, until the optical density of the culture reached 0.6 units at 600 nm and the expression of the fusion protein induced by the addition of 20µl 0.1M IPTG. Samples of 1 ml were taken at 0, 1, 2, 3, and 4 hours after induction. These were centrifuged, resuspended in 100 µl of PBS and sonicated in 30 second bursts before adding 20 µl of reducing solution. The samples were boiled for two minutes and centrifuged at maximum speed on a microfuge before loading 10 µl onto a 20% SDS-PAGE gel. After staining with Coomassie blue and comparing the sample lanes against untransformed BL21(DE3) and molecular standards run on the same gel a band was present at about the 17,000 Da region in all the samples. However, the band was not visible in the untransformed BL21(DE3) cells and was the expected weight of the fusion protein. A western blot was performed using antisera generated against a synthetic peptide of the pVIII N-terminal. It was found that the band reacted against the antiserum in all the samples and was not detected in the BL21(DE3) cell extract or molecular standards lane. Therefore it was concluded that the band was the deletion mutant and that it was being expressed constitutively.

The C-Terminal deletion protein was purified from the *E.coli* proteins using a Pro-Bond nickel affinity column. An overnight culture of BL21(DE3) cells, which had been transformed with pSET110ΔC-T, was used to transform 250 ml of LB/Amp. When the culture had an optical density of 0.6 units at 600 nm 1 ml of 0.1M IPTG was added to the sample to induce expression. After one hour the cells were harvested by centrifugation and the pellet resuspended in 5 ml of 50 mM Tris-HCl,

pH 8. The cells were lysed by freeze thawing three times before incubating at 37°C in the presence of lysozyme. After 15 minutes DNAse I was added to the cell extract to cleave the DNA released by cell lysis. The cell extract was centrifuged at 3000 rpm to separate the insoluble material from the soluble protein and a sample of each was analysed by SDS-PAGE which showed a large 17 kDa band in the soluble fraction.

A 2.5 ml nickel affinity column, diameter 0.5 cm, was prepared by removing the storage buffer from the column and equilibrating with 4 volumes of wash buffer. The soluble cell material was loaded onto the column and the column washed with 20 volumes of wash buffer. The protein was eluted with 250 mM imidazole and the fractions analysed by SDS-PAGE and Western blotting. Staining the polyacrylamide gels with Coomassie blue showed a number of bands, the most concentrated being a band at 17 kDa. Western blotting confirmed that the band purified was the C-terminal deletion mutant.

In an attempt to increase the purity of the final product 20 mM imidazole was added to the wash solutions as this would reduce the amount of non-specific binding to the column. Although the addition of 20 mM imidazole to the wash buffer increased the purity of pVIIIΔC-T in the eluted fractions the quantity of protein purified was reduced. When the purification was repeated using 10 mM imidazole in the wash solutions it was found that analysis by SDS-PAGE and staining with Coomassie blue showed only a marginal reduction in the quantity of protein purified. Also, the number of contaminating proteins present in the eluted fractions were about the same as achieved using 20 mM imidazole. Therefore all further purifications were carried with imidazole at a concentration of 10 mM in the wash buffer, see figure 53.

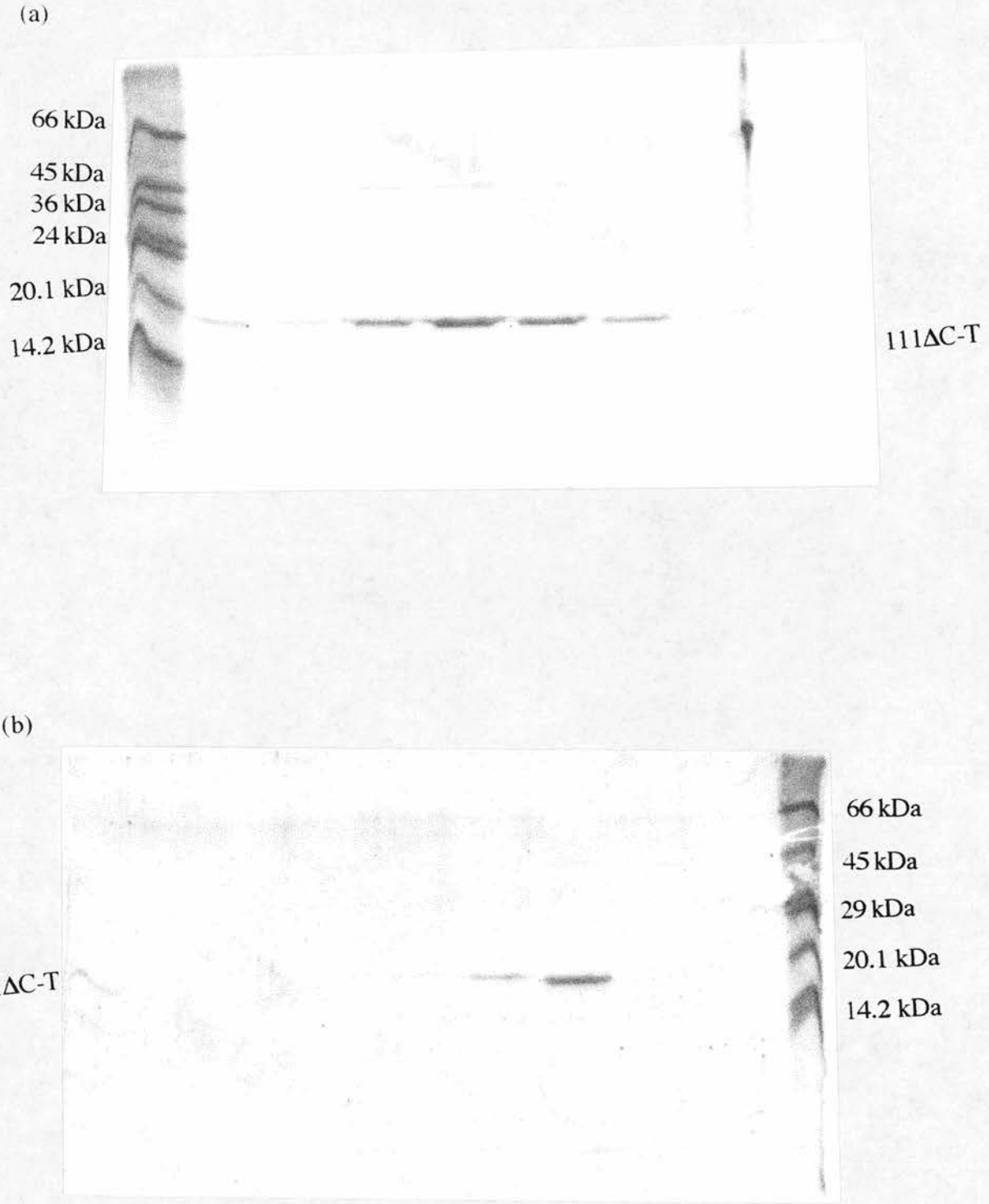


Figure 53: SDS-PAGE analysis of elution fractions obtained from a nickel affinity column. The pVIII deletion mutant 111ΔC-T was eluted with 250 mM imidazole after washing with (a) 10 mM imidazole and (b) 20 mM imidazole in 100 mM sodium bicarbonate, pH 8, 500 mM sodium chloride and the elutions fraction resolved on a 20% polyacrylamide gel and stained with Coomassie blue. Molecular weight standards were resolved in a parallel lane.

3.4 Purification of GST-fusion protein using Pharmacia glutathione Sepharose matrix

A 500 ml culture of BL21(DE3) cells containing the vector pGEX227ΔN-T was used in an attempt to purify the pVIII N-terminal deletion mutant, see method 2.3.9. After analysis by SDS-PAGE and Western blotting it was found that the GST-fusion protein had failed to bind to the column as it was detected in the wash fractions but, not in the elution fractions. The *E.coli* 70 kDa protein which binds to glutathione-Sepharose columns was found, from the SDS-PAGE analysis of elution fractions, not to have bound to the column. This indicated that the failure of the GST-fusion to bind to the glutathione-Sepharose column was probably as result of the purification method rather than a property of the GST-fusion. Therefore, the fusion protein was loaded onto the column using the batch procedure, see method 2.3.10.

Proteins were eluted from the glutathione-Sepharose column using the batch method and analysed by SDS-PAGE and Western blotting. Analysis of the elution fractions by SDS-PAGE revealed that the GST-fusion protein was purified using the batch method with four contaminating proteins. However, Western blotting showed that three of the contaminating proteins were truncated GST-pVIII N-terminal deletion fusion proteins. The remaining impurity was the 70 kDa glutathione binding *E.coli* protein was also present as a contaminating protein see figure 54. An attempt was made to purify the insoluble GST-fusion protein, as the insoluble cell material contained approximately the same quantity of fusion protein as the soluble fraction, using method 2.3.10, except all solutions contained 1 M guanidine chloride. Analysis by SDS-PAGE and staining with Coomassie blue revealed a faint band at the expected molecular weight of the GST-fusion protein. However, as the quantity of protein purified by this approach was significantly less than that purified from the soluble fraction the purification of the insoluble GST-fusion protein was abandoned.

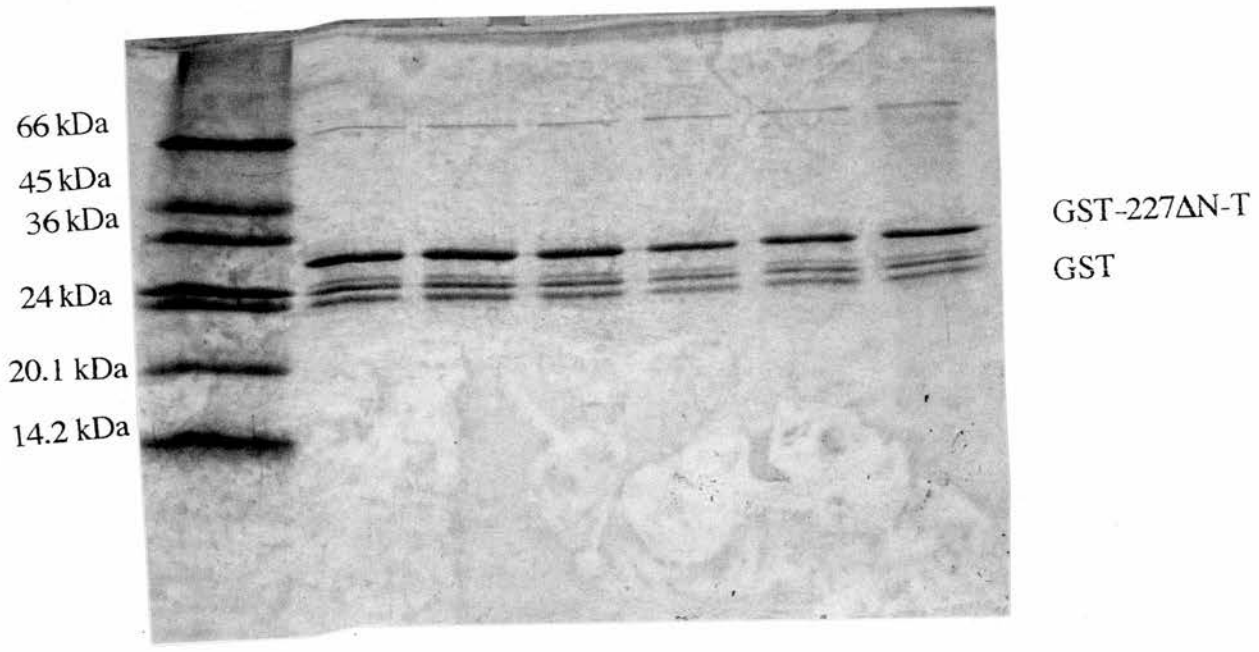


Figure 54: SDS-PAGE analysis of elution fractions obtained from a glutathione affinity column. The pVIII deletion mutant 227ΔN-T was eluted with 10 mM glutathione in PBS. The elutions fractions were resolved on a 20% polyacrylamide gel and stained with Coomassie blue. Molecular weight standards were separated in a parallel lane.

The reason for the decrease in the quantity of fusion protein purified is most probably because the glutathione denatured most of the GST tag and therefore the fusion protein was unable to bind to the column as the activity of the tag had been lost.

3.5 Purification of Fusion Proteins with Maltose Binding Protein Tag

3.5.1 Induction time of MBP fusions expressed by pMALp2 derived vectors

In order to establish the optimum induction time of the culture a single colony was picked from a LB plate and used to inoculate rich broth (L-broth supplemented with 2g/l glucose). The culture was grown, with shaking, at 37°C until it reached an optical density of between 0.6 and 0.7 units at 600 nm. Upon reaching the desired optical density IPTG was added to the culture, mixed, and a sample taken from the

culture. The culture was incubated at 37°C, with shaking and samples taken at 1 hourly intervals, until 5 hours had elapsed. Immediately after taking each sample was spun down in a microfuge and resuspended in 100 µl PBS before freezing. The samples were thawed, 25 µl of reducing solution (see method 2.4.5(b)) added, sonicated for 1 minute and boiled for two minutes prior to loading onto a SDS-PAGE gel and blotting onto nitrocellulose paper. Analysis of the crude extracts by Western blotting, using an antisera developed against the maltose binding protein (New England Biolabs) at 1: 10 000 revealed that there was no significant increase in the production of the fusion protein after 1 hour, therefore an induction time of one hour was used in all further experiments. The Western blot analysis also revealed that the pGSVpVIII construct was expressing a truncated protein.

The truncated protein may have been a result of the harvesting procedure as fusion proteins are at greatest risk of degradation by cellular enzymes during the harvesting and lysis of cells. The time taken to harvest the cells was reduced from 20 minutes to 5 minutes by increasing the centrifuge speed from 5000 rpm to 10000 rpm and therefore possibly decrease the quantity of degraded recombinant protein. Also, in order to inhibit cellular enzymes that may have been responsible for degradation Pefabloc was added to the resuspension buffer. However, it was found that this did not eliminate or reduce the appearance of truncated proteins. Therefore it is possible that the truncated proteins were due to the pre-mature termination of protein synthesis brought about by a depletion of the codon pools for arginine and proline.

3.5.2 Purification of MBP fusions from pMALp2 derived vectors

The plasmids pGSVpVIII and pMAL158ΔN-T were used to transform the competent cells and the contents of the cell cytoplasm and periplasm obtained as in methods 2.3.4 and 2.3.5 respectively. An attempt was made to purify the N-Terminal deletion mutant and full length pVIII by affinity chromatography with an amylose Sepharose

column, see method 2.3.6. Originally the cells were sonicated in a ice-water bath in 15 seconds pulses for two minutes. However, this was altered as analysis by SDS-PAGE and staining with Coomassie blue showed that the fusion protein failed to bind to the amylose column. Prolonged sonification can denature the maltose binding protein thus preventing the fusion protein binding to the column so the lysozyme/DNAse was used as it was less likely to denature the MBP fusion.

Initially the N-Terminal deletion mutant and full length pVIII were expressed in XL1-BLUE however, after staining with Coomassie blue the induced samples did not appear different to uninduced samples. Therefore expression in XL1-BLUE cells was discounted as a possible source of recombinant protein, no western blots were performed and JM105 cells were used as an alternative cell strain. Although expression of the recombinant proteins was detected using western blots probed with pVIII antisera generated in rabbits truncated proteins were also detected using pVIII antisera generated in rabbits. In an attempt to overcome this and increase the quantity of fusion protein expressed by the pGSVpVIII and pMAL158ΔN-T vectors the *E.coli* strain BL21(DE3) was selected as Sun *et al.,* (1995) had found a high level of expression of MBP fusion protein in BL21(DE3). However, analysis revealed no visible difference between BL21(DE3) and JM105. As there was no significant difference between the two strains BL21(DE3) was selected as it is a fast growing *E.coli* strain..

The plasmid pMAL158ΔN-T expressed a protein which was detected by pVIII rabbit antisera only in the cytoplasm. pGSVpVIII expressed a sample present in the periplasm and in the cytoplasm. Due to the low quantity of protein being expressed by the two different vectors it was concluded that scaling the purification procedure up was impracticable and it was decided that expressing the proteins using the pMAL-c2 vector would be a better option as it was capable of producing greater quantities of protein than pMAL-p2 and the derivative pGSV.

3.5.3 Purification of MBP fusions from pMALc2 derived vectors

The plasmids pBSV111ΔC-T, pBSVpVIII, and pBSV227ΔN-T were created by subcloning the inserts from pGSV111ΔC-T, pMALVIIIp2, and pGSV227ΔN-T into the pBSV vectors. Competent ER2058 cells were transformed with the plasmids and expression of the recombinant proteins induced for one hour by the addition of IPTG to a final concentration of 3 mM. Analysis was carried out by running a SDS-PAGE gel and staining with Coomassie blue. Protein bands, which were not present on the untransformed samples were observed at the expected molecular weight for the fusion proteins, 71 kDa and 58 kDa, for the pVIII and 111ΔC-T fusion proteins. However, no band was detected in the 222ΔN-T fusion protein lane. Analysis by Western blotting revealed that every lane of the induced cells contained a MBP band, and the pMALVIII lane contained a further truncated band. The vector pBSV111 was used to establish a purification technique as the C-terminal deletion mutant did not express a truncated protein product.

The C-terminal deletion mutant of pVIII fusion protein was attempted to be purified as method 2.3.4. It was found that the protein produced by the vector pBSV111ΔC-T did not bind to the amylose column. It is possible that this was because maltose present in extract was preventing the MBP from binding to amylose, this was despite growing the cells in a glucose rich media and diluting the cell extract prior to loading onto the column. As several attempts at this method failed to produce reproducible results it was decided to attempt purifying the protein by binding to a DEAE column as the association of MBP to the DEAE column is independent of maltose concentration.

3.5.4 DEAE column

Purification of the MBP-pVIIIΔC-T fusion was attempted using FPLC with a DEAE Sepharose column. A cell extract was prepared as in methods 2.3.7 and loaded onto a DEAE Sepharose column. The FPLC was run with 90 mM NaCl until the optical density at 280 nm had returned to the baseline, this took approximately 45 minutes at a flow rate of 1 ml/min. The protein was then eluted from the column using a gradient starting at 90 mM and ending at 150 mM and fractions collected. After analysis by SDS-PAGE and Western blotting it was observed that two fractions contained MBP and no fusion protein. However, the bulk of MBP was eluted with the fusion protein and the quantity of cellular proteins present in these fraction was too great to be able to use any fractions in an experiment. Therefore, in an attempt to purify the fusion protein from the cellular protein the fractions containing the fusion protein were pooled and dialysed at 4°C overnight in order to reduce the salt concentration present in the sample. The dialysed sample was loaded onto a freshly prepared amylose column however, analysis of fractions collected revealed that the fusion protein failed to bind to the column. Possible explanations of why the protein failed to bind to the amylose column in this case are that the concentration of NaCl remaining in the dialysed solution inhibited binding or that the MBP was mutated or folded incorrectly as both it and the MBP fusion failed to bind to the amylose resin.

3.5.5 Hydroxyapatite Chromatography

A hydroxyapatite column was prepared as in methods 2.3.8 and crude cell extract loaded onto the column. The coulmn was washed with 50 ml of 20 mM Tris-HCl, pH 7.4, before eluting with 500 mM sodium phosphate, pH 7.2. Fractions were collected and analysis by SDS-PAGE revealed that the MBP and MBP fusions could not be detected visually with Coomassie blue staining from any other band, see figure 55. A Western blot was made of the column fractions and probed with anti-MBP antiserum.

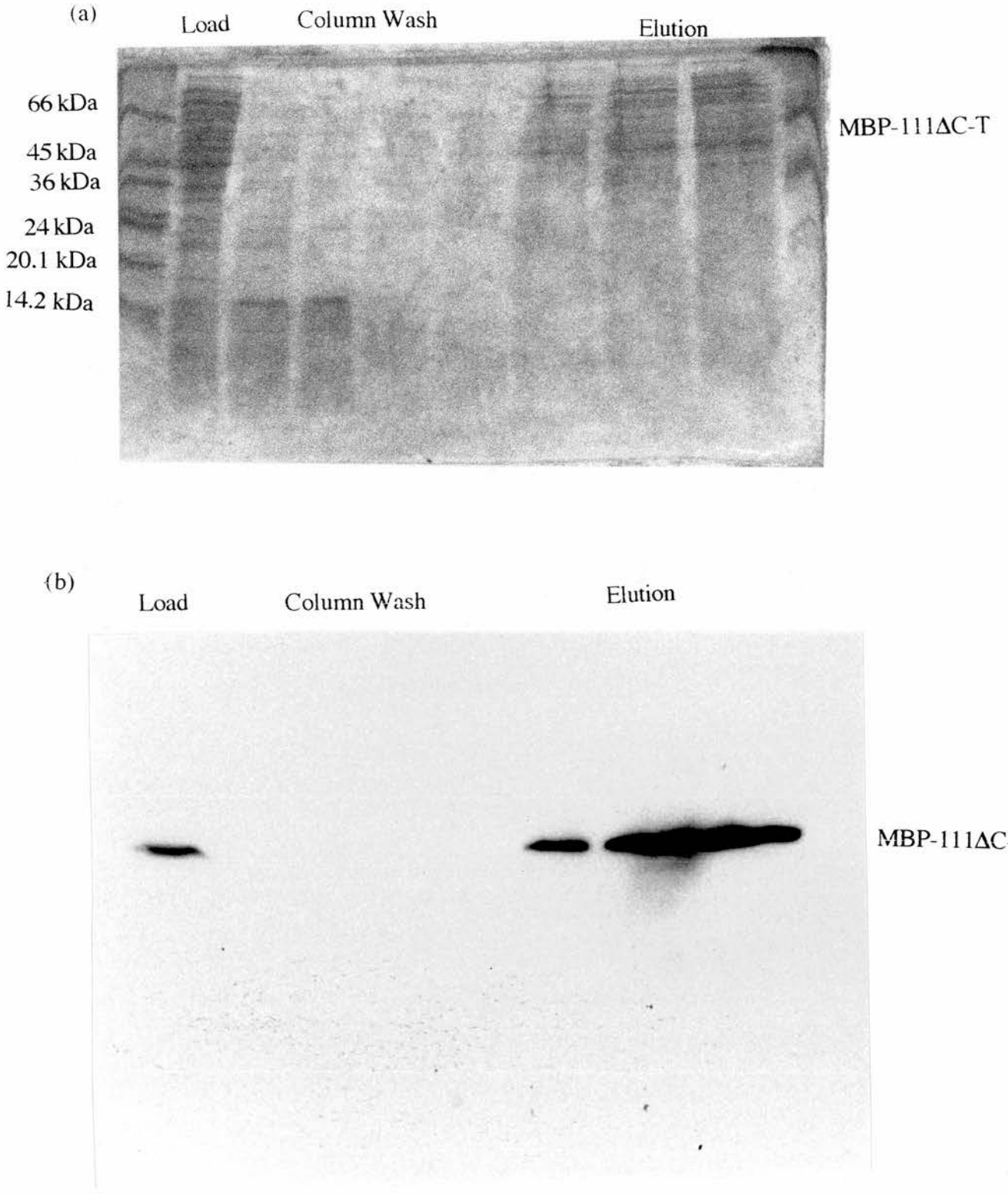


Figure 55: Purification of the MBP-111ΔC-T fusion protein by hydroxyapatite Chromatography. The column load, washings and elution fractions were resolved on a 20% SDS-PAGE gel. The polyacrylamide gels (a) stained with Coomassie Blue and (b) blotted onto Hyperbond-PVDF and probed with anti-pVIII N-Terminal antisera.

It was found that the MBP had bound to the column, see figure 55. However, as the amount of fusion protein bound to the hydroxyapatite column was minimal, and could only be detected by Western blotting, this method was discounted as a possible step prior to purifying with an amylose affinity column.

3.6 Purification of recombinant adenovirus 23 kDa protease

The adenovirus protease was expressed in BL21(DE3) cells which had been transformed with the plasmid p175, a pET11c derivative. The protease was purified as in methods 2.3.3 using a FPLC. The flow through fractions of the DEAE-Sepharose column were analysed by SDS-PAGE for the presence of the adenovirus protease, see figure 56. It was found that most of the proteins had bound to the DEAE column and that the protease was present in fractions 19-35, which corresponded to a peak on trace of optical density at 280 nm of the column flow through. The fractions 19-35 contained only a few contaminating proteins and so was a major purification step in itself. The protease containing fractions were pooled and loaded onto a CM-Sepharose column on a FPLC. The protease bound strongly to the column and was eluted with 150 mM NaCl in 50 mM Tris-HCl pH 8. As the protease bound to the CM column the second column increased the purity of the protease, see figure 56, and, at the same time, concentrated the protease into a few fractions.

3.6.1 Activity of recombinant adenovirus protease

The activity of the purified recombinant adenovirus protease was assayed by monitoring the cleavage of the synthetic peptide LSGAGFSW. Fractions which contained the protease were assayed for activity using 10 µl of the fraction which was incubated at 37°C for ten minutes with 10 µl (7.4 nmol) activating peptide and 25 µl of 50 mM Tris-HCl pH 8, 10 mM EDTA and 2 mM ß-mercaptoethanol prior to the addition of 5 µl (120 nmol) of substrate. Samples, of volume 10 µl, were taken at 5, 10, 15 and 30 minutes. Digestion of the synthetic peptide terminated by adding to an

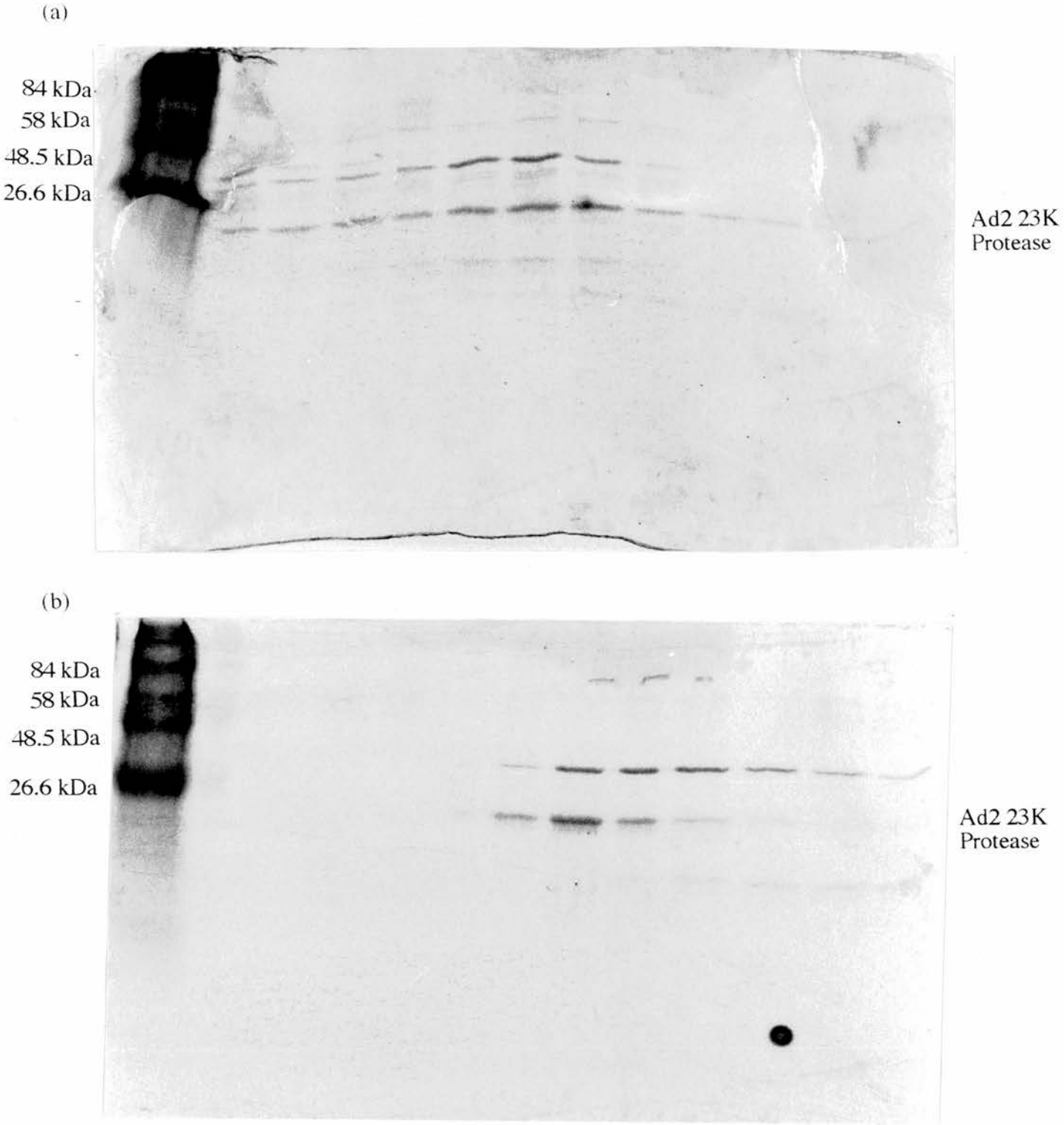


Figure 56: Purification of recombinant Ad2 protease. The crude cell extract was loaded on a DEAE-Sepharose and the flow through fractions collected before analysing on 20% SDS-PAGE gel and staining with Coomassie blue (a). Protease containing fractions were loaded onto a CM-Sepharose column and the protease eluted with 20 mM salt, the elution fractions were analysed on 20% SDS-PAGE gel and stained with Coomassie blue (b).

equal volume of 1% (v/v) TFA and freezing the samples in liquid nitrogen prior to storing at -20°C until analysing by capillary electrophoresis. A control digest was also carried out for each fraction, by replacing the activating peptide with 10 µl 50 mM Tris-HCl pH 8, in order to confirm that the proteolytic activity was a result of the adenovirus protease.

Fractions that were found to contain adenovirus protease which were capable of digesting the synthetic peptide LSGAGFSW were alliquotted in 60 µl aliquots and stored at -70°C. It was found that protease activity was usually restricted to the first three fractions in which protein was eluted.

3.7 Expression and Purification of EDS L3 23 kDa Protease

3.7.1 Expression of EDS Protease in *E.coli*

The gene encoding the EDS 23 kDa protease was cloned into the vector pET11c by Lewis Murray and the plasmid named pETEDS23K. Expression of the recombinant protease in BL21(DE3) was analysed by inducing a 250ml M9/Amp culture with 1ml of 0.1M IPTG when the optical density at 600nm reached 0.6 units. Samples were taken every hour for 5 hours and analysed by SDS-PAGE, staining with Coomassie blue. The results obtained showed that EDS was not being expressed as all the induced samples produced the same result as untransformed BL21(DE3) cells. The experiment was repeated twice, both produced the same results as the original.

As a result of the failure of the vector pETEDS23K to express recombinant protease the gene encoding the protease was subcloned into the vector pRSETA using the Xba I and Bam HI cleavage sites, the vector was named pSETEDS. The expression of the protease was detected by inducing three 10ml LB/Amp cultures with 40 µl of 1M IPTG each when the optical density of the culture was greater than 0.6 units at

600nm. Three cultures of Ad2 protease induced in BL21(DE3) cells were treated in an identical manner as controls. After inducing for 1 hour samples were analysed by SDS-PAGE, staining with Coomassie blue. A thick band was observed at approximately 23 kDa in the EDS protease samples just below the thick band present in the Ad2 protease samples. This is as expected as the EDS protease is two amino acids shorter than the Ad2 protease. In order to verify that the band being expressed was the EDS protease a Western blot was made of the samples and probed with an N-terminal antisera, supplied by Lewis Murray, against the EDS protease. It was found that the antisera detected a band in the EDS lanes and the Ad2 lanes. The band expressed in the EDS lanes was confirmed as the EDS protease by amino acid sequencing of the first five amino acids.

3.7.2 Purification of Recombinant EDS L3 23 kDa Protease from *E.coli*

(a) Purification by FPLC

The predicted isoelectric point of the EDS 23 kDa protease is approximately the same as that of the human adenovirus serotype 2 protease. Therefore the initial method used to purify the protease was based on that of Webster *et al.*, 1993.

E.coli strain BL21(DE3) was transformed with the pSETEDS plasmid. The products obtained from an one hour induction were obtained by disrupting the cells with lysozyme and DNase I treatment and centrifuged, as described in the methods section. As the pI of the EDS protease and the Ad2 protease are almost identical (9.06 and 8.95 respectively) the same protocol used to purify the Ad2 protease was used. The supernatant was loaded onto a FPLC DEAE-Sepharose column that had been equilibrated with 50 mM Tris-HCl, pH 8 and the flow through fractions were collected. The fractions were analysed by SDS-PAGE, see figure 57, and a Western blot was also prepared of the samples which showed a single band present in fractions

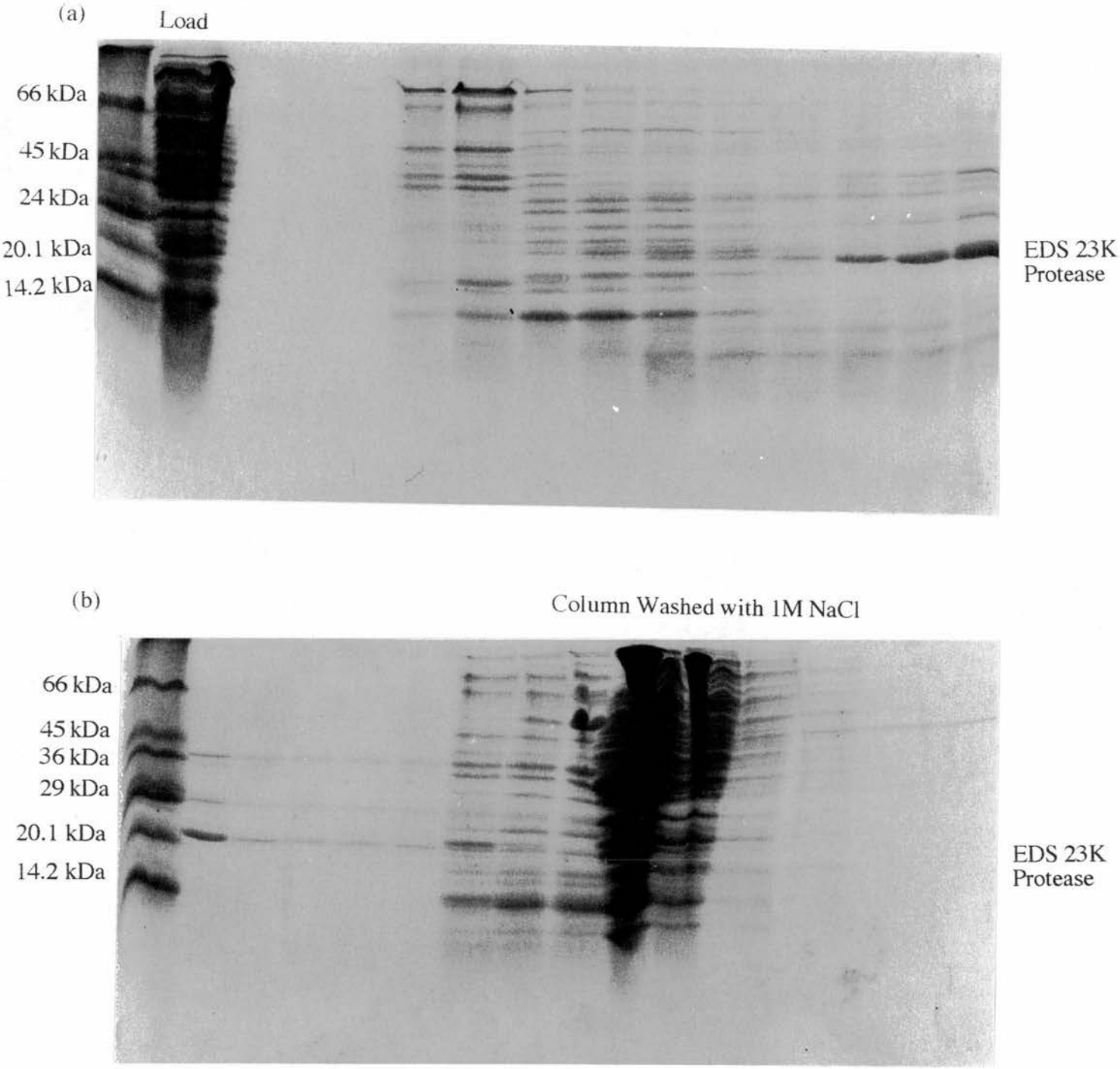


Figure 57: Purification of recombinant EDS protease. The crude cell extract was loaded on a DEAE-Sepharose and the flow through fractions 7-45 collected before analysing on 20% SDS-PAGE gel and staining with Coomassie blue (a). The DEAE-Sepharose column was washed with 1 M and the elution fractions 46- 74 were analysed on 20% SDS-PAGE gel and stained with Coomassie blue (b).

25 to 45. Despite the similarity in the theoretical pI's of the proteins the EDS protease is eluted later than the Ad2 protease which is eluted in fractions 19-35. The EDS protease was also detected at the start of the 1 M salt wash of the DEAE-Sepharose but, this is probably because the salt wash started at fraction 46 and a longer elution period would probably remove the presence of the protease from the salt wash. Fractions 25 to 45 were loaded onto a FPLC CM-Sepharose column that had been equilibrated with 50 mM Tris-HCl pH 8, as in theory the EDS protease would bind to the CM-Sepharose column at pH 8. Flow through fractions were collected and separated on a SDS-PAGE gel and a Western blot also made of the samples. Bound proteins were eluted from the CM-Sepharose column initially with 200 mM NaCl and then 1 M NaCl. The elution fractions were collected and separated on a SDS-PAGE gel, see figure 58, and a Western blot performed. However, both Western blots failed to detect the presence of protease in any sample, including the load. Two proteins were observed to have bound to the CM-Sepharose column after staining with Coomassie blue, see figure 58, both have molecular weights less than 20 kDa and therefore are not potential candidates to be the EDS protease.

Although the EDS protease was not purified by this method it is possible that this procedure could used in part as the DEAE-Sepharose column is a major step in the purification of the protease. However, the final step requires further work as the protease does not appear to bind to the CM-Sepharose column. It is possible that lowering the pH of the protease buffer prior to loading the CM-Sepharose column and using a buffer of the same pH to equilibriate and wash the column may result in the protease binding to the column.

(b) Zinc Affinity Column

A zinc column was used by Webster *et al.,* 1993 to purify the human adenovirus serotype 2 protease. Therefore an iminodiacetic acid-Sepharose column was loaded

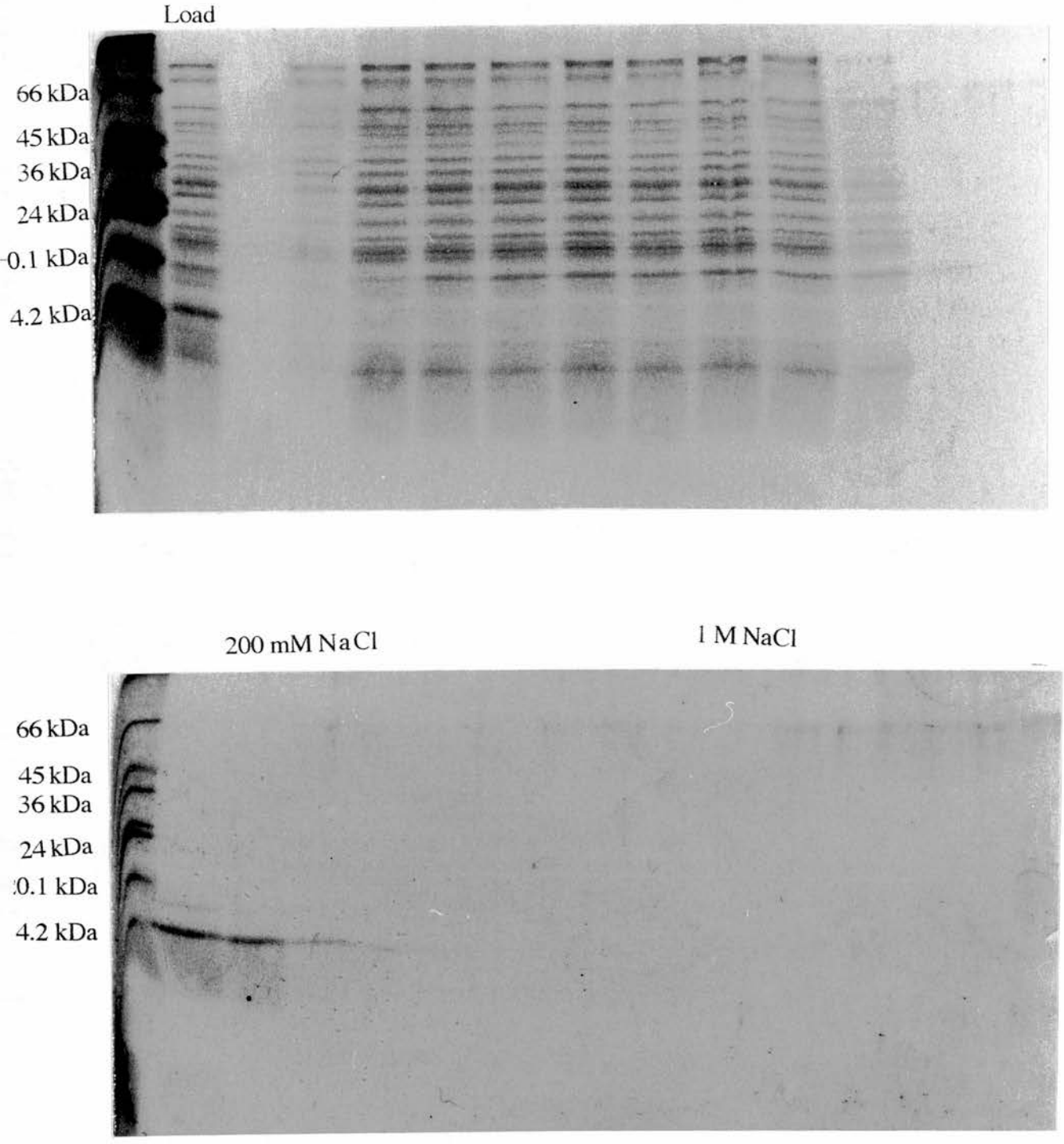


Figure 58: Purification of recombinant EDS protease. Protease containing fractions were loaded onto a CM-Sepharose column and flow through analysed on 20% SDS-PAGE gel and stained with Coomassie blue (a). Protein which had bound to CM-Sepharose column was eluted initially with 200mM NaCl and finally with 1 M NaCl. The elution fractions were analysed on 20% SDS-PAGE gel and stained with Coomassie blue (b).

with zinc and equilibrated with 50mM Tris-HCl pH 8, as described in the methods section. The soluble cell extract obtained from a one hour induction of BL21(DE3) cells transformed with pSETEDS, see methods section for details, were diluted one in four products obtained from a one hour induction were obtained by disrupting the cells with lysozyme and DNase I treatment and centrifuged, as described in the methods section. The column was washed with 50 mM Tris-HCl pH 8 and proteins eluted with 50 mM Tris-HCl pH 8, 1M NaCl and 50 mM EDTA.

Unlike the human adenovirus protease in Webster *et al.*, 1993, the EDS protease was not purified using a zinc column. Analysis by SDS-PAGE revealed that although the EDS protease was present in the soluble fraction of the cell extract it did not bind to the zinc column. Coomassie blue staining of the SDS-PAGE gel also revealed that no proteins were eluted by the column in sufficient concentrations to be detected. Western blots did not detect the presence of the EDS protease in any of the column fractions or the EDS protease present in a control sample.

A further attempt was made to purify the EDS protease using the zinc column by initially passing the cell extract through a 30 kDa molecular weight filter. Analysis by SDS-PAGE revealed that although there were a number of large molecular weight proteins still present in the filtrate there was a reduction in the protein concentration. Also present, in the fraction that had passed through the filter, was a large band running at the expected location of the EDS protease. The filtrate was diluted 1 in 4 and loaded onto a zinc column. However, analysis by SDS-PAGE did not reveal any protein bands present in elution fractions.

An attempt was made to characterise the EDS protease using the filtrate, produced from passing the cell extract through a 30 kDa molecular weight filter, the EDS protease activating peptide (supplied by Dr. Sarah Jones) and synthetic peptide substrates. Cleavage of the synthetic peptides were monitored by capillary electrophoresis but, the traces of digests with and without the activating peptide did not differ significantly. It is possible that the EDS protease could be activated by the *E. coli* cellular proteins as active protease has been detected in insect (sf9) cells

(Webster *et al.*, 1993). However, when the results were compared to digests of purified Ad2 protease the cleavage products were significantly different. Although it is possible that in the EDS protease cleavage occurs at either a different location or at another undefined recognition site it is extremely unlikely as the EDS structural proteins still retaining the consensus cleavage sites (M,L,IXGG-X and M,L,IXGX-G). It is most probable that the observed cleavage of the synthetic peptides is the result of action by cellular enzymes.

3.8 Proteolytic Degradation of recombinant pVIII by the 23 kDa Ad2 Protease

Digestion of a control peptide, which contained an adenovirus protease cleavage site, was carried out in a volume of 50 µl composed of 1 M guanidine chloride, 300 mM NaCl, and Tris-HCl pH 8 solution at 37°C for 1 hour with 10 µl of adenovirus protease (supplied by H. Murray) and 10 µl of 20 µg/ml activating peptide. Analysis of the digestion products by capillary electrophoresis showed no production of cleavage products, suggesting that 1 M guanidine chloride inhibited the protease. The pVIII fusion protein was obtained from the nickel affinity column purification in elution buffer, which is at the same concentrations as above. In order for digestion of the fusion protein to occur the solution in which recombinant pVIII was dissolved was changed by dialysis. The sample was diluted 1:1 in refolding buffer (8 M urea, 20 mM DTT, 80 mM Tris-HCl pH 6.5, 2 mM EDTA and 1% NP-40) and placed into dialysis tubing. The solution was dialysed in dialysis buffer (4 M urea, 50 mM sodium phosphate pH 6.5, 1 mM EDTA and 300 mM NaCl) at room temperature. After 45 minutes the dialysis buffer was replaced with a dialysis buffer 2 (2 M urea, 1 mM ß-mercaptoethanol, 1 mM EDTA, 300 mM NaCl, and 50 mM sodium phosphate buffer pH 6.5). Dialysis was carried out for a further 3 hours at room temperature. Digestion of the control peptide was carried out in a volume of 50 µl composed of 2 M urea, 1 mM b-mercaptoethanol, 1 mM EDTA, 300 mM NaCl and 50 mM sodium phosphate buffer pH 6.5 at 37°C for 1 hour with 10 µl of protease and 10 µl of 20 µg/ml activating peptide. Analysis by capillary electrophoresis showed that the control peptide was cleaved by the protease in buffer 2. The transfer of fpVIII from elution buffer to buffer 2 could also be achieved by precipitating fpVIII out of the

elution buffer by adding an equal volume of water. After precipitation the sample was centrifuged at 10,000 rpm and recombinant pVIII redissolved in buffer 2.

Protease digests were set up using 10 µl protease 10 µl activating peptide, and b-mercaptoethanol, which were incubated together for 10 minutes before the addition of 30 µl recombinant pVIII and 10 µl buffer 2. Analysis of protease digests on a 20% SDS-PAGE gel showed the production of several cleavage products, none of which could be easily identified as the theoretical cleavage products (see figure 59). A Western blot of a 20% SDS-PAGE gel was probed with N-terminal antiserum and bands that corresponded to recombinant pVIII, cleaved pVIII and the 23 kDa protease were observed. Eight other bands were detected (see figure 60) which when compared to standards had the weights 25 kDa, 24.3 kDa, 21.3 kDa, 19.4 kDa, 18.1 kDa, 17.2 kDa, 16 kDa and 15 kDa cleavage products (see figure 60). The Western blot was stripped and reprobed with C-terminal antisera which detected pVIII, cleaved pVIII and two bands with the weights of 23.8 kDa and 14.5 kDa when compared to standards (see figure 61).

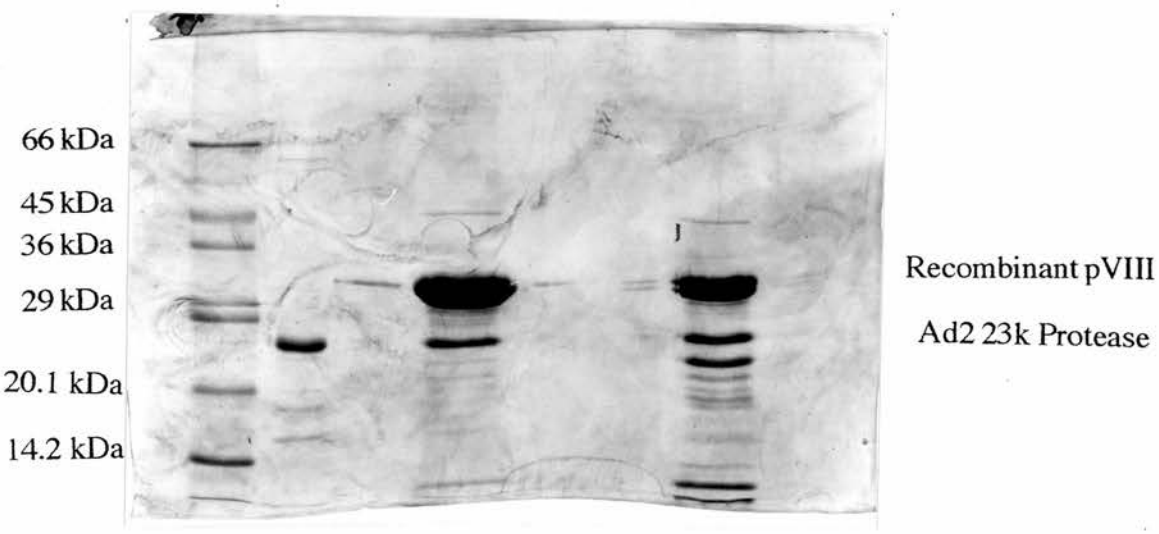
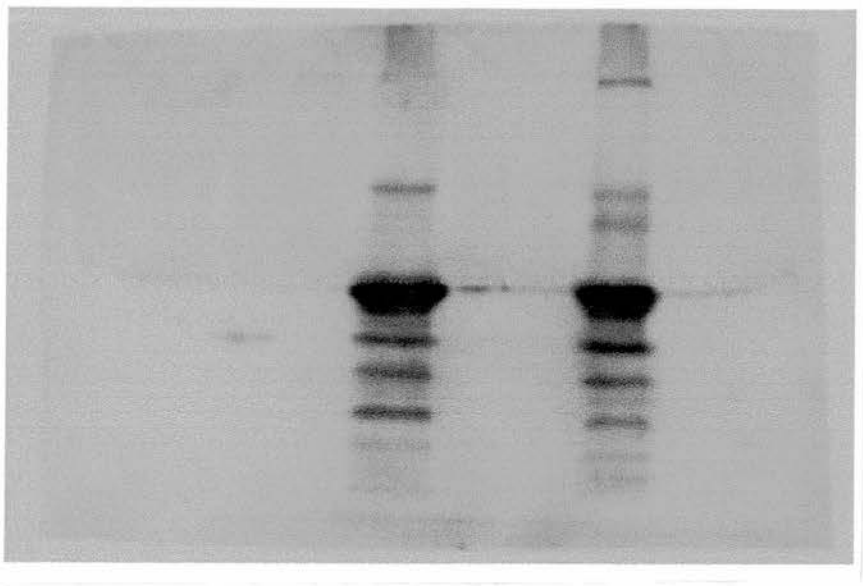


Figure 59: Digestion of recombinant pVIII by the adenovirus protease. Purified pVIII was digested for 8 hours by activated adenovirus protease and loaded onto a 20% polyacrylamide gel which was stained with Coomassie blue. The adenovirus protease and Dalton VII markers were ran as molecular weight standards in parallel lanes.

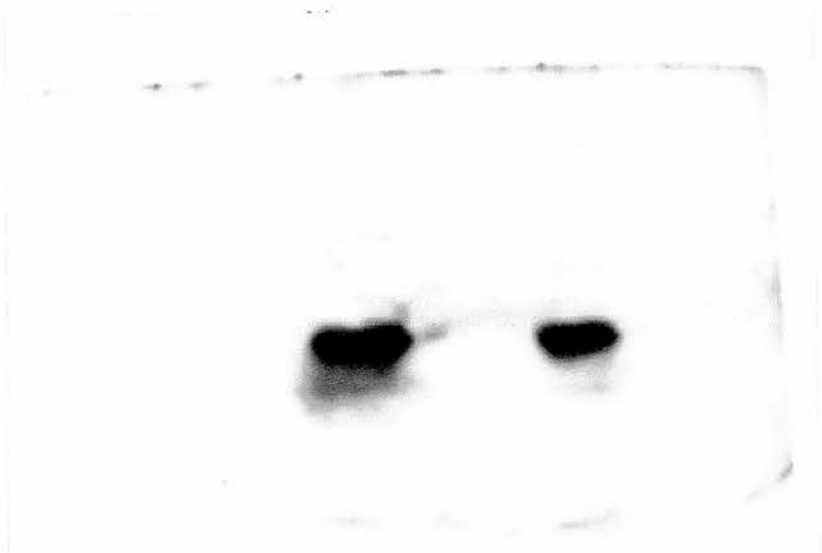
(a)



Recombinant pVIII

Ad2 23K Protease

(b)



Recombinant pVIII

Ad2 23K Protease

Figure 60: Western blot analysis of recombinat pVIII digestion by the adenovirus protease. The products of the 24 hours digestions of purified pVIII, by activated adenovirus protease, were electrophoresed, blotted onto Hyprebond-PVDF and probed with (**a**) pVIII N-Terminal antisera and (**b**) pVIII C-Terminal antisera.

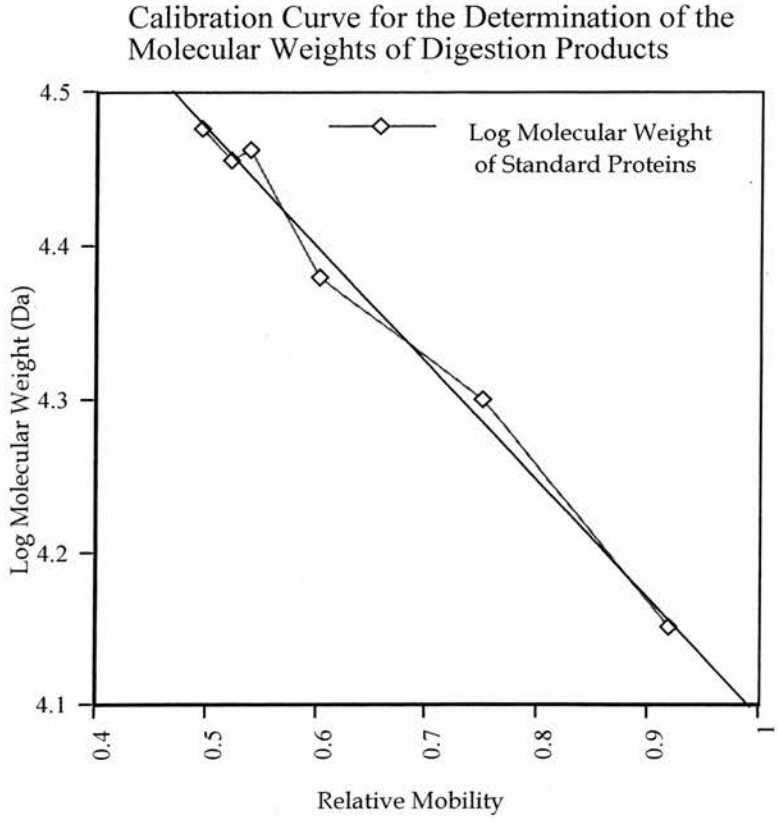


Figure 61. Calibration Curve for Determining the Molecular Weights of Digestion Products.

3.9 Identification of the pVIII Sequence Which When Cleaved Forms The Mature Protein VIII

An attempt at identifying the sequence of Ad2 pVIII which forms the mature protein VIII using antisera generated from peptides composed of the N and C-terminal sequences of Ad2 pVIII was made. Two 20% SDS-PAGE gels loaded with Ad2ts1, Dalton VII markers (supplied by Sigma) and Ad2 were ran at 40 mA until the dye-front was 5 mm from the bottom of the gel. A gel was stained with Coomassie blue and the other gel used to make a Western blot. After probing the blot (see figure 62) with N-terminal antisera a band was found in the Ad2 lane which corresponded to the band identified as VIII on the Coomassie stained gel (see figure 63). The Western blot was stripped and reprobed using the C-terminal antisera (see figure 62).

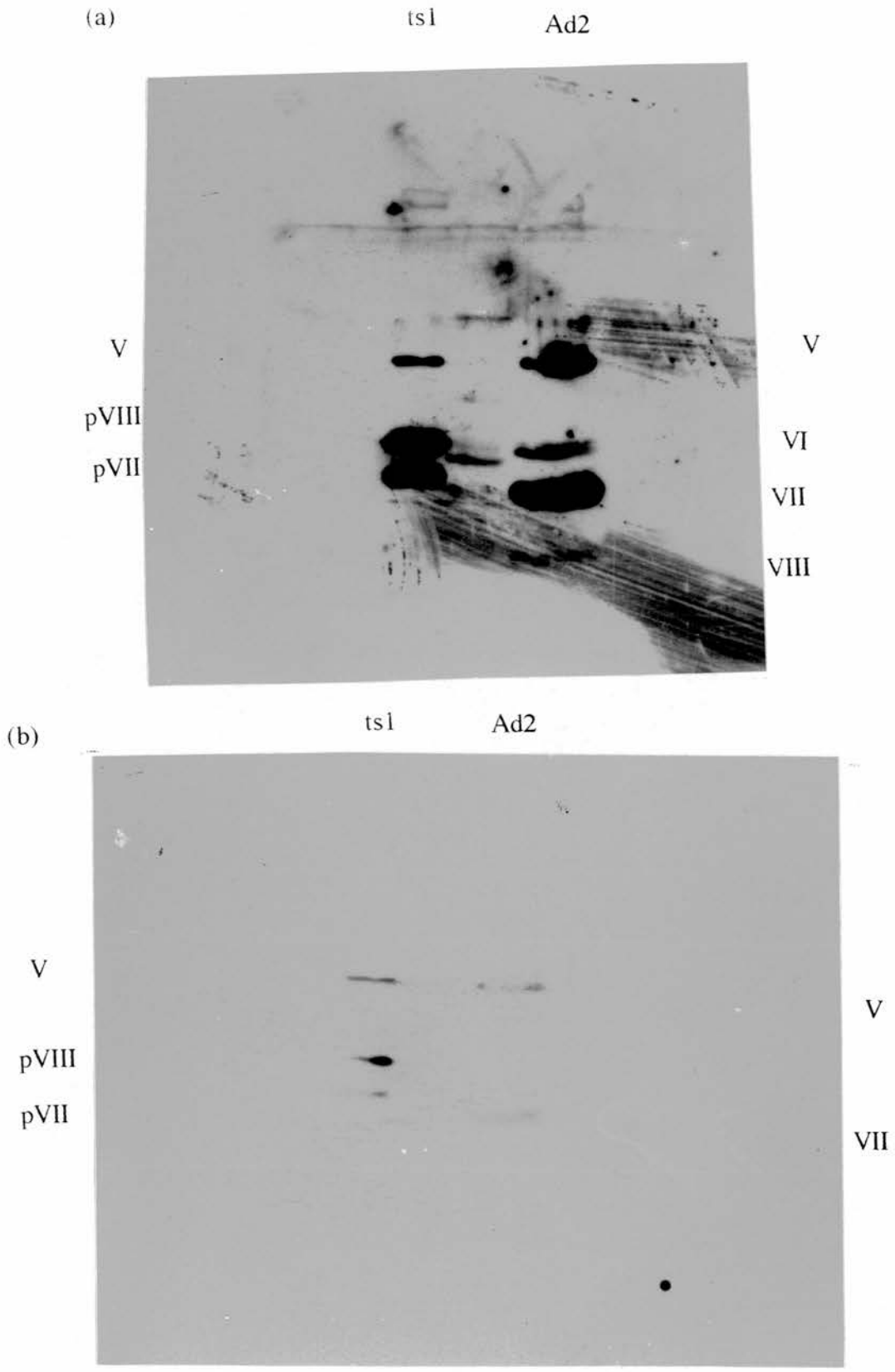


Figure 62: Western Blot analysis of adenovirus serotypes ts1 and 2, and Dalton VII markers. Adenovirus serotypes ts1 and 2, and Dalton VII markers were loaded onto a 20% polyacrylamide gel, electrophoresed, blotted onto nitrocellulose and probed with (a) pVIII N-Terminal antisera and (b) pVIII C-Terminal antisera.

The C-terminal antisera failed to detect any band in the region of VIII in Ad2, but did pick out the pVIII band in Ad2ts1, showing that the antisera was active.

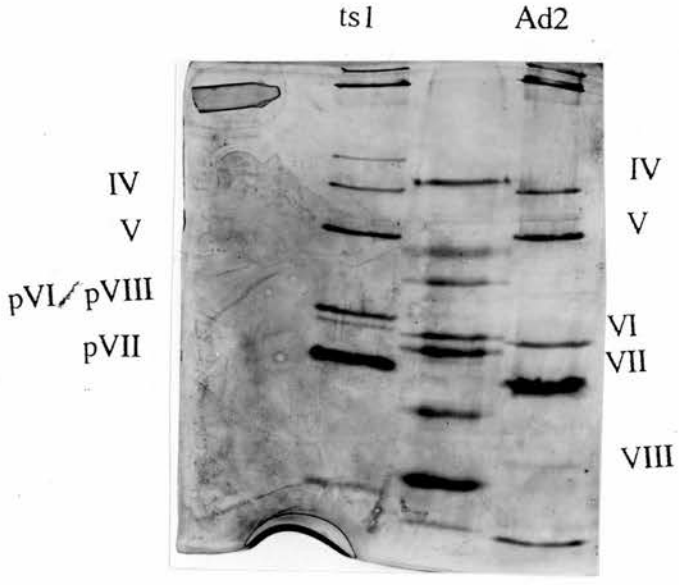


Figure 63: SDS-polyacrylamide gel electrophoresis of adenovirus serotypes ts1 and 2, and Dalton VII markers. Adenovirus serotypes ts1 and 2, and Dalton VII markers were loaded onto a 20% polyacrylamide gel and stained with Coomassie blue.

3.10 Amino acid Sequencing of recombinant pVIII digestion products

Recombinant pVIII was digested with the adenovirus 23 kDa protease and the digestions products seperated by SDS-PAGE as in methods 2.4.10(a). The digestion products were blotted onto a Pro-bond PVDF membrane as in methods 2.4.10(b). After staining with Amido black and drying the protein bands were sequenced for a minimum of five cycles.

The amino acid sequencing of all the bands in the pVIII digest identified a protein with the sequence SESML, this was provisionaly identified as a contaminating *E.coli* protein. Four proteins were identified with the sequence QQMGR, these would included recombinant pVIII, and recombinant pVIII N-terminal cleavage products that

had cleaved at 111 a.a, 132 a.a. and 158 a.a. The adenovirus protease was identified as a protein with the residues MGSSE was sequenced. Also identified were the pVIII C-Terminal cleavage products GRSSF and GIQL.

3.11 Protease affinity to immobilised pVIII

Recombinant pVIII was immobilised on Sepharose as in methods 2.4.15. In order to examine whether the adenovirus protease bound to pVIII activated and unactivated protease was passed through the column and eluted sequentially with 50 mM Tris-HCl pH 8 containing 0.05 M, 0.1 M, 0.15 M, 0.25 M, 0.5 M, 2.5 M NaCl. The elution fractions were analysed on by SDS-PAGE and Western blotting.

Coomassie blue staining of the SDS-PAGE gels did not reveal any protein bands. However, probing of the Western blot with an anti-protease monoclonal antibody revealed that the unactivated protease did bind to the column but, it was eluted by the first elution buffer which contained 50 mM NaCl. The activated protease also bound to the column and was eluted by the elution buffer containing 500 mM NaCl, see figure 64. Therefore the affinity of the protease for pVIII is increase at least 5 fold upon activation.

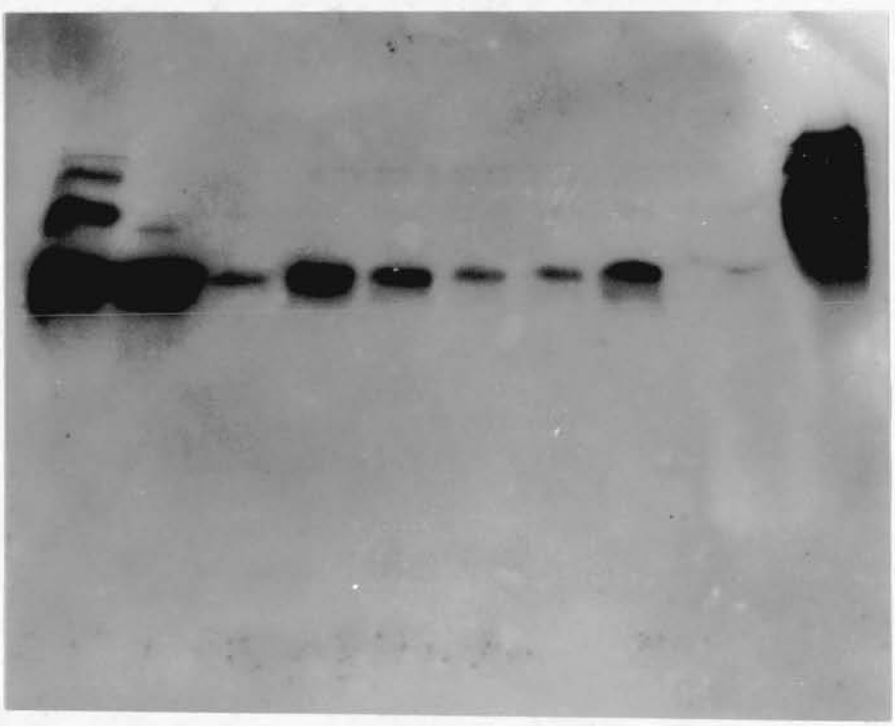
3.12 The amino acid sequence of Adenovirus 17 pVIII gene

The human adenovirus type 17 genome, accession no. AF108105, submitted by Chillon *et al.,* 1999; Armentano and Souza 1998 was used to determine the sequence of pVIII. In order to limit the search area for the pVIII gene the area of the Ad17 genome initially examined was from 25000bp to 30000bp as the start codon for pVIII in Ad2 is at 27215bp. The Ad17 genome sequence was translated by the GCG programme MAP in the three forward reading frames and saved in a file. Microsoft Word was used to search the file for the sequence MSK as this is a highly conserved

(a)

Ad2 23K Protease

(b)



Ad2 23K Protease

Figure 64: Western blot analysis of affinity of the adenovirus protease to immobilised recombinant pVIII. The load, wash and elution fractions varying NaCl concentrations of (a) unactivated protease and (b) activated protease were separated in parallel lanes, blotted onto Hyperbond-PVDF and probed with anti-protease monoclonal antibodies.of all human adenovirus serotypes, the probably of this sequence occurring at random is 1in 8000.

sequence at the start of pVIII. A sequence was identified starting at 25471bp with similarity to Ad2 pVIII which produced a protein with 100 amino acid residues (see figure 65). However, this sequence was 127 amino acids shorter than Ad2 pVIII and although homology to the Ad2 amino acid sequence was strong for the first 70 residues there was little correlation to the Ad2 sequence in the final 30 residues.

M S K E I P T P Y M W S Y Q P Q M G L A A G A S Q D Y S T
R M N W L S A G P S M I S R V N G V R N H R N Q I L L E Q
A A V T S T P R Q S S T R V I G P P P W C I R K S P G R L P
Y Y F R V T H W P K S A

Figure 65. Sequence of Ad17 pVIII as translated by GCG programme MAP from the Ad17 genome (accession no. AF108105).

As this sequence would have a number of implications in the role of pVIII as only the N-terminal sequence was expressed a further search was carried out using Microsoft Word for the sequence SGGIG, a conserved region in the C-terminal region of pVIII. The sequence was identified in a different frame appoximately 550 bp downstream of the start codon. Like the N-terminal sequence this sequence only lasted about seventy amino acid residues before becoming random. However, from this it was possible to identify the middle section of pVIII which was expressed a frame different to both N-terminal and C-terminal regions. From this it is possible to conclude that the sequence of Ad17 pVIII is as in figure and that there is an error in the sequencing of the Ad17 genome between 25670 and 25676 where an extra base has been inserted. There is probably also an error between 25971 and 25974 where either an extra base has been included or two base pairs are missing. It is mosre likely that it is the latter as the Ad2 genome codes for two amino acid residues at this point.

M S K E I P T P Y M W S Y Q P Q M G L A A G A S Q D Y S T
R M N W L S A G P S M I S R V N G V R N H R N Q I L L E Q
A A V T S T P R ? L N P R N W P S T L V Y Q E I P G P T T V
L L P R D A L A E V R M T N S G V Q L A G G A S R C P L R P
Q S G I K T L V I R G R G T Q L N D E L V S S S I G L R P D
G V F Q L A G A G R S S F T P N Q A ? ? L T L Q S S S S E P
R S G G I G T L Q F V E E F V P S V Y F N P F S G S P G L Y P
D E F I P N F D A V R E A V D G H D

Figure 66. Proposed sequence of pVIII translated from Ad17 genome (accession no. AF108105).

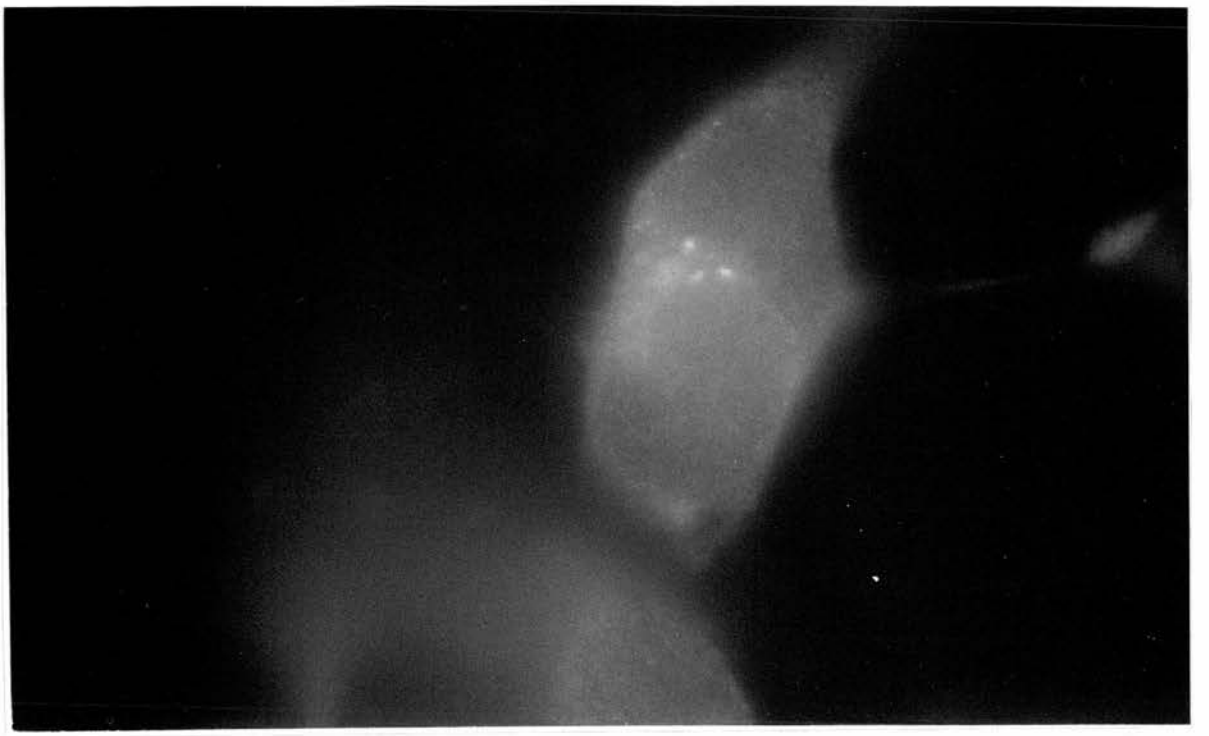
3.13 Immunofluorescence Results

3.13.1 Double Labelling of pVIII-CT and the fiber

At twenty four hours post infection (p.i.) a HeLa monolayer grown on a multispot slide was fixed as in the methods 2.5.6(a) and stored at 4°C. The cells were blocked and permeabilised as described in the methods section. The cells were probed for the presence of pVIII with a polyclonal antiserum raised in sheep against a synthetic peptide and for the fiber with a polyclonal antiserum raised in guinea pigs against the fiber and supplied by W.C. Russell. It was observed that pVIII was located in the cytoplasm of the cells and around the nuclear membrane but, not inside the nucleus whereas the fiber was observed to be in the cytoplasm but, unlike pVIII it did not accumulate around the nuclear membrane.

In certain cells pVIII and the fiber appeared to appeared to exhibit a similar distribution and may interact with each other. In figure 67 which was taken at sixteen hours p.i. there is a group of four dots on the fiber photograph that appear to be in an identical position to a group of dots in the pVIIIC-T photograph. An experiment on a

(A)



(B)

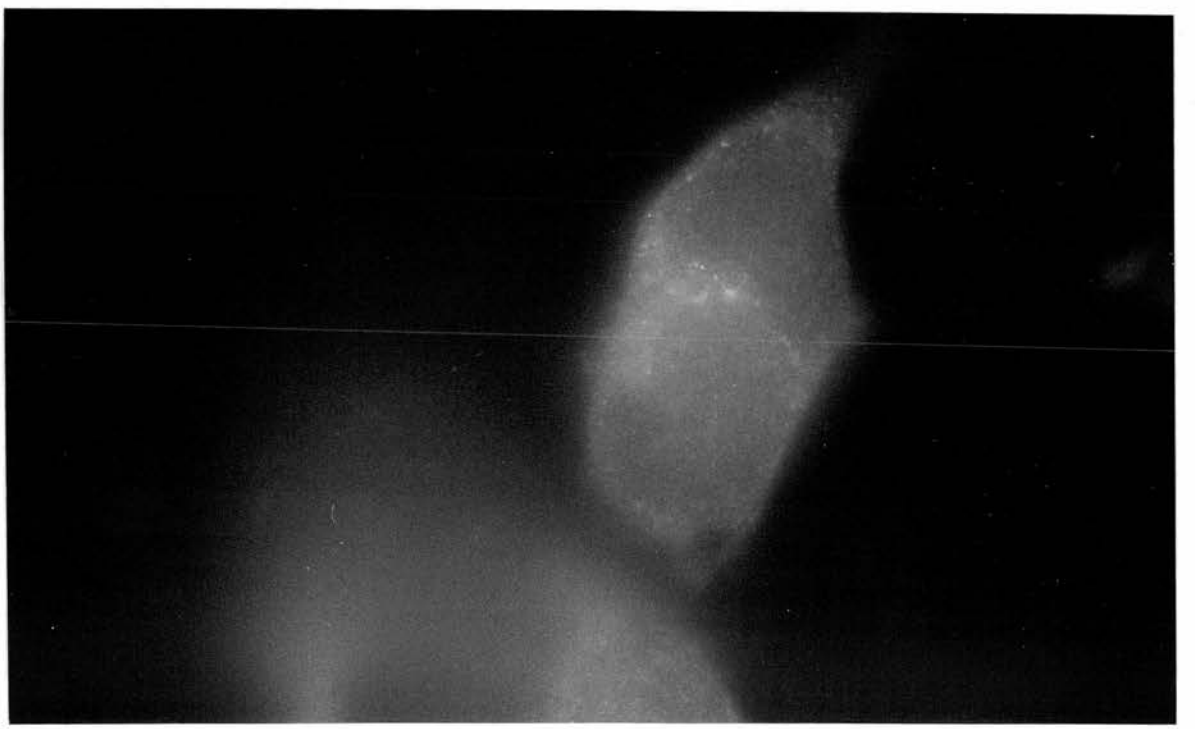


Figure 67. Immunofluorescence staining of pVIII and Fiber proteins. Monolayer cultures grown on multispot slides were infected with Ad2 at a multiplicity of approximately 100 p.f.u per cell and analysed at 16 hours post infection for viral protein expression by indirect immunofluorescence. (A) HeLa cells probed with antisera against pVIII N-Terminal polypeptide. (B) HeLa cells probed with antisera against adenovirus type 5 fiber.

confocal microscope did show interaction between the two proteins but, the cell had a high background caused by non-specific antibody binding so it is possible the interaction was a product of the high background.

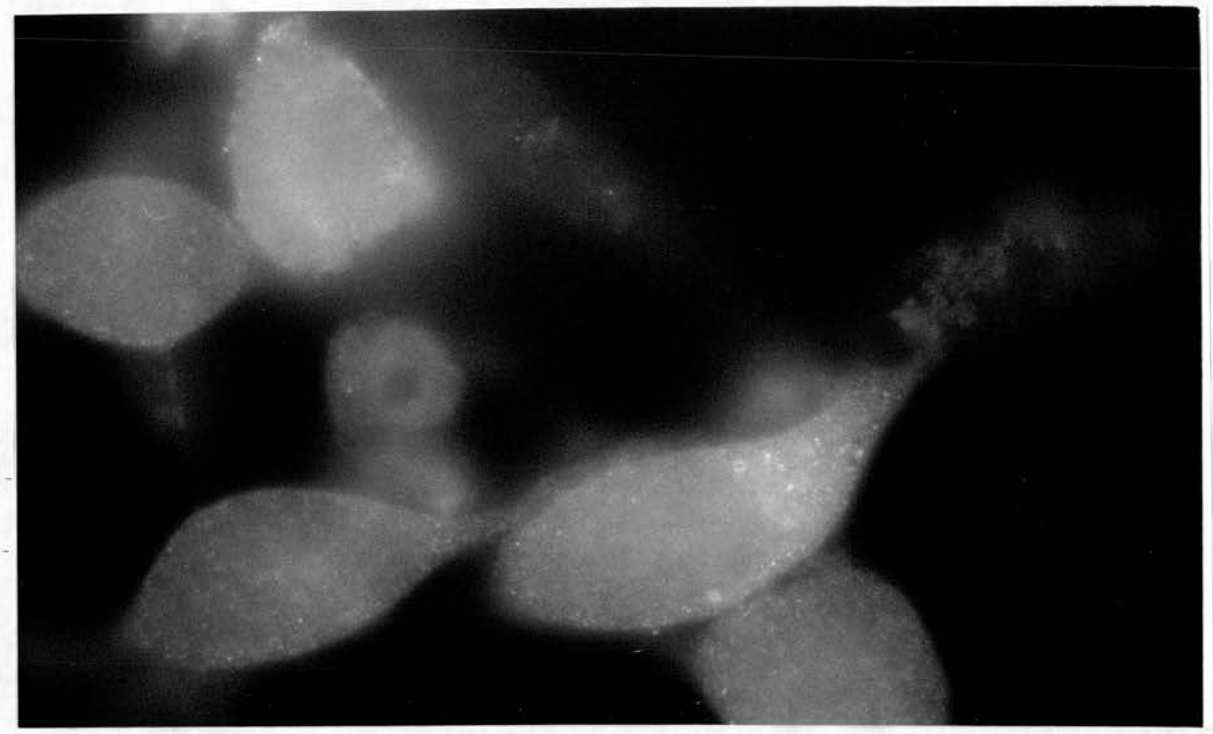
3.13.2 Double Labelling of the adenovirus protease and the fiber

A HeLa monolayer grown on a multispot slide and infected with Ad2, at twelve hours p.i. the monolayer was fixed as in the methods 2.5.6(a) and stored at 4°C. The cells were blocked and permeabilised with Triton X-100 as described in the methods section. The protease was probed for using the monoclonal antibody OA10B3, supplied by O.A. Vaughan and the fiber probed for using the guinea pig antisera. The protease
appeared randomly distributed throughout the cytoplasm of the cell, as was the fiber and was not detected in the cell nucleus. Occasionally cells were observed to contain sites where there was a potential interaction. In figure 68 the two spots in the cytoplasm of centre cell, which analysed for the presence fiber correspond closely to the location of two sites of fluorescence caused by the presence of protease.

3.13.3 Double Labelling with the adenovirus protease and pVIII

At twenty eight hours post infection (p.i.) a HeLa monolayer grown on a multispot slide was fixed and stored at 4°C. The cells were blocked and permeabilised as described in the methods section. It was observed that pVIII and the protease both displayed a group of three spots in the same orientation and area of a HeLa cell, see figure 69. This was taken as an indicator of possible interaction between pVIII and the protease. Further work by O.A. Vaughan using a confocal microscope showed an interaction between the adenovirus protease and pVIII

(A)



(B)

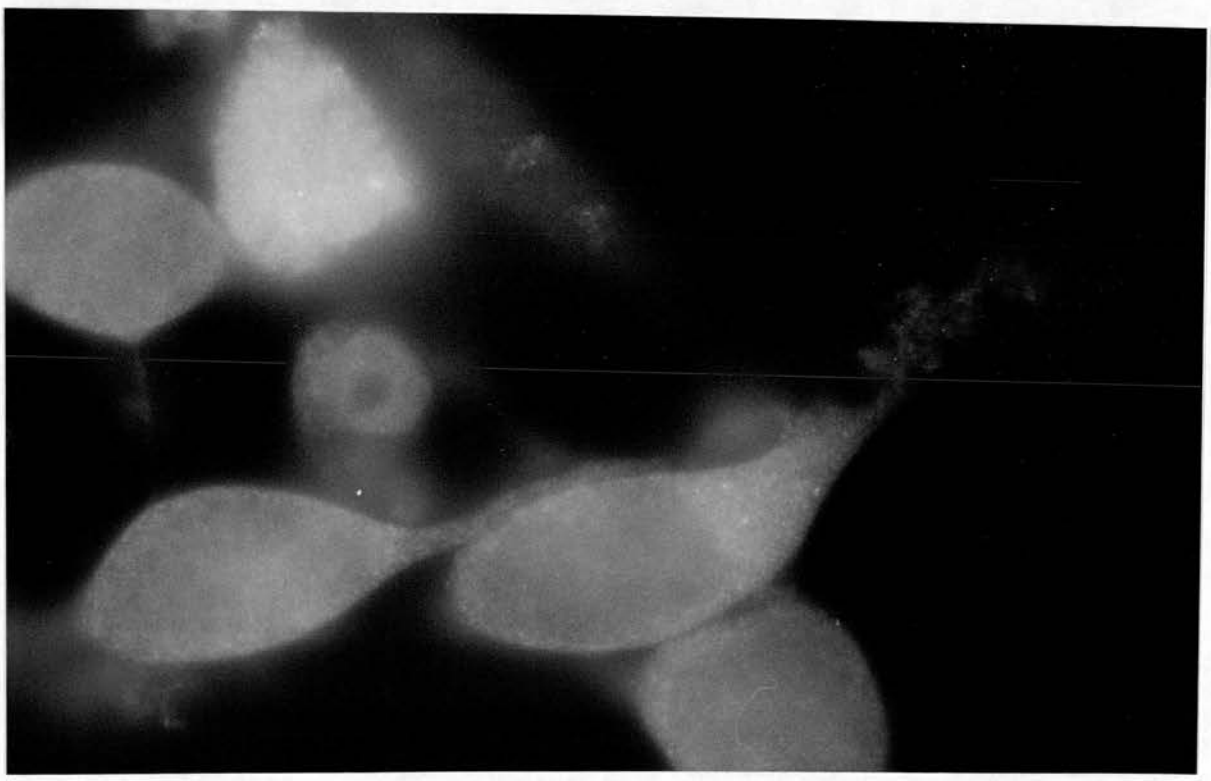
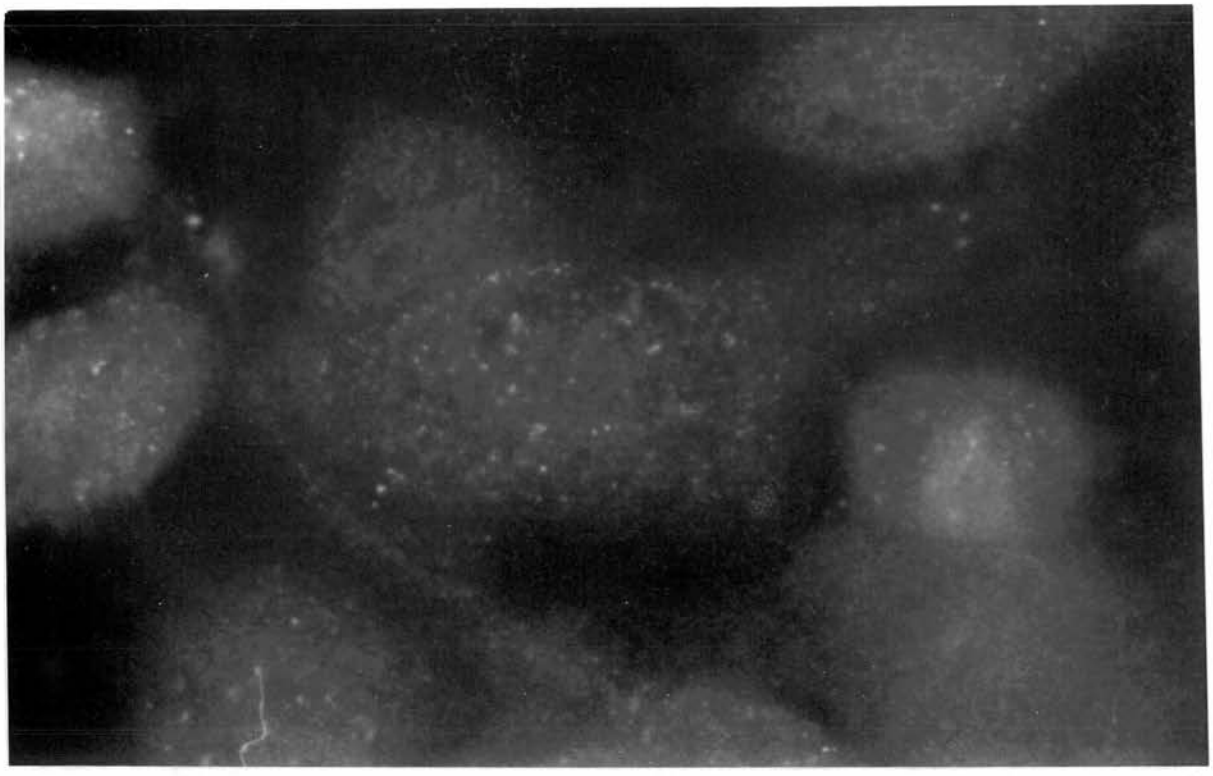


Figure 68. Immunofluorescence staining of protease and Fiber proteins. Monolayer cultures grown on multispot slides were infected with Ad2 at a multiplicity of approximately 100 p.f.u per cell and analysed at 12 hours post infection for viral protein expression by indirect immunofluorescence. (A) HeLa cells probed with monoclonal antibodies against the adenovirus protease. (B) HeLa cells probed with antisera against adenovirus type 5 fiber.

(A)



(B)

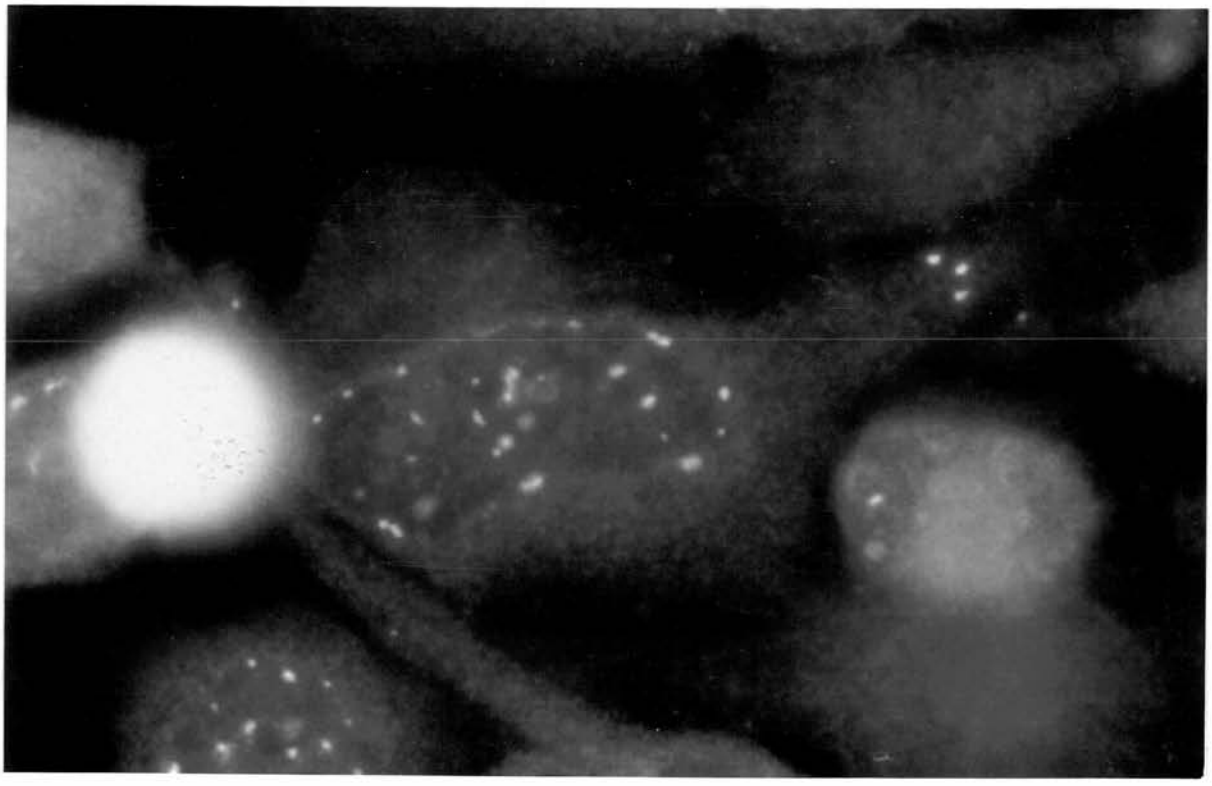


Figure 69. Immunofluorescence staining of protease and protein pVIII. Monolayer cultures grown on multispot slides were infected with Ad2 at a multiplicity of approximately 100 p.f.u per cell and analysed at 28 hours post infection for viral protein expression by indirect immunofluorescence. (A) HeLa cells probed with monoclonal antibodies against the adenovirus protease. (B) HeLa cells probed with antisera against recombinant pVIII.

3.13.4 Distribution of Selected viral antigens at 24 hours post infection

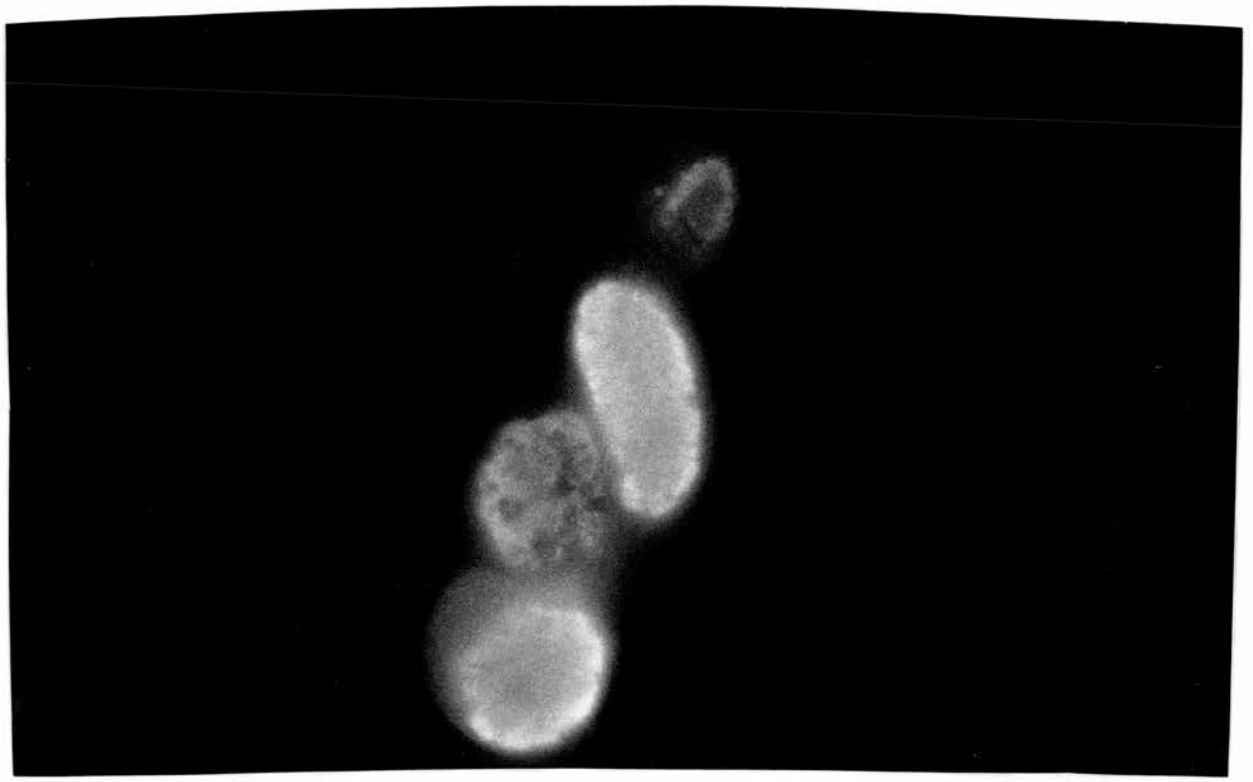
(a) Hexon and Penton

At twenty four hours post infection (p.i.) HeLa monolayers grown on a multispot slides were fixed and stored at 4°C. The cells were blocked and permeabilised as described in the methods section before being probed with antibodies raised against the hexon and the penton which were donated by W.C. Russell. At 24 hours post infection the hexon and the penton, see figure 70, were observed to be localised in the nucleus of the HeLa cell. The hexon also appears, in some cells, to be detected in the cytoplasm around the nuclear membrane but, this may just be background caused by the intense fluorescent response or it may be the antibodies detecting virions in the cytoplasm. Owing to the intense fluorescence observed in the cells at 24 hours it was not possible
to observe any pattern to the hexon location in the nucleus. The penton also produced a strong fluorescent response at twenty four hours p.i. however, some structure was still visible. The strongest response to the penton antigen was from around the nuclear membrane but in some nuclei areas could be observed that were free of fluorescence indicating that the penton may be aggregating with itself or another protein. It is also possible that the penton may be aggregating around another protein in the nucleus which is responsible for the 'holes' observed.

(b) Adenovirus protein pVI

At twenty fours hours post infection (p.i.) a HeLa monolayer grown on a multispot slide was fixed before storing at 4°C. The cells were blocked and permeabilised as described in the methods section. The adenovirus protein pVI was probed for using a polyclonal antiserum donated by W.C. Russell that was generated in rabbits against a recombinant pVI fusion protein. Also used was a polyclonal antiserum raised in

(A)



(B)

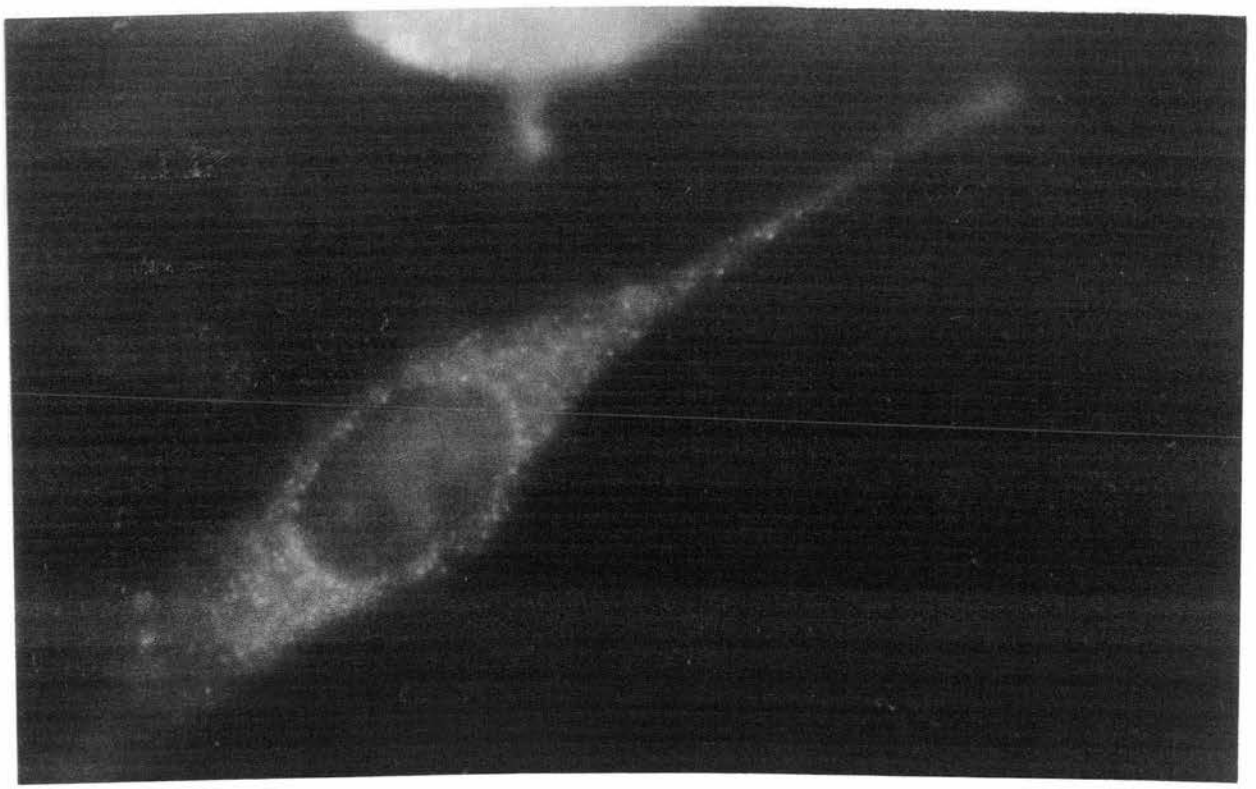


Figure 70. Immunofluorescence staining of penton and hexon protein . Monolayer cultures grown on multispot slides were infected with Ad2 at a multiplicity of approximately 100 p.f.u per cell and analysed at 24 hours post infection for viral protein expression by indirect immunofluorescence. Cells were fixed with paraformaldehyde and permeabilised with Triton X-100. (A) HeLa cells probed with antiserum against the adenovirus penton. (B) HeLa cells probed with antiserum against adenovirus hexon.

rabbits against a synthetic peptide of the protease activating peptide (pVIct). Unfortunately, the response at 24 hours p.i. of both antisera, see figure 71, masked much of the structure in the nucleus but, it appears that the fluorescence is composed of circles and dots within the nucleus. Although both antisera detect pVI in the cytoplasm and the nucleus there is a difference in the specificity. As expected the antisera against pVI is more efficient at detecting pVI in the cytoplasm due to a larger number of epitopes available, it is the antiserum raised against the activating peptide which displays the strongest response in the nucleus. The strong reaction of the anti peptide antiserum in the nucleus may be because after fixing and permeabilising it is the c-terminal epitopes which are the most exposed.

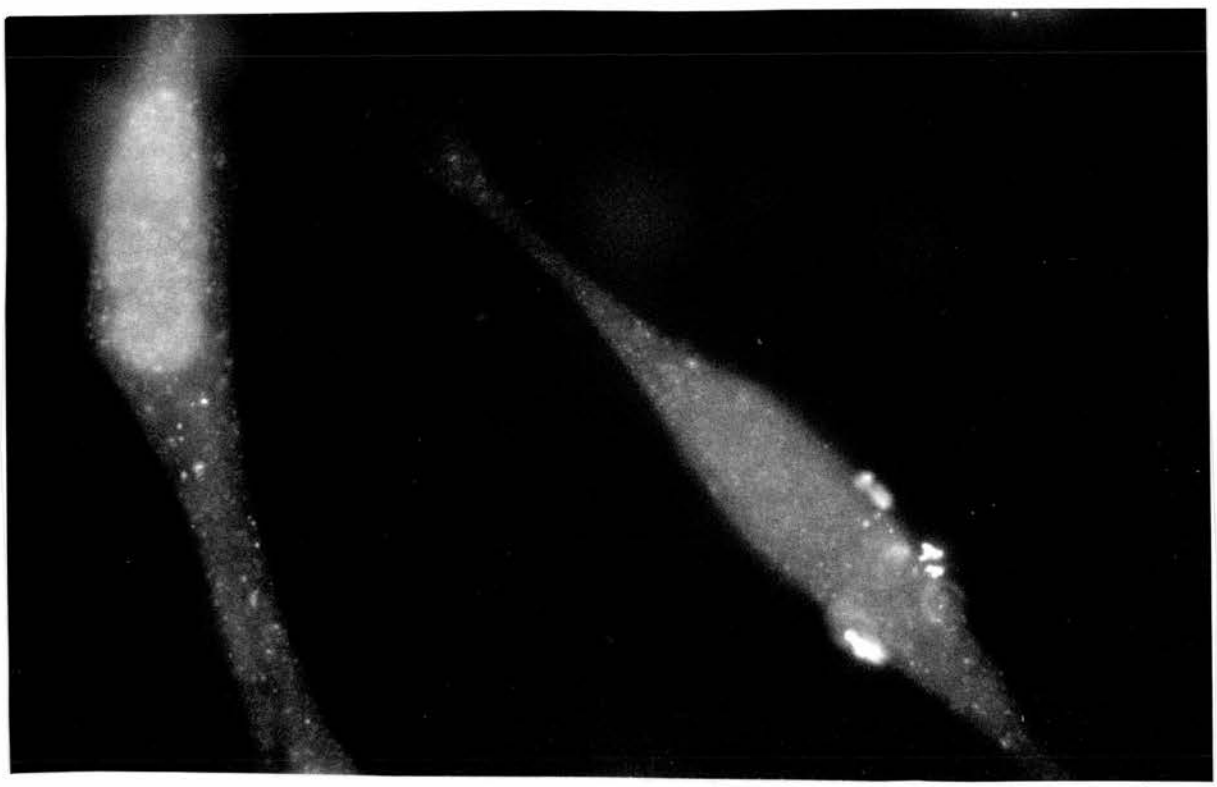
(c) The Adenovirus DNA Binding protein and L1 52 kDa protein

Twenty four hours after infecting the a HeLa monolayer with adenovirus serotype 2 the cells were fixed and stored at 4°C until they were permeabilise, see methods section 2.5.6 for fixing and permeabilising procedures. At twenty four hours p.i. the DNA binding protein (DBP) is observed in the nucleus of the cell, see figure 72, forming large globular structures which are present throughout the nucleus. The L1 52 kDa protein is also observed in the cell nucleus and although there are sites in the nucleus where structures are formed they are irregular unlike the DBP structures.

3.13.5 Development and localisation of fluorescent antigens against pVIII

A series of infections were prepared to examine the development and localisation of the adenovirus protein pVIII. At 12, 16, 24 and 28 hours post infection (p.i.) HeLa monolayers grown on a multispot slides were fixed as in the methods 2.5.6(a) and stored at 4°C. The cells were permeabilised with Triton X-100 and blocked as described in the methods section. At twelve hours sporadic spots are visible in the

(A)



(B)

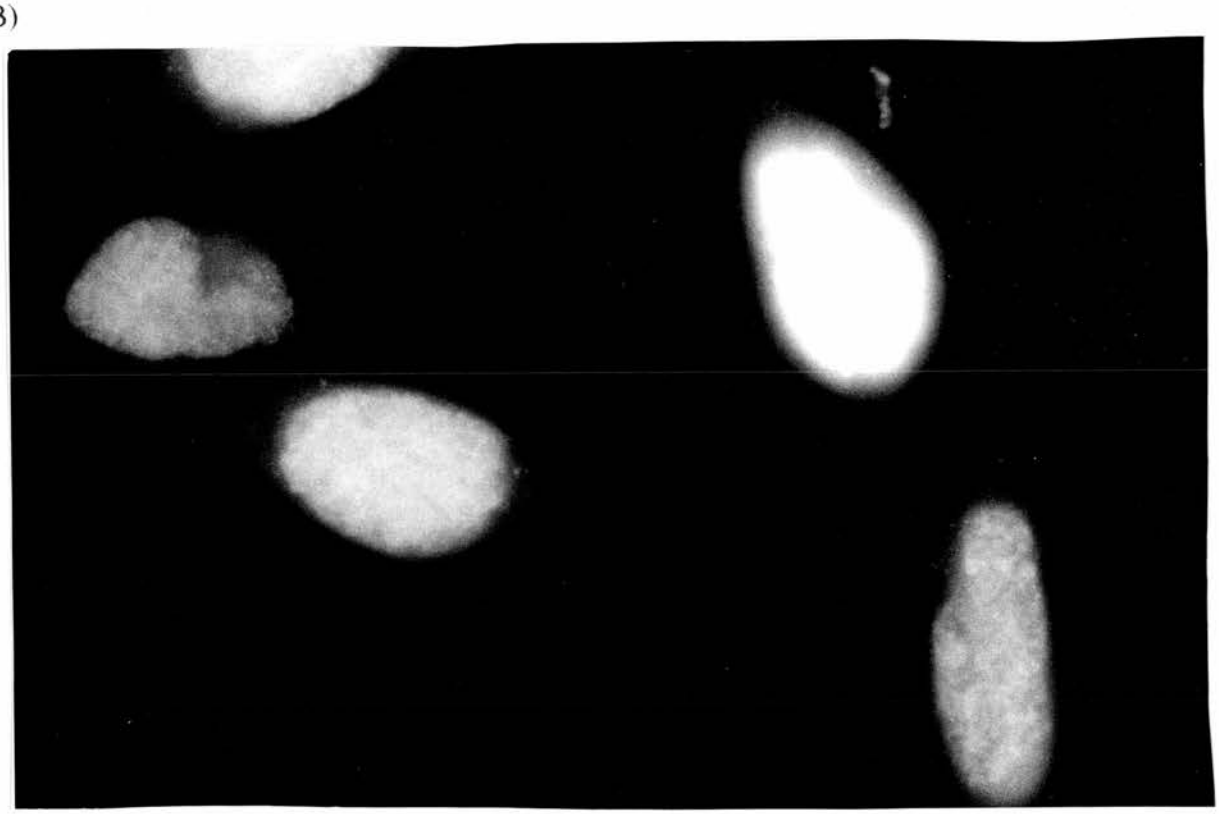
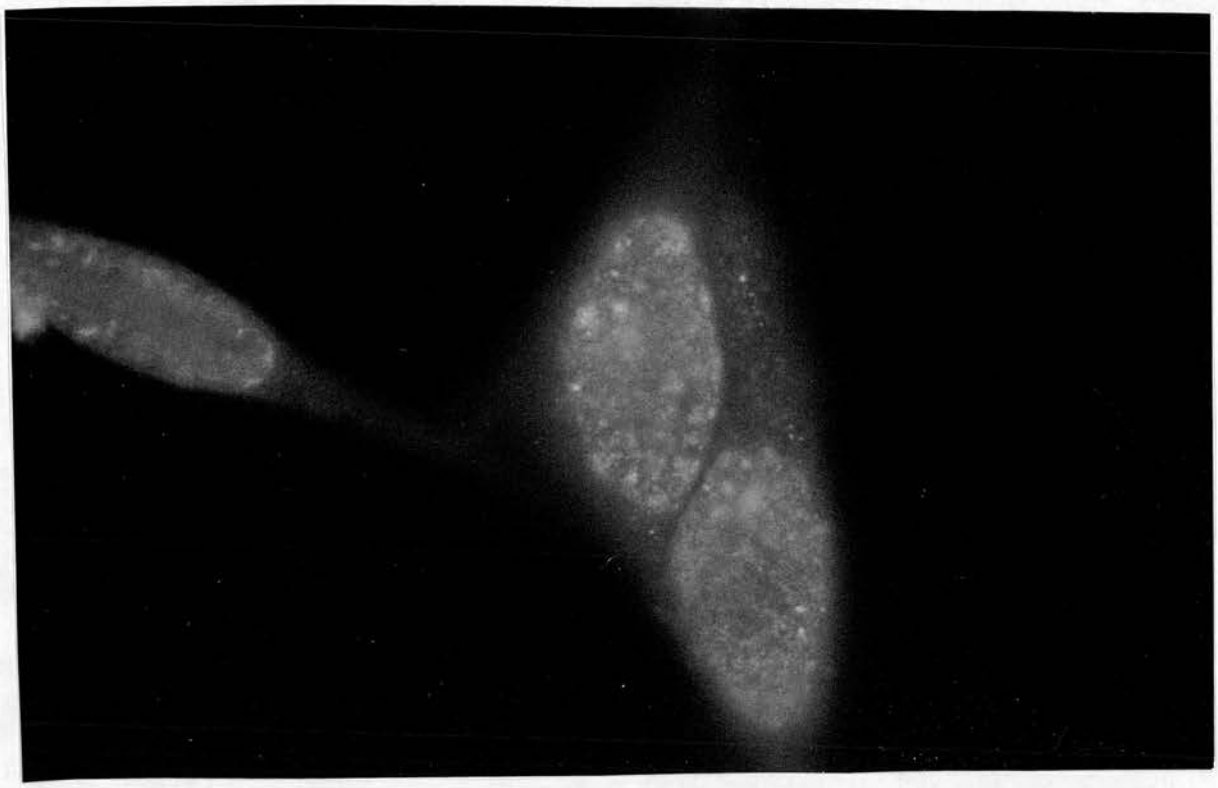


Figure 71. Immunofluorescence staining of pVI proteins. Monolayer cultures grown on multispot slides were infected with Ad2 at a multiplicity of approximately 100 p.f.u per cell and analysed at 24 hours post infection for viral protein expression by indirect immunofluorescence.(A) HeLa cells probed with antiserum against the adenovirus protein pVI. (B) HeLa cells probed with antisera against a synthetic peptide of the pVI C-terminal.

(A)



(B)

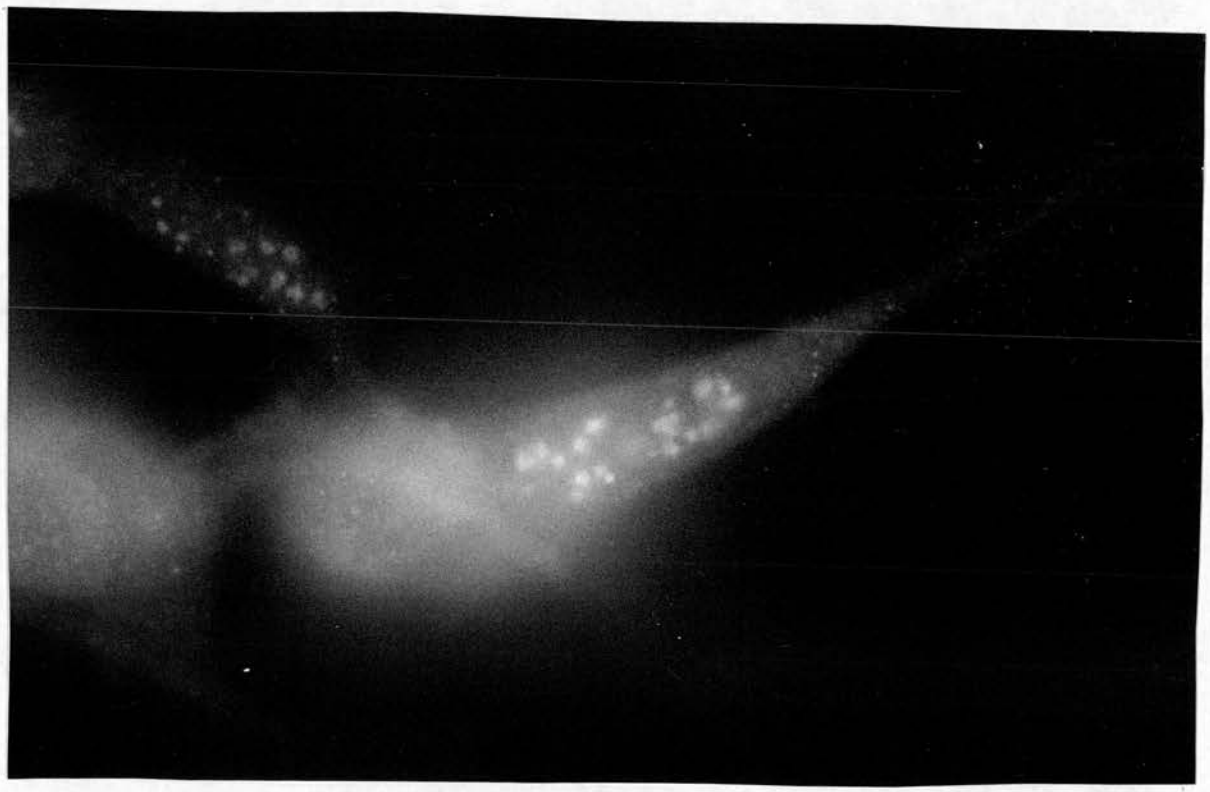


Figure 72. Immunofluorescence staining of L1 52 kDa and DBP proteins. Monolayer cultures grown on multispot slides were infected with Ad2 at a multiplicity of approximately 100 p.f.u per cell and analysed at 24 hours post infection for viral protein expression by indirect immunofluorescence. (A) HeLa cells probed with antiserum against a synthetic peptide of the adenovirus L1 52 kDa protein. (B) HeLa cells probed with antiserum against the adenovirus DBP.

cytoplasm and the concentration increases over time, see figure 73, until twenty four hours post infection. At twenty four hours p.i. as well as the random spots in the cytoplasm small rods are also visible in the cytoplasm. However, at no point was pVIII detected in the nucleus of the cell but, by sixteen hours p.i. fluorescence was detected around the nuclear membrane.

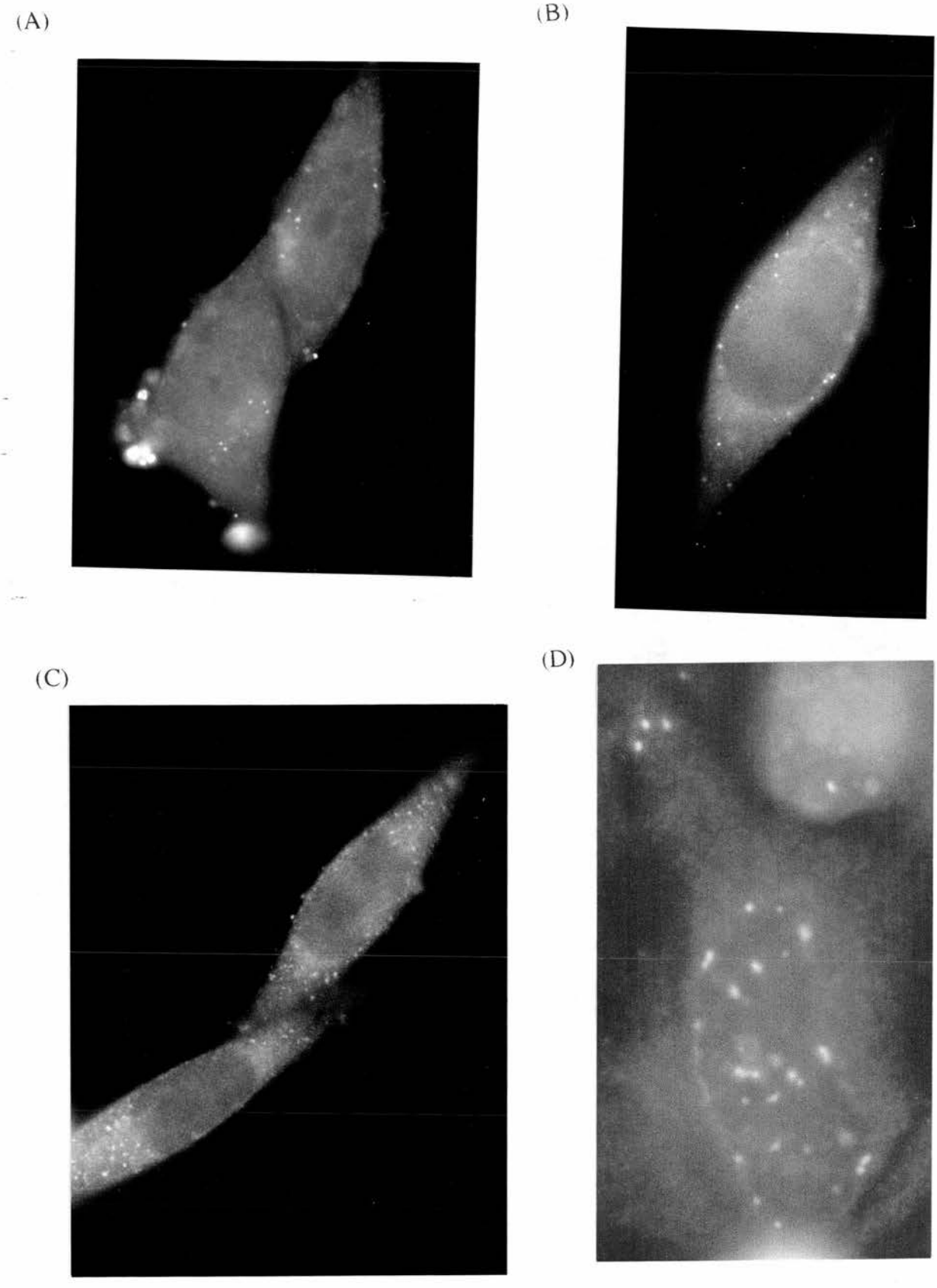


Figure 73. Immunofluorescence staining of pVIII over time. Monolayer cultures grown on multispot slides were infected with Ad2 at a multiplicity of approximately 100 p.f.u per cell and analysed at 24 hours post infection for viral protein expression by indirect immunofluorescence. The antiserum used was generated against a synthetic peptide in the pVIII C-terminal region and raised in sheep. Fluorescence observed in HeLa cells at (A) 12 hours p.i., (B) 16 hours p.i., (C) 24 hours p.i. and (D) 28 hours p.i.

4. Discussion

4.1 Analysis of pVIII Digestion Products

4.1.1 Western Blot Analysis of pVIII Digestion Products

Digestion of recombinant pVIII with adenovirus protease theoretically results in the production of twelve digestion products (see figure 74). Eight possible digestion products were detected by the N-Terminal antiserum (25 kDa, 23.8 kDa, 21.3 kDa, 19.4 kDa, 18.1 kDa, 17.2 kDa, 16 kDa, and 15 kDa) and another two other possible cleavage products by the C-Terminal antiserum (23.8 kDa and 14.5 kDa). The 23.8 kDa band was identified as the protease as it appeared on both blots and therefore could not be a cleavage product, it also had the same mobility as the protease. The 25 kDa band detected by the N-Terminal Blot could not be identified as any of the theoretical cleavage products. Therefore the 25 kDa band is probably an impurity from the pVIII or protease purifications. The 21.3 kDa and 19.4 kDa products are probably the fusion protein cleaved at 158 a.a. and -23 and 158 a.a. as the expected molecular weight of these products are 22.1 kDa and 20.6 kDa respectively. The 18.1 kDa and 17.2 kDa bands are likely to be pVIII fusion protein cleaved at 131 a.a. and -23 and 131 a.a. respectively, their molecular weights are 19 kDa and 17.6 kDa. respectively. The 16 kDa and 15 kDa products are probably recombinant pVIII cleaved at 111 a.a. and -23 and 111 a.a. as the expected molecular weight of these products are 16.6 kDa and 15.2 kDa respectively.

This leaves five theoretical cleavage products unidentified. Three of the unidentified cleavage products (5.4 kDa, 3.0 kDa, and 2.4 kDa) are not detected as neither antiserum is able to detect the fragments and that the proteins are too small to be resloved on an ordinary SDS-PAGE gel. The two putative C-Terminal proteins which are not detected by Western blotting with the C-Terminal antiserum are the

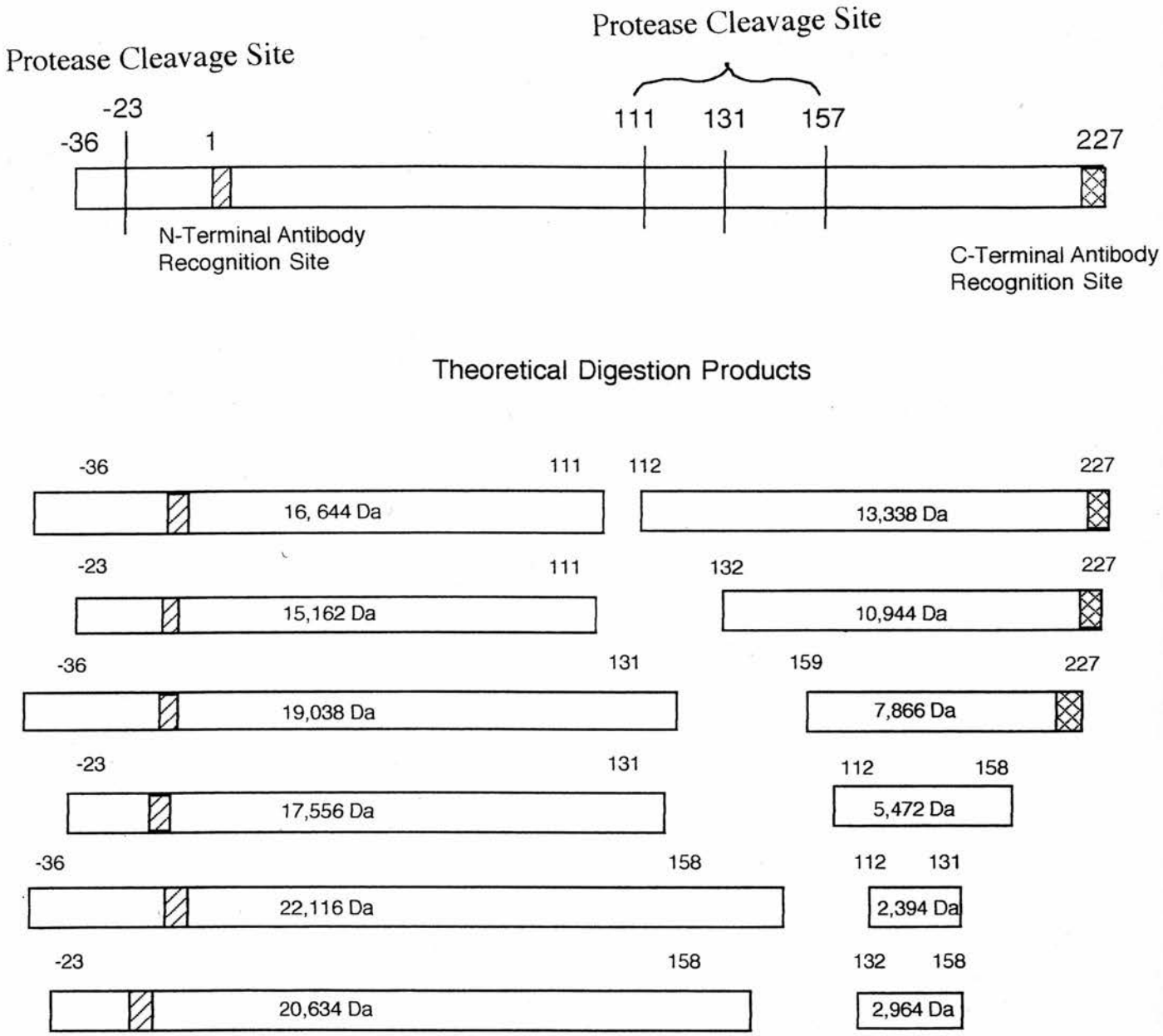


Figure 74: Digestion Products of recombinant pVIII

10.9 kDa and 7.8 kDa cleavage products. However, both cleavage products are too small to be sperated on an SDS-PAGE gel. It may be possible to detect the proteins using low molecular weight SDS-PAGE but, if the 158 cleavage site is cleaved faster than the 131 cleavage site then the 10.9 kDa fragment would not appear.

The data indicates that the 111 (LAGG-F), 131 (IRGR-G) and 158 a.a. (IGGA-G) cleavage sites were cleaved and the -23 a.a. (MTGG-Q) cleavage site in the fusion protein tag was cleaved, as in agreement with the cleavage sites predicted by Webster *et al.,.,* (1989) and Anderson (1990). The existence of the 158 a.a. cleavage site agrees with Anderson (1990), who sequenced a protein with the first five amino acids identical to that of the theoretical cleavage product produced by cleavage at 158 a.a.

4.1.2 Amino Acid Sequencing of pVIII Digestion Products

The products of a digestion reaction of recombinant pVIII with the adenovirus protease were analysed using a N-terminal amino acid sequencer. Eight protein bands of different molecular weights were separated by SDS-PAGE (30 kDa, 25k Da, 23 kDa, 22 kDa, 18 kDa, 17 kDa, 14 kDa and 11kDa). N-terminal sequencing of the 30 kDa band produced the sequence QQMGR, which is the sequence present in the polypeptide tag attached to recombinant pVIII. From the molecular weight and the sequence it was assumed that this protein band was recombinant pVIII. The 25 kDa band had an N-Terminal sequence of SESML, this sequence does not belong to any adenovirus protein and so was assummed to be an *E.coli* contaminating protein. However, after using the gcg program FASTA to search the Swiss Protein database no direct match was found. The closest matches, by sequence, for *E.coli* proteins are the maltose operon periplasmic protein precursor and phenylalanyl-tRNA synthetase ß chain. However, both proteins do not contain a protease cleavage site prior to the sequence and both have theoretical isoelectric points of less than 6. The 23 kDa band was confirmed as the adenovirus protease as it had the N-terminal sequence MGSEE which corresponds to the first five amino acid residues in the adenovirus serotype 2 protease.

N-terminal sequencing of the 22 kDa, 18 kDa, 17 kDa all produced the same sequence of QQMGR, which is a sequence present in the polypeptide tag attached to

recombinant pVIII. From the molecular weight of the bands it was concluded that the 22 kDa band was recombinant pVIII cleaved at 158 a.a., the 18 kDa band was pVIII cleaved at 132 a.a. and that the 17 kDa band was pVIII cleaved at 111 a.a. Sequencing of the 14 kDa and 11 kDa bands confirmed cleavage of recombinant pVIII at the 132 and 158 theoretical cleavage sites as the sequences GIQLN and GRSSFTP were obtained respectively. However, there was no evidence of a band corresponding to the cleavage product 111-227 and therefore confirmation of this cleavage site has not been obtained but, given the presence of a theoretical cleavage site and the apparent molecular weights of the cleavage products it is probable that the site is cleaved by the protease. A possible reason as to why no evidence of the 111-227 cleavage product was found is because the 132 a.a. is cleaved preferentially to the 111 a.a cleavage site.

4.1.3 Evidence for VIII being formed by cleavage of pVIII at 111 a.a

The observed cleavage products of the 111 a.a. cleavage site shows that the protein can be cleaved at this site *in vitro* , although at low levels. The cleavage of the 111 a.a. cleavage site may only be possible *in vitro* because of the digest conditions altering the conformation of recombinant pVIII allowing the protease access to cleavage site. However Greber *et al.,.,* (1993) claim that during the dissociation of VIII from the viral capsid VIII is not cleaved. Should the 111 a.a cleavage site be cleaved to form VIII then this would account for the observation. It could also explain why the pVIII protein in murine adenovirus serotype 1 (Adm1) lacks the cleavage site at 131 a.a. but retains the cleavage sites at 111 and 158 a.a. if the cleavage of pVIII at 111 a.a. forms VIII. Cleavage at 111 a.a. would result in a protein with a molecular weight of 12.65 kDa which is less than the expected molecular weight of 15 kDa obtained from SDS-PAGE analysis. Although the SDS-PAGE gel molecular weight of VIII indicates that VIII is formed from the 1-131 amino acid sequence of pVIII photographs of gels in Liu *et al.,.,* (1985) show VIII

migrating to a position of 14 kDa which would indicate that VIII is formed by cleavage at 111 a.a. However, it should be noted that the SDS-PAGE gel molecular weight of pTP indicated that this protein had a molecular weight of 55 kDa but, DNA sequencing showed that its molecular weight was only 38 kDa.

4.1.4 Evidence for VIII being formed by cleavage of pVIII at 131 a.a.

The 15.2 kDa theoretical cleavage product of pVIII (cleaved at 131 a.a.) agrees with the molecular weight of VIII of 15 kDa which was proposed by Stewart *et al.,.,* (1993) and with the molecular weight of VIII found from a 20% SDS-PAGE gel of 15 kDa. In the event of VIII being formed by cleavage at 131 a.a. then it is possible that the 111 a.a. cleavage site is involved in the disassembly of the adenovirus capsid, during infection, as an alteration in the structure of VIII would cause capsid instability. However, Greber *et al.,* (1993) claim VIII disassociates from the capsid without being cleaved. Should the 111 a.a. cleavage site only be accessible to the protease after cleavage of pVIII it would account for the conservation of this cleavage site in the protein as cleavage of this site would allow the infection process to start/proceed. However, should the 111 a.a cleavage site not be cleaved it is a puzzle as the virus has conserved the 111 a.a. cleavage site in all the known pVIII sequences but it remains functionally redundant.

4.1.5 Analysis of Adenovirus 2 with Antipeptide Antisera

Verification that VIII was formed from the N-Terminal sequence of pVIII was carried out by probing a Western Blot of disrupted purified ad 2 virus. Although the SDS-PAGE gel molecular weight of VIII indicates that VIII is probably formed from the 1-131 amino acid sequence of pVIII there is no direct evidence that this conclusion is correct. Probing of the Western blot with antiserum, raised against a sythetic peptide of pVIII's N-terminus, produced a band which corresponded to the band produced by

VIII in SDS-PAGE gels. Afterwards the antibodies were stripped from the Western blot and the blot probed with antiserum, raised against a sythetic peptide of pVIII's C-terminus. However, no band was detected in the region where VIII was located. Therefore it can be concluded that the N-terminal sequence of pVIII forms VIII and that the cleavage site is probably 131 amino acid (from recombinant pVIII digests and observed molecular weight). This could be confirmed by the production of synthetic antibodies for the 110-131 region and 132-158 regions of pVIII. The N-terminal antiserum required a long exposure time to detect VIII, which may be because VIII is only present in low quantities in the mature capsid. The N-terminal antiserum exhibits some non-specific binding with for pVI, pVII, V, VI, and VII. However, the non-specific binding of the N-terminal antiserum could be prevented by using milk powder as the blocking agent, instead of 5% bovine serum albumin, in the blocking buffer. The C-terminal antiserum, for pVIII, did not show any evidence of cross reaction with other structural proteins.

4.1.6 Conclusion

The available evidence indicates that VIII is formed from pVIII amino acids 1-111. This would explain why Adm1 can still form a stable capsid despite it lacking the 131 a.a. cleavage site. It also explains the large number of amino acid changes in the sequence 112-131. The only evidence which points towards VIII being formed by 1-131 a.a. is the molecular weight of 15 kDa (Edvardsson *et al.,* 1976). However, as the molecular weight of VIII was obtained from SDS-PAGE gels, this can only be taken as an approximate and viral preparations by Liu *et al.,* (1985) migrated on an SDS-PAGE gel to the position of a 13 kDa protein. Therefore, it is reasonable to conclude that VIII is formed by the first 111 N-terminal amino acids of pVIII.

4.2. Cross Reaction of the pVIII N-Terminal antiserum with other structural proteins

The occurrence of cross reacting antibodies has been frequently observed and 3.5% of antiviral monoclonal antibodies were shown to cross react by (Srinivasappa *et al.*, 1986). This is probably because an antiserum is able to recognise antigenic determinants 3 amino acids long (Houghten, 1985). Statistically the probability of a trimer occurring by chance is 20^3 (once every 8,000 residues) and given the coding capacity of the adenovirus any amino acid trimer would be expected to occur more than once. Therefore it is not unlikely that a cross-reaction would be observed. However, as four different proteins were detected by a specific antiserum, it is possible that the sequence may have a common function in the four proteins. The sequences of pVI, pVII, pVIII, V, VI, VII and VIII were searched for homology and it was found that there was a common sequence in the precursor proteins and the mature proteins (see table 7). Protein V exhibits the greatest homology to the synthetic peptide used to create the N-terminal antiserum. Protein V possesses two P-T-P trimers as well as a Q-I-P-T-T-X-X-X-S sequence, which may be the main antiserum binding site. Precursor VI and the mature protein VI does not possess the P-T-P trimer but does contain an L-P-T-T-X-X-X-A sequence which may form the antiserum binding site. Protein VII possesses the trimer P-T-P and a Ser residue located for bases downstream. As this is the only segment of pVII/VII which has any significant homology to the synthetic peptide it is probable that this sequence is the binding site for the antiserum. From the homology comparisons it is probable that the cross-reaction of the N-terminal antiserum was due to the trimer Pro-Thr-(Pro,Thr) and a Ser/Ala residue located four amino acids further downstream (P-T-[P,T]-X-X-X-[S,A]).

Protein(s)	Amino Acid Sequence
Synthetic Peptide	Met Ser Lys Glu Ile Pro Thr Pro Tyr Met Trp Ser Tyr Glu
pVIII/VIII	Met Ser Lys Glu Ile Pro Thr Pro Tyr Met Trp Ser Tyr Glu
pVII/VII	Ala Arg Asp Tyr Thr Pro Thr Pro Pro Pro Val Ser Thr Val
pVI/VI	Lys Glu Glu Gly Leu Pro Thr Thr Arg Pro Ile Ala Pro Met
V	Val Asp Val Glu Ile Pro Thr Thr Ser Ser Thr Ser Ile Ala

Table 7: Homologous Regions of pVIII, pVI, pVII, V.

4.3. Purification method used to obtain the fusion protein pVIII

The purification method used to obtain the fusion protein pVIII from cellular extract is unorthodox due to the characteristics it possesses. The procedure was developed in order to overcome the insolubility of recombinant pVIII and to take advantage of its six histidine tag and solubility in 1M guanidine chloride. The initial sonication and centrifugation of the cellular extract were intended to purify pVIII from the insoluble proteins. However, as it was found that recombinant pVIII was itself an insoluble protein the initial sonication and centrifugation were used to remove the soluble proteins from the cellular extract. As a result of this, guanidine chloride, at 1M, was added to dissolve recombinant pVIII and the sonication and centrifugation repeated with recombinant pVIII being present in the supernatant. Originally recombinant pVIII was bound to the nickel affinity resin (via the six histidine tag) by mixing together at 4˚C for 30 minutes before loading into a column. This procedure was later changed in order to maximise the binding of recombinant pVIII to the nickel affinity resin by packing the resin by gravitational sedimentation into the column. After the affinity column had settled, the semi-purified recombinant pVIII was loaded into the nickel column and was passed through the column. The affinity column was washed with wash buffer to elute any proteins which had bound to the column as a result of its ionic charge. Elution buffer was added to the column to elute any proteins which

were bound to the nickel column. However, analysis of the elution products by SDS-PAGE showed that the pVIII fusion protein was not eluted by the elution buffer. It was concluded, from the SDS-PAGE gel, that recombinant pVIII was still on the affinity column because either the concentration of imidazole used was not sufficient to elute it or, it had come out of solution. The concentration of imidazole (250 mM) in the elution buffer was sufficient to expect elution of all bound protein, therefore, it was concluded that recombinant pVIII had come out of solution and would be resolubilised by adding guanidine chloride to 1M in the wash and elution buffers. Washing and elution of the affinity column without guanidine chloride in the wash and elution buffers was retained in the purification procedure as it removed all the proteins which were soluble in the wash and elution buffers. After washing the affinity column with wash buffer containing guanidine chloride the proteins bound to the affinity column were eluted with elution buffer (containg 1 M guanidine chloride). Analysis of the elution products by SDS-PAGE showed that only recombinant pVIII with two negligible contaminating proteins were eluted with the elution buffer containing guanidine chloride.

4.4 Computer Software Analysis of the EDS L3 23 kDa Protease Gene

The amino acid sequence of the EDS L3 23 kDa protease obtained from the translation of the DNA sequence showed a minimum of 37.5% similarity in a Pearson and Lipman search with the other known adenovirus sequences. The adenovirus serotypes which most closely matched that of the EDS protease were bovine serotype 7 (b7) and ovine adenovirus 287 (OAV287), similarities of 57.7% and 56.7% respectively. The degree of similarity between the EDS and these serotypes indicates that it may be an intermediate between aviadenoviruses and mastadenoviruses as the protease shows only a 40% similarity with the protease of the CELO (FAV1) adenovirus.

In a study of the EDS genome Hess *et al.*, (1997) found that overall the EDS genome resembled that of OAV287 from close to the beginning of the genome until 22 kbp into the genome, the homology between the EDS genome and that of CELO is reportedly much lower. This result is surprising as EDS belongs to a different genus than that of OAV 287. The EDS genome did have some characteristics in common with the CELO genome in that it does not encode for proteins pV and pIX, however these are also shared with OAV 287. In a comparison with the amino acid sequences of genes of the EDS virus and other adenoviruses Hess *et al.*, 1997 found that for all proteins apart from mu and the fiber EDS was most similar to OAV 287, see table 8. Protein mu and the fiber exhibit highest homology with FAV10 and CELO respectively. The similarity between the fiber of EDS and the aviadenovirus CELO is to be expected as the fiber determines which cell types the virus will penetrate.

Alignments of the known adenovirus protease sequences show that the active site residues of His-55, Asp/Glu 72 and Cys 122 are conserved, as is the Cys 104 activating peptide binding site. In general the sequences around these sites are well conserved but, the amino acid sequence in the Ad4 protease is significantly different to the other around Cys 122. In Ad4 there is an insert of 5 amino acids prior to the active site residue which is not present in other serotypes. Presumably any changes this insert causes in secondary structure is corrected by the 8 residue deletion which occurs after the active site allowing the cystine residue to form part of the catalytic triad.

4.5 EDS L3 23 kDa Protease Antiserum

The antisera used to detect the EDS protease was generated by Lewis Murray by injecting a rabbit with a synthetic peptide of the first 15 N-terminal residues of the protease coupled to HSA. The optimal concentration of the antiserum, as determined by performing an ELISA, against the synthetic peptide used to generate it was

	CELO	FAV10	Ad 2	Ad 12	Ad 40	OAV28	BAV3	CAV1	MAV1	HEV
E1BS	*	x	20	18	22	**32**	x	24	21	x
E1BL	*	x	24	23	24	**41**	x	21	22	x
IVA2	36	x	42	41	41	**68**	x	42	42	x
POL	40	x	46	46	48	**56**	x	49	x	x
pTP	35	35	35	37	38	**54**	x	36	x	x
52K	36	x	32	34	32	**45**	x	32	x	x
IIIa	27	x	34	34	34	**53**	x	31	x	x
III	55	53	57	56	56	**69**	x	57	x	52
pVII	46	44	37	39	39	**49**	x	30	x	32
mu	52	**58**	45	46	48	55	x	51	x	43
pVI	36	x	33	34	33	**49**	x	24	30	x
II	52	51	54	55	56	**73**	53	56	52	x
23K	41	x	40	41	42	**57**	41	41	x	x
DBP	30	x	35	33	34	**53**	x	35	17	x
100K	39	36	36	36	34	**56**	x	34	35	x
pVIII	29	x	34	35	36	**53**	34	38	33	x
IV	**35**/32	x	24	28	34/28	29	32	25	24	x
E4	*	x	18	22	25	**28**	x	22	20	x

Table 8: Amino acid Sequence Identity Between Identified Genes of EDS Virus and Known Genes of Other Avian and Mammalian Adenoviruses. The proteins with greatest similarity to the EDS virus are highlighted. * - Gene not identified, x-sequence unavailable. CELO – fowl adenovirus serotype 1; FAV10 – fowl adenovirus serotype 10; OAV287 – ovine adenovirus serotype 287; BAV3 – bovine adenovirus serotype 3; CAV1 – canine adenovirus serotype 1; murine adenovirus serotype 1; HEV – hemorrhagic enteritis virus. Taken from Hess *et al.,* 1997.

determined to be 1: 125 (oral communication Dr Sarah Jones). This concentration indicates that the specificity of the antisera may be lower than desirable as a high concentration is required for a detectable reaction. Use of the antiserum found that while it didn't cross react with *E.coli* proteins it did cross react with the Ad2 protease. As antibodies can detect epitopes as small as three amino acids this result was not unexpected as homology exists between the N-terminals of the two proteases, see figure 75. It is probably the middle section of the peptide, ELKA, which is responsible for the cross reaction between the two adenovirus proteases.

```
                1               15
vprt_adeeds     MSGTS ESELKALMKS
vprt_ade02      .MGSS EQELKAIVKD
```

Figure 75. N-terminal sequences of the EDS protease and the Ad2 protease.

Unfortunately the EDS protease antiserum proved to be unreliable when used to detect the protease by Western blotting and therefore made attempts at the purification of the protease more time consuming as it was impossible to monitor at which steps the protease was being lost during purification.

A major problem in protein purification is the identification of the protein being purified from all the other cellular proteins. When the DNA or amino acid sequence of a protein is known synthetic peptides can be created and used to produce antisera. However, the problem with this is that the antisera tends to be serotype specific and therefore expensive if other related proteins are investigated. Using the GCG programme PILEUP all available sequences of the adenovirus protease were examined for homology and a sequence of 15 amino acids residues was identified as being highly conserved, see figure 76.

```
              42              56
 vprt_adeeds  AIINT GSRAS GGLHW
  vprt_ade02  AIVNT AGRET GGVHW
  vprt_ade03  AIVNT AGRET GGEHW
  vprt_ade04  AIVNT AGRET GGEHW
  vprt_ade05  AIVNT AGRET GGVHW
  vprt_ade12  AIVNT AGRET GGVHW
  vprt_ade17  AIVNT AGRET GGVHW
  vprt_ade40  AIVNT AGRET GGVHW
  vprt_ade41  AIVNT AGRET GGVHW
  vprt_adeb2  AIVNT AARET GGAHW
  vprt_adeb3  AIVNT AGRET GGVHW
  vprt_adeb4  AIVNT GPREK GGVHW
  vprt_adeb7  AIINT GPREQ GGIHW
 vprt_adeb10  AIVNT ASRET GGVHW
  vprt_adem1  AIVNT ASRET GGVHW
  vprt_adecu  AVVNT AGRET GGVHW
vprt_adeo287  AIINT GPREQ GGIHW
```

Figure 76. Sequence alignment of adenovirus protease sequences starting at Ala 42 and ending at Try 56 of the EDS protease.

The sequence AIVNTAGRETGGVHW contains nine a.a. residues which are conserved in all the known sequences of the adenovirus protease. Of the remaining six residues only two do not occur in more than 75% of known sequences. The sequence includes Histidine 54, one of the catalytic residues, and according to the crystal structure of Ad2 protease the rest of the sequence is accessible for antibody binding.

As three amino acid residues of a protein are capable as acting as an epitope (Houghten, 1985) and assuming that this is still possible for synthetic peptides it is calculated that there is only a 1.54% chance of an antisera generated against the synthetic peptide AIVNTAGRETGGVHW not reacting against any protease sequence assuming that unidentified sequences remain consistent with the known pattern. Given that a protease has been sequenced from each human adenovirus subgroup and various animal sources it is unlikely that the probably of failure would increase by more than 2-3%. It should be noted that for statistically purposes each residue is considered independent of each other whereas, in reality the residues cannot be totally independent otherwise structural changes may occur which destroy the catalytic ability of Histidine 54.

4.6 Purification of the EDS Protease

Analysis of the EDS protease sequence by the GCG programme Isoelectric predicted that the EDS had a similar pI to the Ad2 protease. As purification system had been developed for the Ad2 protease using FPLC columns this procedure was used to purify the EDS protease. However, it was found that the EDS protease was not purified by this method. Unfortunately due to the unreliability of the EDS protease antiserum it was not possible to determine whether the protease was remaining bound

to the DEAE or CM columns or passing through the columns in fractions different to those observed with the Ad2 protease.

The develop of an antiserum capable of detecting the EDS protease at low concentrations would be a major step forward in attempts to purify the protease. It was observed that fractions collected from the flow through of the DEAE column during purification contained more proteins than samples collected during the purification of the Ad2 protease. The most probable explanation of this is because L-broth was used as the growth media in EDS protease purifications but, the minimal media M9 was used for Ad2 protease purification. L-broth was used as the growth media for the EDS protease as the cells were found not to grow well in M9 media, this could have been because the protease was being expressed prior to induction. A possible solution to this would be to transform BL21(DE3)pLysE cells with the vector pSETEDS and increase the induction time from 1 hour to 5 hours. However, the use of different media does not explain the difference in behaviour between Ad2 protease and the EDS protease and it might be the case that the pH of the 50mM Tris-HCl buffer requires altering. Although, the pH of the Tris-HCl buffer was dropped to a pH of 7 in an attempt to ensure the EDS protease was eluted the protease wasn't detected due to the unreliable EDS protease antiserum, too many protein bands were present to attempt amino acid sequencing and the results from activity assays were inconclusive due to the presence of cellular enzymes.

The zinc column was used to try and purify the protease as a number of divalent ions are known to interact with exposed imidazole and thiol groups (Porath *et al.,* 1975). Zinc, copper, nickel and iron have all been shown to inhibit the Ad2 protease and as there is are His and Cys residues in the protease catalytic triad it is possible that this is the cause of their inhibitory effect. It was hoped that the zinc column would be able to bind the protease by an interaction with a thiol group. However, it was found that the protease did not bind to the column. A possible explanation of this may be that

because the protease was in an inactivated state so the active site was not exposed and therefore the thiol group was unable to bind the protein to the column. Therefore it may still be possible to purify the protease using a zinc column providing the activating peptide of the EDS protease, is added to the sample prior to loading onto the column. However, the addition of the activating peptide at this stage may restrict further use of protease purified by this method.

4.7 Immunofluorescence

4.7.1 Immunofluorescence Controls

Owing to the wide range of antisera used in the immunofluorescence studies a number of controls were carried out. The secondary antibodies were preadsorbed and diluted to their recommended concentration for use before testing for non-specific binding to uninfected and infected HeLa monolayers. It was found that the secondary antibodies did not bind non-specifically to the monolayers which had been fixed with paraformaldehyde and permeabilised with Triton X-100.

The primary antibodies were preadsorbed and serial dilutions prepared in order to establish the concentration at which the antisera detected the viral antigens. After the concentration was established for the primary antiserum the antiserum was checked for non-specific interactions against the cellular proteins by examining non-infected monolayers which had been prepared in an identical manner to the infected monolayers. It was found that all antisera, at their working concentrations, did not cross react with uninfected cells.

Experiments were carried out to ensure that the pre-immunisation bleeds, where available, did not exhibit a reaction against cellular proteins. The secondary antisera used in double labelling experiments were assayed to ensure that non-specific binding to antibodies of a different species did not occur.

During the course of the experiments the blocking procedure was altered from using 1% (v/v) New Born Calf Serum (NBCS) in PBS as a blocking agent to 1% (v/v) Foetal Calf Serum (FCS) in PBS. The change in blocking procedure was made as it was found that the blocking with NBCS masked some of the viral epitopes, according to Harlow and Lane (1988) this is because NBCS contains higher levels of antibodies than FCS.

4.7.2 Double labelling of pVIII, the fiber and the protease.

As the function of pVIII is unclear, it was hoped that immunofluorescence studies may aid in the understanding of the protein. The colocalisation between pVIII and the protease was unexpected as pVIII is cleaved in the mature virion. It is possible that pVIII is associating with the protease and preventing premature activation by pVI. However, *in vitro* digests of synthetic peptides with and without the presence of pVIII did not show any significant inhibitory effects. Although pVIII and the protease were not detected in the nucleus it does not mean that the proteins are not present in the nucleus. The absence of fluorescence in the nucleus merely indicates that the protein is not concentrated in localised areas in the nucleus and that the two proteins are most probably present but in low concentrations. It is possible, as pVIII is seen to accumulate around the nuclear membrane, that pVIII plays a role in the transport of certain proteins to the nucleus for assembly into capsids but, there is no evidence to support or contradict this observation.

In vitro experiments with immobilised pVIII have shown that the adenovirus protease does have a degree of affinity towards pVIII in unactivated and activated states. However, the activated protease has a stronger affinity for pVIII than the unactivated protease. Therefore, assuming these results are valid for *in vivo* experiments it is probable that pVIII is associating with activated protease. If the protease is activated

then it is possible that the pVIII-protease complexes are also associated with the cytoskeleton and that pVIII may be playing a role in the transport of protease to the cytoskeleton or in the regulation of cytoskeleton breakdown by the protease.

The detection of the fiber protein in the cytoplasm was unexpected as the fluorescence due to fiber antiserum has been detected as occurring in the nucleus of infected cells (Hayshi and Russell, 1968; Velicer and Ginsberg, 1970) . It is possible that because the antiserum used had been raised against Ad5 fiber instead of Ad2 fiber, as the cells were infected with Ad2 virus, that the antibodies bound non specifically to another protein. However, there is a 80% identity between Ad2 and Ad5 fiber which makes this unlikely. The only possible explanation that could account for the failure of the fiber antiserum to detect the fiber in the nucleus is that all the epitopes were masked due to the fixing and permeabilising procedures. It is most likely that the observed fluorescence of the anti-fiber antiserum is due to a non-specific cross reaction of the antiserum with the adenovirus protease as Weber *et al.,* (1989) reported a cross reaction between an antiserum raised against a synthetic peptide of the protease with the fiber.

The possible colocalisation of pVIII with the fiber in the cytoplasm is not readily explainable and probably does not occur. However, it is possible that pVIII may act as a scaffolding protein in the formation of the penton complex but, as pVIII does not display any interaction with the penton base this must be considered unlikely. The role of pVIII has been speculated upon but no conclusive arguments can be made other than it has a role in capsid stability as an Ad5 ts strain contains a mutation in the pVIII gene.

4.7.3 Fluorescence of selected viral antigens

At twenty four hours post infection the fluorescence observed with the penton and the hexon was the same as that by Hayashi and Russell, 1968. Unfortunately the intensity of the fluorescence prevented the observation of any structures in the nucleus. No fluorescence was detected in the cytoplasm but, this was as expected as most viral polypeptides are transported to the nucleus within 3-6 minutes of being released from the ribosomes (Horwitz *et al.*, 1969; Velicer and Ginsberg, 1970). The hexon is assembled in the cytoplasm in the presence of the L4 100 kDa scaffolding protein before being transported to the nucleus (Oosterom-Dragon and Ginsberg, 1981). It is unknown whether the penton requires the presence of a scaffolding protein or whether it occurs before or after transport to the nucleus.

The protein precursor pVI is identified as being located in the cell nucleus and in the cytoplasm at twenty four hours. The presence of pVI in the cytoplasm may indicate that the protein is colocalising with the adenovirus protease prior to transport into the nucleus as Hasson *et al.*, , 1992, found that the protease and pVI were transported into the nucleus as a complex.

DNA binding protein is detected in the cell nucleus at 24 hours post infection in globular structures. It is thought that these globular structures may represent sites of AdDNA replication (Sugawara *et al.*, 1977; Ginsberg *et al.*, 1977). In addition to DBP, the replicating Ad2 DNA and AdPol were also colocalised in these globular sites. The L1 52k Da protein is also detected in the nucleus at twenty four hours p.i. which, considering the nucleus is the site of viral assembly is not unsurprising as the protein is a scaffolding protein required for viral construction. The L1 52k Da protein was localised to areas distinct from the viral DNA replication centres by Hasson *et al.*, 1992.

4.7.4 Development and localisation of fluorescent antigens against pVIII

The adenovirus protein precursor pVIII is observed at twelve hours post infection in the cytoplasm of infected HeLa cells and fluorescence is observed to increase until twenty four hours p.i. when the increase in fluorescence levels out. The localisation of pVIII around the nuclear membrane may be as a result of protein transport. Although pVIII does colocalise in the cytoplasm with the protease it is unlikely that this as part of the viral assembly pathway as capsids can be formed with pVIII and lacking the protease. Interestingly capsids that are lacking the viral core do not form proteins VI and VIII but, instead retain the precursor proteins. This would indicate that the pathway responsible for inserting the protease in the capsid is connected to the viral core pathway.

5. Areas for Further Research

The data obtained so far upon pVIII and VIII could be increased by the production of synthetic peptides in order to raise an antiserum against the sequences 111-131 and 132-158. This would result in positive conformation on whether VIII is formed the sequence 1-110 or 1-131 of pVIII. At present pVIII is in an *E.coli* vector which may explain the insoluble nature of recombinant pVIII. Cloning into an insect vector may result in the production of a soluble pVIII fusion protein with greater structural homology to that of viral pVIII as the protein more likely to be folded correctly in insect cells than in the reducing cytoplasm of *E.coli*. Also, if pVIII does undergo post translational modification then it would be modified in the eukaryotic cell as opposed to remaining unmodified in the prokaryotic cell. The location of the hexon binding site of pVIII could be determined using the deletion mutants of pVIII and by the use of West-Western blots. However, this would require the purified proteins to have the same structural orientation as the corresponding sequence in pVIII, in order for the binding domain to be presented to the hexon and therefore, may not be possible. Another approach to this would be to immobilise recombinant hexon on a Sepharose column and observe whether or not pVIII and pVIII deletion mutants are able to bind to the hexon.

Further immunofluorescence studies can be carried out on the roles of proteins in adenovirus infections. However, the first step should be to generate an antiserum against the Ad2 fiber. Although all antigens would benefit from confocal studies the interaction of the DBP with the cellular protein p53 would be worth investigating as herpes virus, which also produces a DBP, has centres of DNA replication where p53 and the herpes virus DBP colocalise. The full developmental picture of pVIII could be established as it is not known when pVIII first becomes visible in the cell, although this will be at a point after 6 hours post infection. Also, electron microscopy work

could be carried out in order to observe where pVIII is accumulating in the cell and whether or not it may be interacting with cellular proteins.

The EDS protease purification procedure requires further refinement and the possibility of growing the cells in M9 media should be investigated as this may reduce the quantity of cellular proteins which are purified with the protease on the DEAE-Sepharose column. Once purified recombinant EDS protease is obtained the consensus cleavage site should be confirmed as whether or not it is the same as for the other adenovirus proteases and if it is capable of cleaving precursor proteins form another serotype. It would also be interesting to compare the kinetic data of the EDS protease with that of the Ad2 protease as the two proteases are distantly related to each other and the structure of the EDS activating peptide differs significantly from the human adenovirus activating peptides.

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