U90, A TUMOUR ASSOCIATED POLYPEPTIDE ALTERED BY HSV INFECTION

by

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SUMMARY

Herpes simplex virus (HSV) has been associated with cervical neoplastic disease for a number of years and has been shown to induce morphological transformation of rodent cells. HSV infection alone is not thought to induce cancer but rather it is proposed to act as a co-factor initiating one of the many steps towards a potentially cancerous phenotype. A number of possible mechanisms of HSV transformation have been proposed, but the oncogenic properties of the virus are still not fully understood. The subject matter of this thesis will be concerned with one of these mechanisms, namely the postulate that transformation may occur by altering the expression of host cell polypeptides.

A set of polypeptides called transformation associated polypeptides was previously found to be recognised in immunoprecipitation reactions by both sera from tumour bearing animals and antisera raised against HSV infected cells. This observation implicated the induction of such cellular polypeptides by HSV infection in the changes involved in the progression from a normal cell to that of a transformed phenotype. One of these transformation associated polypeptides a 90,000MW polypeptide called the U90, has been investigated.

The U90 polypeptide was previously thought to be specific to the transformed state as it could only be immunoprecipitated from radiolabelled transformed cells and not from primary control cells. Characterization studies have now shown that the U90 is, in fact, a highly conserved polypeptide and as such is likely to play an important role in the cell.

HSV infection was found to increase the expression of the U90 by up to 8 fold (at 3 hours post-infection) and resulted in the accumulation of the U90 in the cytoplasmic fraction - as opposed to the membrane associated fraction from where it is normally isolated in the uninfected tumour cell.

The accumulation of the U90 in the cytoplasm following HSV infection, enabled the protein to be purified with much greater ease as a consequence of (1) the increased amount of the protein and (2) the soluble nature of the U90. The purified U90 was subsequently used to generate partial internal amino acid sequence (after enzymatic digestion) and also used to generate a polyclonal mono-specific antibody.

Comparison of the amino acid sequences of the seven different U90 peptides with sequences entered in the NBRF databases resulted in no significant homology being found with any of the entries and confirmed

that the U90 was not a stress protein such as hsp90 or GRP94 which had previously been suggested. This indicated that the U90 is a novel transformation associated polypeptide. The sequence data generated was insufficient to allow searches for functional domains to be completed and therefore the function of the U90 is still not known.

By applying the U90 purification strategy to control RE cells and using the U90 monospecific antibody it was found that control cells did in fact express a homologue of the U90, which was recognised by the monospecifc U90 antibody. This 90K polypeptide behaved in an highly similar manner to the U90 - HSV infection resulted in the accumulation of the polypeptide in the cytoplasmic fraction and it could be purified in an identical manner to the U90 using the U90 purification strategy.

Comparison of the U90 isolated from the Bn5T tumour cells to the U90 homologue isolated from control primary RE cells showed that in the transformed cells the U90 had a much slower turnover than in the control cells (approximately 13 hours as opposed to 31 minutes). If the U90 is implicated in regulation of cell growth or differentiation as suggested previously by the detection of the U90 in rat embryos up to 14 days gestation, increasing the total amount of the protein in the cell by HSV infection could have a significant affect on the cell regulation. A similar affect is seen in the transformed cell where the increased half life of the U90 means that more of this protein is present in the cell for extended periods.

During these studies HSV-1 but not HSV-2 infection, was found to increase the levels of a second host polypeptide, a glucose regulated polypeptide GRP94. The increase in the total amount of GRP94 in the cell following HSV-1 infection appears to be a consequence of a translational or post-translational change, but in contrast to HSV induction of U90, immediate early gene expression was found to be inadequate to increase GRP94. Due to time limitations studies were concentrated on the U90. However the increase of GRP94 by HSV type 1 but not type 2, marks an interesting distinction between the host cell interactions of the two viruses.

ABBREVIATIONS

A	adenine
AFP	alpha foetoprotein
AIDS	acquired immunodeficiency syndrome
ALV	avian leukosis virus
AS	ammonium sulphate
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
BHK	baby hamster kidney
BL	Burkitt lymphoma
bp	base pairs
C	cytosine
CAPS	3 cyclohexylamino-1- propanesulphonic acid
cDNA	complementary DNA
CEA	carcinoembryonic antigen
CIN	cervical intraepithelial neoplasia
CMI	cell mediated immunity
CMV	cytomegalovirus
c-onc	cellular oncogene
cpm	counts per minute
CRPV	cottontail rabbit papillomavirus
DATD	N,N'-diallyltartardiamide
dATP	deoxyriboadenosine triphosphate
dNTP	deoxyribonucleoside triphosphate
DMEM	Dulbecco's minimal essential medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E	early
EBV	Epstein-Barr virus
EBNA	Epstein-Barr virus nuclear antigen
EDTA	ethylenediamine tetra-acetic acid
ER	endoplasmic reticulum
EV	epidermodysplasia verruciformis
g	gravity
G	guanine
GRP	glucose regulated polypeptide
FPLC	fast protein liquid chromatography
Helu	human embryonic lung
HIV	human immunodeficiency virus

HCMV	human cytomegalovirus
HHV-6	human herpes virus type 6
HHV-7	human herpes virus type 7
HPLC	high performance liquid chromatography
HPV	human papillomavirus
HRP	horse radish peroxidase
hsp	heat shock protein
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HTLV	human T cell lymphotrophic virus
IE	immediate early
IP	immunoprecipitate
K	kilo
L	late
LAT	latency associated transcript
LMP	latent membrane protein
LTR	long terminal repeat
MAB	monoclonal antibody
mg	milligram
MHC	major histocompatibility complex
mRNA	messenger RNA
MTR	morphological transforming region
mu	map unit
MW	molecular weight
ng	nanogram
NK	natural killer cell
NPC	nasopharyngeal carcinoma
NPT	non-permissive temperature
N-terminus	amino terminus
ori	origin of replication
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pfu	plaque forming units
PITC	phenylisothiocyanate
pmole	picomole
PMSF	phenylmethylsulphonyl fluoride
p-onc	proto-oncogene
PAA	phosphonoacetic acid

PT	permissive temperature
PVDF	polyvinylidene difluoride
Rb	retinoblastoma
RE	rat embryo
RIPA	radio immunoprecipitation assay
RNA	ribonucleic acid
RNasin	ribonuclease
rpm	revolutions per minute
RR	ribonucleotide reductase
RSV	Rous sarcoma virus
SDS-page	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SV40	simian virus 40
syn	syncytial
T	thymine
TBS	tumour bearing sera
TCA	trichloroacetic acid
TEMED	N,N,N',N'tetramethylethylenediamine
TFA	trifluroacetic acid
ts	temperature sensitive
TSTA	tumour specific transplantation antigen
v-onc	viral oncogene
UV	ultra violet
VZV	varicella zoster virus
WT	wild type
ug	microgram
U90	upper 90

CHAPTER 1 : INTRODUCTION

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CHAPTER 1 : INTRODUCTION

SECTION 1. TUMOUR VIRUSES AND VIRAL ONCOGENES

Investigations of age specific cancers which showed an exponential increase with age (Armitage & Doll, 1954) provided the initial basis for the concept that multiple events causing damage to the cell, can result in tumourigenesis. Carcinogens which induce such events can be chemical, physical or viral (reviewed by Doll & Peto, 1981). Exposure to such factors can result in the alteration of a normal cellular gene (termed a proto-oncogene) to an activated oncogene (termed a cellular oncogene or c-onc). A proto-oncogene has recently been defined as a gene where "if manipulation of any sort engenders a transforming allele (or oncogene), convention admits the normal counter part to the ranks of proto-oncogene" (Bishop, 1991). These proto-oncogenes frequently have functions associated with growth (Hunter, 1989), and therefore, alteration to their regulation or function can have important consequences for the cell. Alternatively, if the oncogene is introduced to the cell by a virus, it is termed a viral oncogene or v-onc. Such v-oncs are transduced cellular genes lacking introns which are normally present in the gene in the normal cell environment. In addition the v-onc are usually mutated in some way and/or contain rearrangements.

A number of carcinogens have been directly linked to specific human tumours. Over two hundred years ago, exposure to soot was found to be the cause of cancer of the scrotum in chimney sweeps, as documented by Pot in 1775 (Cairns, 1978). In the 1850's, the development of a new industry producing synthetic dyes resulted in the exposure of workers to the extremely carcinogenic agent 2-naphthylamine. This was the starting material for the production of synthetic dyes. Exposure to 2-napthylamine resulted in a number of cases of cancer of the bladder- although it took over 50 years for the use of this chemical to be stopped (Cairns, 1978). A third well documented carcinogen is asbestos, which causes asbestosis and can also result in a specific lung cancer, mesothelioma. Asbestos and its involvement with cancer of the lung, illustrates the proposal that multiple factors are involved in cancer, as lung cancer in patients exposed to asbestos occurs almost exclusively in people who

smoke. Certain viruses have also been demonstrated to act as carcinogens and have a direct involvement in tumour induction (Bishop, 1991). Examples of such viruses include adenoviruses, herpesviruses, papovaviruses and retroviruses.

In 1892 Koch compiled postulates to evaluate the relationship of a casual agent to the disease with which it was associated. These postulates were based on the following points:

1. The micro-organism should be found in all cases of the disease in question, and its distribution in the body should be in accordance with the lesions observed.

2. The micro-organism should be grown in pure culture in vitro (or outside the body of the host) for several generations.

3. When such a pure culture is inoculated into susceptible animal species, the typical disease must result.

4. The micro-organism must again be isolated from the lesions of such experimentally produced disease. (Koch, 1892).

Koch's postulates have been used to establish if an infection with a particular virus is associated with the development of a specific cancer. Some viruses however, implicated in cancer do not fulfil these postulates. This is because of the particular way in which they are implicated in tumour progression.

Evans (1976) revised these conditions to enable them to be applied more readily to viruses and cancer. This was required because of the complications which arise during infection and carcinogenesis, i.e. the nature of the host, the long incubation period, the possibility of cofactors, and the inability to fulfil the third condition in humans, that is, to induce tumour formation using the isolated virus. Aurelian et al, (1987) subsequently revisited the postulates with specific reference to HSV and cervical cancer. The modified Koch's postulates are listed in Table 1.

Some of the tumour viruses identified by these rules, and some which do not fulfil all the conditions will be discussed in this section. These viruses, and the diseases with which they are associated, are summarized in Table 2. These viruses can be divided into two groups: the first group of RNA viruses, consists of only one family, the retroviruses, and the second group of DNA viruses, consists of the majority of DNA virus families, the exceptions being the parvo

TABLE 1

KOCH'S POSTULATES:- ADAPTED FOR VIRAL CARCINOGENISIS

1. Cancer patients should be exposed more than controls to the putative virus and cancer patients should have greater virus specific antibody or cell mediated responses than age/sex matched controls within that geographical area.
2. Cancer associated immune response should be indicative of viral expression.
3. The immunological response should be specific for the virus in question and a similar response should not be seen with other viruses or cancers.
4. The disease should follow exposure to the virus.
5. Prospective sero-epidemiological studies should show virus specific antibody present prior to the onset or recurrence of the disease.
6. Virus or genetic material of the virus should be present in the affected tissue.
7. Expression of viral information should occur in the affected tissues.
8. Virus or genetic information from the virus should be able to induce neoplastic transformation of cells in vitro.
9. Reproduction of the disease should be possible in animal models.
10. Elimination or modification of the putative cause should reduce the incidence of the disease.
11. The whole thing should make biological and epidemiological sense.
12. If the association between virus and cancer is seen in some populations but not in others, then the presence of cofactor(s) should be sought.

(adapted from Aurelian et al. 1987)

TABLE 2

SOME VIRUSES ASSOCIATED WITH TUMUORS IN ANIMALS AND MAN

VIRUS	ASSOCIATED DISEASE
RETROVIRUSES	
Rous sarcoma virus	Sarcomas and leukaemias in chickens
FLV	Leukaemia in cats
HTLV-1	Adult T cell leukaemia/lymphoma
PAPOVAVIRUSES	
Polyoma virus	Carcinomas and sarcomas in mice
SV40	Sarcomas in rodents
BK and JC viruses	Neural tumours in rodents and monkeys
Papillomavirus	Genital and skin warts in man, progressing to cervical or skin carcinoma In cattle, genital, alimentary and skin warts progressing to carcinomas
HEPADNOVIRUSES	
Hepatitis B virus	Primary hepatocellular carcinoma in man
ADENOVIRUSES	
Adenovirus types 2, 5 + 12	Sarcomas in hamsters
HERPESVIRUSES	
Lucke Herpes virus	Renal adenocarcinomas of the frog
Mareks disease virus	Neurolymphomatosis in fowl
Herpes virus saimiri	Leukaemia and T cell lymphomas in monkeys
Epstein Barr virus	Burkitt's lymphoma, nasopharyngeal carcinoma and immunoblastic B cell lymphoma in man
Herpes simplex virus (types 1 and 2)	Cervical neoplasia (?)
Human cytomegalovirus	Kaposi's sarcoma and cervical neoplasia (?)

and poxvirus families. The different mechanisms by which tumour viruses can transform cells are illustrated in Figure 1.

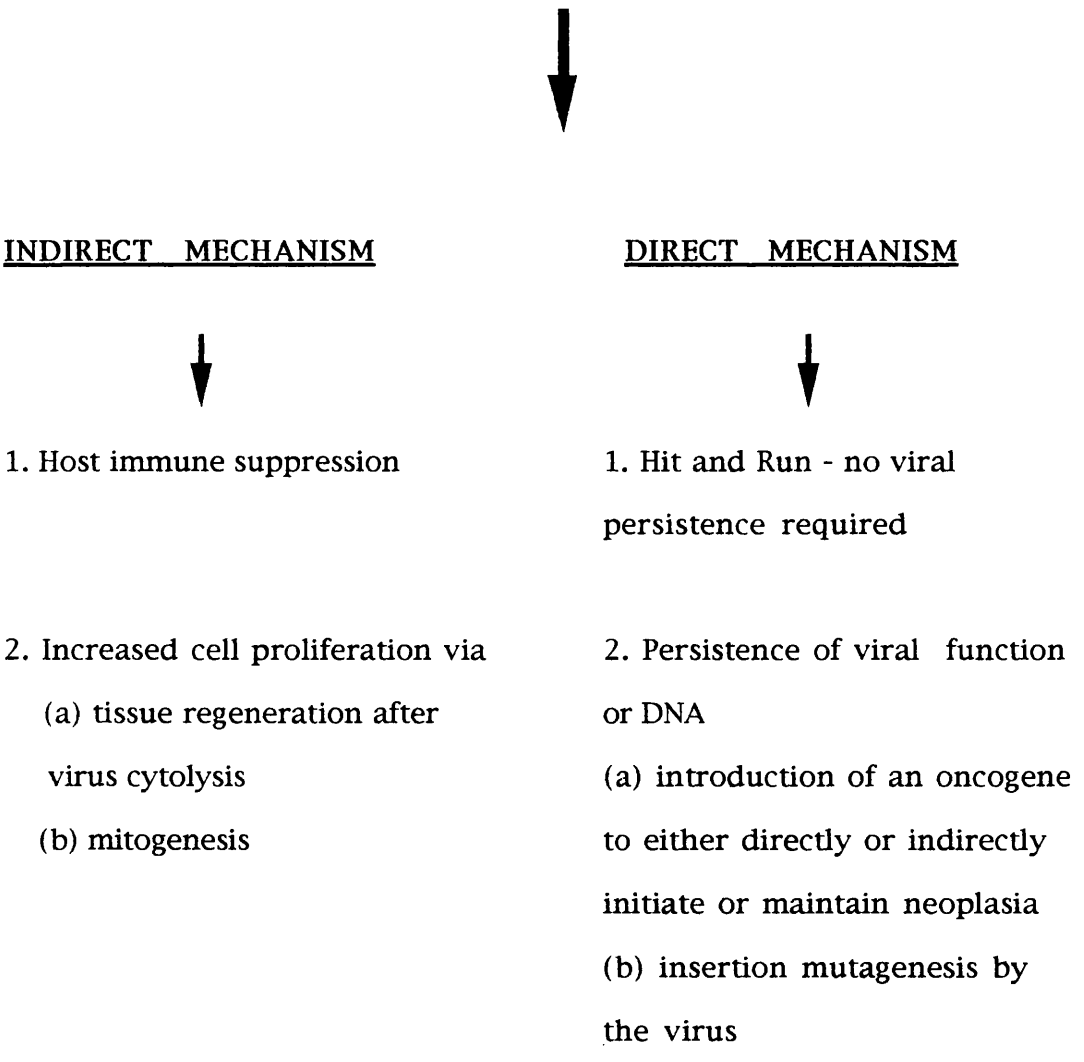
1(a) RETROVIRUSES

Retroviruses have a small infectious diploid RNA genome of between 5-10,000 nucleotides and code for an RNA dependent DNA polymerase activity known as reverse transcriptase (Fenner, 1975). This enzyme is crucial in the unique replicative cycle of the virus which involves the production of a double stranded DNA intermediate called the provirus, which is covalently inserted into the host's genome (Weiss et al, 1984). Retroviruses can either act as acutely transforming retroviruses, inducing tumour formation within a very short period of time, or as non-acute retroviruses which can result in the induction of slow forming tumours.

Acute retroviruses transduce v-oncs into the host genome resulting in cell transformation, whereas the non-acute retroviruses transform the cell by inserting the provirus into the host genome in a position such that the long terminal repeats (LTRs) of the virus which contain strong promoters, act "in trans" and alter the regulation of proto-oncogenes. This can account for the difference in the length of time for which a tumour takes to develop. Ellerman and Bang first induced leukaemia in chickens in 1908 by the injection of avian leukosis virus (ALV). Peyton Rous followed this some 3 years later by inducing leukaemias and later sarcomas in chickens with Rous sarcoma virus (RSV). Since then, studies involving retroviruses have facilitated the discovery of more than 20 cellular oncogenes largely by using retrovirus induced tumours in animal models.

A number of retroviruses have been isolated from animals and are found to be associated with a variety of diseases including cancer. However, it has only been in the last 10-15 years that retroviruses have been found to be the cause of specific human diseases. Human T cell lymphotrophic virus (HTLV-1), which causes adult T cell human leukeamia or lymphoma (Poisez et al, 1980) was first discovered in southern Japan. A second retrovirus HTLV-II, was isolated from a T cell variant of hairy cell leukaemia (Kalyanaraman et al, 1982). The third human retrovirus to be discovered was the human immunodeficiency virus

FIGURE 1 : VIRUS INDUCED NEOPLASIA



(HIV), which is not contained within the oncovirus sub-group of retroviruses, but instead is classified within the lentivirus sub-group. HIV, the causative agent of acquired immunodeficiency syndrome (AIDS), unlike other lentiviruses is associated with tumour formation, but this is only as a consequence of the ability of the virus to destroy the immune system of the patient. HIV patients frequently develop the normally very rare mesenchymal tumour, Kaposi's sarcoma (Fauci, 1988) or B cell non-Hodgkin's lymphoma (Ziegler et al, 1984).

The oncovirus sub-group of the retroviruses transforms cells by a number of mechanisms which, due to the unique replicative cycle of retroviruses, are specific for these viruses. These mechanisms will be discussed in Section 3 of this chapter.

1(b) PAPOVAVIRUSES

The Papovavirus family is a group of small double stranded DNA tumour viruses of 5-8Kb which includes polyomavirus, simian vacuolating virus (SV40), and papillomaviruses. Papillomaviruses will be discussed in this category as they have historically been classified as papovaviruses, however recent genetic and molecular studies indicates that papillomaviruses should constitute a sub-group of their own. In contrast to RNA viruses DNA tumour viruses do not carry viral oncogenes which have direct cellular counterparts. The evolutionary origins of the oncogene products of DNA tumour viruses remains obscure, but the function of these products is becoming clearer, with many of them interacting directly with specific host cell proteins (Lane, 1989). This aspect will be discussed in Section 4.

Specific immortalizing genes have been isolated from each of the papovaviruses: large tumour (T) antigen from polyomavirus (Rassoulzadegen et al, 1983; Jat & Sharp, 1986), large T antigen from SV40 (Petit et al, 1983) and E6 and E7 from papillomaviruses (Matlashewski et al, 1987; Munger et al, 1989).

Polyomavirus, which induces various carcinomas and sarcomas in the mouse, encodes three proteins of particular interest, large T, middle T and small t. These are 100, 55, and 22Kd in size respectively and are produced by alternative splicing of the mRNA, with all three species having the same 5'

sequence. Large T antigen will immortalize cells in culture, resulting in indefinite growth and diminished requirements for cell growth factors and middle T antigen will complement large T, by transforming immortalized cells (Rassoulzadegen et al, 1982, 1983). Middle T antigen which has a protein-tyrosine kinase activity (Courtneidge & Smith, 1983) is found to complex with various host proteins including c-src, c-yes and c-fyn (which are tyrosine kinases). Large T antigen is also found to complex with cell polypeptides, binding the retinoblastoma gene product p110 (Rb), a tumour suppressor (Lane, 1989). Although small t is not essential for transformation it appears to effect the cell's cytoskeleton and augments both transformation of cells in culture and tumourigenesis in animals (Sleigh et al, 1978; Lewis & Martin, 1979; Martin et al, 1979).

SV40 was discovered in rhesus monkey kidney cell cultures which were used to produce the polio vaccine (Sweet & Hilleman, 1960). This virus is a 5,200bp closed circular virus which becomes covalently integrated into the transformed cell's genome. Differential splicing of SV40 RNA produces large T and small t antigen RNA. These mRNA have the same 5' and 3' ends, but the presence of a stop codon in the small t antigen mRNA prevents translation of the entire RNA. The large T antigen in isolation is able to transform cells and maintain the transformed state (Kriegler, 1984), and in a similar manner to the small t antigen of polyomavirus, SV40 small t can enhance both immortalization and transformation stages (Bikel et al, 1987). Large T antigen is a multi-domain protein with an ATP dependent DNA helicase activity. This function however, is not thought to be essential for transformation, as mutations affecting this function do not affect the ability of large T antigen to transform cells. Large T antigen can also bind to both p53 and Rb110 (both tumour suppressor genes), a function which is thought to play an important role in transformation. The large T antigen is reviewed by Rigby & Lane (1983) and Livingstone & Bradley (1987).

Papovaviruses BK and JC which are similar to SV40 and polyomavirus have been isolated from human patients. These viruses are associated with the development of neural tumours in rodents and monkeys (reviewed by Wyke, 1991).

The papillomaviruses, of which there are in excess of 62 types (DeVillers, 1989; DeVillers, personal communication) are found in 90% of all cervical cancers tested world-wide, with types 16 and 18 being present in 70-80% of cases (zur Hausen, 1989; Knebel Doeberitz et al, 1990). The papillomaviruses have a complex transcription pattern, and unlike SV40 and polyomaviruses, the mRNAs are transcribed from only one strand of viral DNA. The host cells of papillomaviruses are restricted to squamous epithelial cells with productive infection occuring only in terminally differentiated epithelial cells (reviewed by Pfister, 1987). Until recently this has prevented advances in the study of papillomaviruses as no in vitro tissue culture system was available which allowed permissive replication of the virus. The development of new model systems such as the raft system (McCance et al, 1988) which allows epithelial cells to progress from an undifferentiated to a differentiated state in vitro has enabled papillomaviruses to be grown in culture. A mouse model system is also currently being developed using the European harvest mouse (Micromys minutus) and the harvest mouse papillomavirus. This mouse papillomavirus has been identified in a variety of benign and malignant tumours and the virus has been isolated, cloned and characterized. Attempts are being made to transmit the harvest mouse papillomavirus to inbred laboratory strains to enable the development of a well defined mouse system for immunological studies of papillomavirus-induced disease (reviewed by Sundberg & O'Banion, 1989).

Papillomaviruses have long been associated with benign tumour production and infect a variety of animals and birds. The cottontail rabbit Shope papillomavirus (CRPV) was the first oncogenic papilloma tumour virus to be isolated and characterized (Shope & Hurst, 1933). Other known viruses resulting in malignant progression include bovine papillomavirus type 4, canine oral papillomavirus, colobus monkey papillomavirus types 1 and 2, equine venerial papillomavirus and rhesus monkey papillomavirus (Sundberg & O'Banion, 1989).

Papillomaviruses are associated with a number of diseases in humans, but only a sub-set of the HPV types are involved with malignant progression. HPV types 6, 11, 31 and 35 are associated with condyloma accuminata and dysplasias such as low grade cervical intraepithelial neoplasia (CIN), ie CIN I and possibly type II. These types of papilloma viruses are considered to be "low risk" viruses,

whereas HPV types 16, 18 and 33 are associated with high grade CIN, ie type III and carcinoma of the cervix (Tidy & Farrel, 1991) and are "high risk" viruses.

Squamous cervical carcinoma arises in the area of the uterine cervix named the transformation zone. During the reproductive era this region undergoes a differentiation process known as squamous metaplasia which results in the formation of squamous epithelium. The transformation zone is situated between the endocervix and ectocervix of the uterine cervix as illustrated in Figure 2. In the transformation zone a large number of cells are in the premitotic or mitotic phase and are therefore probably more susceptible to progression to a cancerous phenotype than quiescent epithelial cells. Such dysplasia can occur resulting in CIN which is graded into three precancerous stages I, II and III as described previously. In a normal healthy patient the squamous epithelium covering the vagina and ectocervix is composed of 4 distinct levels: three - four layers of parabasal cells are separated from the basement membrane by a single layer of basal cells, below which is the underlying stroma. Dysplasia of the parabasal cells can result in CIN I to III, with the latter being the most severe and corresponding to dysplasia of the entire epithelium which is also known as carcinoma in situ. A cancerous progression from CIN III results in invasive carcinoma when the basement membrane is breached.

Identification of adenocarcinomas of the columnar epithelium has recently increased, with up to 10% of all cervical tumours being of this nature. It is possible that the increase in the development of such adenocarcinomas could be linked with the widespread use of oral contraceptives (Kitchener, personal communication)

HPV has been associated with malignancy in epidermodysplasia verruciformis (EV) which is characterized by chronic widespread warts which often progress to squamous cell carcinoma (Orth et al, 1978). HPV has also been identified in flat condylomas of the uterine cervix which frequently occur in women who subsequently develop cervical carcinoma (Meisels & Fortin, 1976). However, it was not until 1983 that HPV-16 was shown to be in 61% of cervical tumours in a study using DNA-DNA hybridization techniques (Durst et al, 1983) and then cloned from a cervical tumour (Gissman et al, 1983). The percentage of samples positive for HPV varies from study to study however, 80-100% of

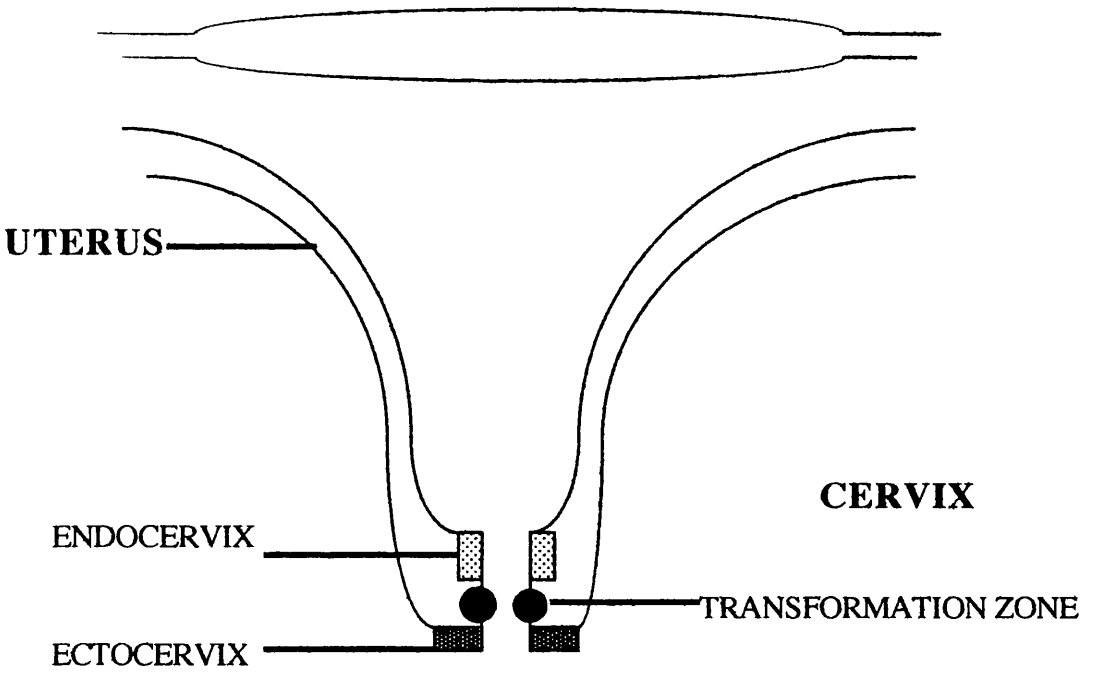


FIGURE 2 : Anatomy of the uterine cervix

invasive squamous cancers and most high grade CIN lesions have been found to be positive for HPV (McCance et al, 1985; Young et al, 1989). HPV-16, 18 and 31 are most commonly isolated in CIN III and cervical cancer (Tidy & Farrel, 1991). Geographical distribution however may influence this as in the West of Scotland HPV-16 is the most prevalent, even in low grade CIN (Murdoch et al, 1988). Head-to-tail dimeric episomes of HPV-16 DNA have been found in genital cancer biopsy samples (Kennedy et al, 1987) but HPV-16 DNA has also been found to be integrated in control tissues (Macnab et al, 1986; Murdoch et al, 1988). Cases of HPV infections were found to be very frequent when compared with precancers and cancers suggesting that HPV alone may be insufficient to produce malignant change (Macnab et al, 1986). This suggestion has been substantiated by studies which show that E6 and E7 of HPV can only immortalize cells (Woodworth et al, 1989). More recent studies have shown no clear correlation between the detection of HPV-16 either by DNA/DNA hybridization or by PCR and histological changes in the appearance of the cervix (Kitchener et al, 1991), and it has been stated that additional factors should be taken into consideration in future studies, eg HPV gene expression, tumour associated proteins and HPV cell targeting (Kitchener et al, 1991).

It has been shown that as a consequence of papillomavirus integration, the viral genes E1 and E2 are lost or disrupted (Schneider-Maunoury et al, 1987; Schwarz et al, 1987) and it is proposed that this results in the over-expression of E6 and E7 (the transforming genes of human papillomaviruses). E7 is the most abundant protein in HPV transformed cells and is thought to play a role in transformation via an interaction with the p110 Rb protein. Comparison of the SV40 T antigen, the adenovirus E1A and papillomaviruses E7 coding sequences reveals homology in a region which is implicated in binding to p110 Rb suggesting a common mechanism of action of the different viral transforming proteins (Phelps et al, 1988). In vitro E6 complexes with a cellular tumour suppressor protein p53, resulting in p53 degradation. This degradation is proposed to occur via the action of the ubiquitin-dependent protease system (Scheffner et al, 1990). Such degradation of a host negative regulator protein has been proposed as a novel mechanism of oncogenesis (Scheffner et al, 1990). Other nuclear oncoproteins such as N-myc, c-myc and c-fos have also been proposed to be degraded by the ATP-dependent ubiquitin system, enabling the

cell to alter the levels of their regulatory proteins very quickly (Ciechanover et al, 1991).

Cofactors have been implicated in malignant progression following papillomavirus infection such as UV light, smoking and co-infection with other viruses for example, HSV or cytomegalovirus (discussed in Tidy & Farrel, 1991). In humans, UV irradiation from sunlight can act as a cofactor following HPV-5 or 8 infection resulting in the progression of EV to malignant disease in up to 50% of cases (Orth, 1986). In addition to human cytomegalovirus (HCMV), herpes simplex virus (HSV) (section 1e), has been implicated as a cofactor in anogenital cancer. Immunosuppression has also been suggested as a cofactor with HPV and cervical neoplasia (Jablonska et al, 1987). The immune response to warts in patients is reported to be primarily cell mediated immunity (CMI), and it is thought that the persistence of papillomavirus induced warts may be related to defects in CMI (Jablonska et al, 1987). It has also been shown that patients with persistent anogenital lesions induced by potentially oncogenic HPVs have reduced CMI, and the degree of CMI suppression is also related to the degree of malignancy of any resulting anogenital cancer (Malejczyk et al, 1987).

Papillomaviruses also infect and cause tumour development in cattle. This however appears to occur by quite a distinct mechanism from tumour development in humans. In bovine papillomavirus type 1 the transforming gene is E5, whereas E6 and E7 are involved in transformation by human papillomaviruses. HPV also differs significantly from BPV as E6 and E7 are found to integrate in the human tumours but no BPV DNA has been detected in transformed cells or in alimentary tumours associated with BPV. Consequently, BPV does not fulfil Koch's postulates.

Cofactors are also thought to play a role in tumour development in cattle (Jarret et al, 1978). Although BPV-4 induces papillomatosis, quercetin found in bracken is thought to be required for progression to squamous cell carcinoma (Jarret et al, 1978). In addition it is believed that BPV may alter the host's immune response and this too may play a role in tumour progression. These latter studies have been carried out in cattle where vaccinated cows can be shown to reject BPV induced tumours more quickly than unvaccinated cattle. However the differences between BPV and HPV and how they interact with

their host calls into question the ability to produce HPV vaccines based on the BPV model.

1(c) HEPADNOVIRUSES

The hepadnovirus family includes human hepatitis B virus and hepatitis viruses isolated from ducks, squirrels and woodchucks (Schodel et al, 1989). These small, enveloped, partially double stranded DNA viruses of approximately 3Kb, (reviewed by Tiollais et al, 1985) exhibit a tropism for hepatocytes. Hepatitis B is associated with primary hepatocellular carcinoma in humans, with chronic carriers of the virus being at least 200 times more likely to develop hepatocellular carcinoma than a healthy patient (Beasley et al, 1981). Hepatocellular carcinoma is one of the most common tumours world-wide but is most prevalent in certain regions of Asia and particularly Southern China and Taiwan (Beasley et al, 1981).

How hepatitis B could be involved in the progression of this cancer was unknown until very recently. Persistence of the virus with subsequent inflammatory response was thought to be important. In addition it was previously postulated that virus, by integrating in the host genome may have altered the expression of a cellular oncogene. However, the discovery that the viral transactivator, the product of the X gene (Twu & Schloemer, 1987) when expressed in transgenic mice, induced liver cancer in these animals (Kim et al, 1991) directly implicated a viral gene product in neoplastic progression. Despite this, the persistent nature of hepatitis B infection is still thought to be the most important factor in the development of hepatocellular carcinoma.

Cofactors are also postulated to be implicated with hepatocellular carcinoma, in particular a mutagen aflatoxin B originating from *Aspergillus flavo*. The hotspot of mutations consistently seen in the cellular anti-oncogene p53 in cases of hepatocellular carcinoma in China suggests the action of a single mutagen. This has been postulated to be aflatoxin B (Bressac et al, 1991; Hsu et al, 1991).

1(d) ADENOVIRUSES

Adenoviruses have a linear double stranded DNA genome of approximately 36Kbp and are divided into 6 subgroups, A-F. Sub-group A (eg

Ad12), induce tumours at a high frequency in laboratory rodents, and the viruses of sub-group B (eg Ad3 and Ad7) are weakly oncogenic. The left most 11% of the genome comprises 2 regions, E1A and E1B which are involved in adenovirus transformation (Gallimore et al, 1974; Graham et al, 1974). The integration of these viral genes into the genome of transformed cells is required to maintain the transformed phenotype (Gallimore et al, 1985).

The products of the E1A and E1B regions are multi-functional proteins which in addition to cell transformation, are involved with transcription, post-transcriptional events, DNA stability and replication (reviewed by Boulanger & Blair, 1991). Two of the E1A products are proposed to be involved in the establishment of immortalized cells in vitro, with E1B cooperating with E1A to induce transformation (Houweling et al, 1980; van der Elsen et al, 1983). Transfection studies with E1A indicate that transfected E1A will immortalize cells, but this is 30 fold less efficient than immortalization as a consequence of adenovirus infection (Gallimore et al, 1985). Two of E1A's three conserved domains (Kimelman et al, 1985; Moran & Mathews, 1897) are claimed to be analogous to myc, acting as an immortalizing gene. The oncoproduct of E1B is proposed to act in a similar manner to activated ras, cooperating to give complete and stable transformation (Bernards & van der Eb, 1984).

Cell transformation by adenoviruses may occur as a consequence of viral proteins interacting with host cell tumour suppressor gene products. This is supported by the fact that E1A in a similar manner to E7 of papillomavirus and the large T antigen of SV40, binds to Rb, and E1B binds p53 (reviewed by Boulanger & Blair, 1991).

1(e) HERPESVIRUSES

A number of viruses in the Herpesvirus family have been associated with malignant disease. These include Marek's disease virus which causes lymphoblastosis in chickens (Kato & Hirai, 1985), herpes virus saimiri, isolated from monkeys which induces T cell lymphomas in inoculated marmosets (Melendez et al, 1970) and malignant lymphoma in cotton-top marmosts inoculated with Epstein-Barr virus (Shope et al, 1973), ie animals other than the natural host. In humans, herpes simplex virus (HSV) has been associated with cervical carcinoma, as has human cytomegalovirus (HCMV) (Stevenson &

Macnab, 1989), although the latter association is more controversial (reviewed by Macnab, 1987; Macnab & Kitchener, 1989). Nevertheless, HCMV has been detected in CIN tissue by DNA/DNA hybridization (Fletcher et al, 1986), and using a 20Kb fragment encoding the major IE gene HCMV has been cloned from CIN samples (Fletcher & Macnab, 1988). This fragment has been shown to transform Helu cells (Geder et al, 1976) and human endothelial cells in culture (Smiley et al, 1988). In addition mouse cervices treated with UV irradiated HCMV developed carcinomas and had elevated levels of dysplasia (Heggie et al, 1986).

Epstein-Barr virus (EBV) is a third human herpes virus which is associated with cancer. The association of EBV with a number of diseases is well established (zur Hausen et al, 1970; Dambaugh et al, 1986). These include EBV as the etiological agent of infectious mononucleosis and the association of EBV with endemic Burkitt lymphoma (BL), a B cell lymphoma, nasopharyngeal carcinoma (NPC), an epithelial cell tumour and secondary B cell proliferations in immunosuppressed individuals (zur Hausen et al, 1970; Dambaugh et al, 1986).

EBV has a large genome (176Kb) which is characteristic of a herpes virus and expresses a large number of viral gene products (reviewed by Farrel, 1989). The virus is a lymphotropic human herpes virus which establishes latent infection in B lymphocytes with little or no virus production. However, in latently infected growth transformed B lymphoblastoid cell lines, EBV expresses 6 Epstein Barr nuclear antigens (EBNA), (reviewed by Speck & Strominger, 1989). These are EBNA 1, 2, 3A, 3B, 3C and leader protein LP. Three integral latent membrane proteins (LMP) are also expressed - 1, 2A and 2B (reviewed by Kieff & Leibowitz, 1990).

Expression patterns of EBNAs and LMPs can differ with EBV protein expression frequently restricted to EBNA-1A in Burkitt lymphoma tissue or newly established cell lines (Hitt et al, 1989; Allday et al, 1990). It has been reported that only EBNA-1 transcripts could be found in NPC, whereas in latently infected B lymphocytes the six EBNAs were seen (Hitt et al, 1989). In transplant and B cell lymphomas from cotton tail tamarins Allday et al, (1990) found that the full range of antigens found in EBV immortalized lymphoblastoid cell lines were expressed. This is in contrast to BL and NPC, which were found only to express EBNA-1 (Allday et al, 1990). In NPC (but not in BL) LMP may also be expressed. Two possible explanations as to why only EBNA-1 is expressed

under these circumstances have been proposed. Sample et al, (1991) suggest that although the EBNAs are generated by differential splicing, EBNA-1 may have a different transcription initiation site permitting EBNA-1 transcription in isolation. Allday et al, (1990) suggest that CpG methylation of viral DNA may be responsible for the restricted pattern of EBNA expression.

Experiments to investigate the involvement of LMP in EBV transformation have been reported where LMP was expressed from either of two heterologous promoters and introduced into two different established cell lines. This resulted in the cells being able to grow in an anchorage-independent manner (Wang et al, 1985; Baichwal & Sugden, 1988).

Burkitt's lymphoma (BL), a B cell tumour (Klein et al, 1968), is an excellent example of the multistep development of cancer, as three factors are implicated in the disease (discussed by Rowe & Gregory, 1989). These are firstly the geographical and climatic features coincident with holoendemic malaria resulting in the immuno-suppression of the host and activation of B cells (Lam et al, 1991). Secondly, characteristic chromosomal translocations involving 8q24 are seen (Manolor & Manolora, 1972; Bernheim et al, 1981), with the common breakpoint at c-myc (Dalla-Farera et al, 1982; Neel et al, 1982). These result in a translocation near to the immunoglobulin loci (Rabbitts & Rabbitts, 1989) which was assumed to cause the deregulation of c-myc and has been confirmed by transfection studies (Lambardi et al, 1987) and transgenic experiments (Adams et al, 1985). The third factor is the virus EBV. Although there is little doubt that EBV is involved in BL, its mode of action has been unclear. Possible explanations include the action of viral proteins, as indicated previously, or the involvement of the cellular EBV receptor CR2, which as a result of viral attachment causes B cell proliferation (Gordon, 1989). The order of these three events in the development of BL may be unclear, but EBV is firmly implicated, and furthermore, complies with Koch's postulates.

Herpes simplex virus type 2 (HSV-2) was originally associated with squamous cell carcinoma of the uterine cervix based on seroepidemiological evidence (Rawls et al, 1969; Nahmias et al, 1970; Royston & Aurelian, 1970). These studies demonstrated a higher HSV antibody titre in cancer cases than in controls. Gilman et al, (1980) analyzed sera of such patients but grouped them according to parameters which could influence the presence or absence of HSV-1 or HSV-2 antibodies. This resulted in a significant difference in the

amount of two different HSV-2 antibodies seen in the case versus control samples. Although not all studies agree with the sero-epidemiological studies (Vonka et al, 1984b), it has been proposed that such negative results are due to the overmatching of case and control sera (Reeves et al, 1989). In addition, previously type specific testing could not be performed and it has been proposed that HSV-1 antibodies could neutralize HSV-2 antigens during investigations (discussed by Whitley, 1984).

Current epidemiological studies suggest that HSV is not the major cause of cervical cancer (DiPaolo et al, 1990), but may act in conjunction with human papillomaviruses to result in malignant disease. Indeed Gius & Laimins (1989) previously showed that Vmw 65 of HSV-1 can activate E7 of HPV type 18, and Hildesheim et al (1991) showed a 8.8 increased relative risk fold in the development of cervical cancer if antibodies to both HSV-2 and HPV16 or 18 were present in a patient.

Peto, (personal communication) in a comparative study of cervical cancer patients in Greenland and Denmark, found that although both countries have similar incidences of HPV, patients in Greenland had a high incidence of cervical cancer which correlated to a high incidence of HSV antibodies. In Greenland 46% of patients who were positive for HPV developed cervical cancer, but in Denmark where HSV is less prevalent only 2% of HPV positive patient developed cervical cancer. It has recently been shown that sera of patients with cancer of the cervix contain antibodies against a cellular polypeptide which is induced by HSV-2 infection (Macnab et al, in press). These studies suggest that HSV infection may play an important role as a cofactor in the development of cervical cancer or alternatively they may indicate that HSV induces some proteins also expressed in cervical cancer.

HSV-2 has also been shown to induce cervical neoplasia in the mouse model. The application of uv inactivated HSV-1 or HSV-2 virus (Wentz et al, 1981; Wentz et al, 1983b) and HSV-2 DNA (Anthony et al, 1989) induced cervical carcinoma in the mouse. Moreover, vaccination of such mice with HSV-1 stopped the development of abnormal cervices and the progression to tumours, further suggesting a role for HSV in tumour formation (Wentz et al, 1983a). The possible mechanisms of action of HSV as a tumour virus will be discussed in more detail in Section 5 of this chapter.

2. COOPERATION BETWEEN VIRAL ONCOGENES AND ACTIVATED PROTO-ONCOGENES TO IMMORTALIZE AND TRANSFORM CELLS

As indicated previously, many events in the life of a cell can contribute to what may finally become a cancerous phenotype. Up to 6 events have been proposed to be involved, each of which can cooperate to convert a normal cell to a transformed cell (Peto et al, 1975).

Oncogenes can be divided into two distinct groups - nuclear oncogenes and cytoplasmic oncogenes. These two groups are proposed to have different functions in cancerous progression. Nuclear oncogenes such as E1A, E7 and polyoma and SV40 large T antigens are proposed to act as "immortalizing oncogenes". This is because the expression of these oncoproteins does not alter cell morphology, the requirement for growth factors or anchorage dependence, but may immortalize cells. Cytoplasmic oncogenes conversely are able to induce cell shape changes, lead to anchorage independent growth and reduce growth factor requirements, but cannot immortalize cells. This is an over-simplified view as there are exceptions which can fit into both categories, however, this provides a basis for classification of oncogenes (reviewed by Marshall, 1989; Hunter, 1991).

Cooperation can occur between these nuclear and cytoplasmic oncogenes, with the first such evidence coming from experiments carried out with combinations of polyomavirus oncogenes (Rassoulzadegan et al, 1982). The observation that specific combinations of oncogenes were able to cooperate led to the introduction of the concept that nuclear oncogenes cooperate best with cytoplasmic oncogenes (Weinberg, 1985).

A similar model for progression to a transformed phenotype is reviewed by Balmain & Brown (1988), where the mouse skin carcinoma model is used to define two distinct stages in malignant progression, initiation and promotion. Low levels of a non-carcinogenic agent are thought to initiate the cell, with multiple treatments of a tumour promoter resulting in the eventual morphologically transformed cell. Infection by a tumour virus as described in Section 1 either introduces a virus encoded oncogene or activates a cellular p-onc. In both situations the resultant oncogene(s) can then cooperate with other events which have already occurred or will occur at a later stage so

contributing to a transformed phenotype. Such cellular and viral oncogenes can have a wide variety of functions, some of which are outlined in Table 3.

3. ONCOGENE ACTIVATION AT THE GENETIC LEVEL

Proto-oncogenes are cellular genes encoding proteins which under normal circumstances are functionally important in the growth and development of the cell. "Activation" of these proto-oncogenes to c-onc can result in either an alteration in oncogene regulation or a change in oncoprotein function. Oncogene activation can occur either by infection with a tumour virus or by exposure to carcinogens or radiation. The mechanisms by which proto-oncogenes can be activated are outlined in Figure 3, and described in more detail below (reviewed by Paul, 1987).

TRANSDUCTION of a proto-oncogene occurs by retrovirus infection where the provirus becomes inserted into the host genome adjacent to the p-onc such that co-transcription of the viral and p-onc sequences occur. Genomic rearrangement can result in the virus incorporating the p-onc which is now under viral control and therefore constitutes an activated oncogene. As a consequence of the mechanism of viral replication, the proto-oncogene is more likely to become mutated which is seen in the case of v-src.

INSERTIONAL MUTAGENESIS can also occur following retrovirus infection. The long terminal repeats (LTRs) in the retrovirus are instrumental for this form of oncogene activation as they contain transcription initiation sequences which can act on both the viral genes and the adjacent host genes. The LTRs also contain enhancer elements which can alter host gene expression (proto-oncogene), without being directly adjacent to the gene.

CHROMOSOMAL REARRANGMENTS also result in alteration in the control of proto-oncogenes, but by a mechanism which involves translocation of the proto-oncogene to a site of expression such as the immunoglobulin gene. Chromosomal breaks are required to facilitate such oncogene activation.

POINT MUTATIONS may occur naturally in the host, but occur with higher incidence if the p-onc has become incorporated in a retrovirus because of the more error prone mode of replication of an RNA virus. Mutation hot-spots can be seen in oncogenes and tumour suppressor genes (see following section) such

TABLE 3 : POTENTIAL ONCOGENE FUNCTIONS

FUNCTION	EXAMPLES
Growth factors	v-sis (subunit of PDGF-2/B)
Protein tyrosine kinases	(either transmembrane receptors for growth factors or cytoplasmic proteins associated with membranes)
Transmembrane receptors	v-erb B (truncated epidermal growth factor) v-fms + v-ros
Peripheral membrane proteins	v-abl, v-fes/fps, v-src, v-gfr + v-yes
Adenylate cyclase regulators	Ha-ras, Ki-ras (functionally similar to G proteins)
Protein serine/threonine kinases	v-mil/raf + v-mos
DNA binding activity	c-myc

FIGURE 3

MECHANISMS OF ONCOGENE ACTIVATION

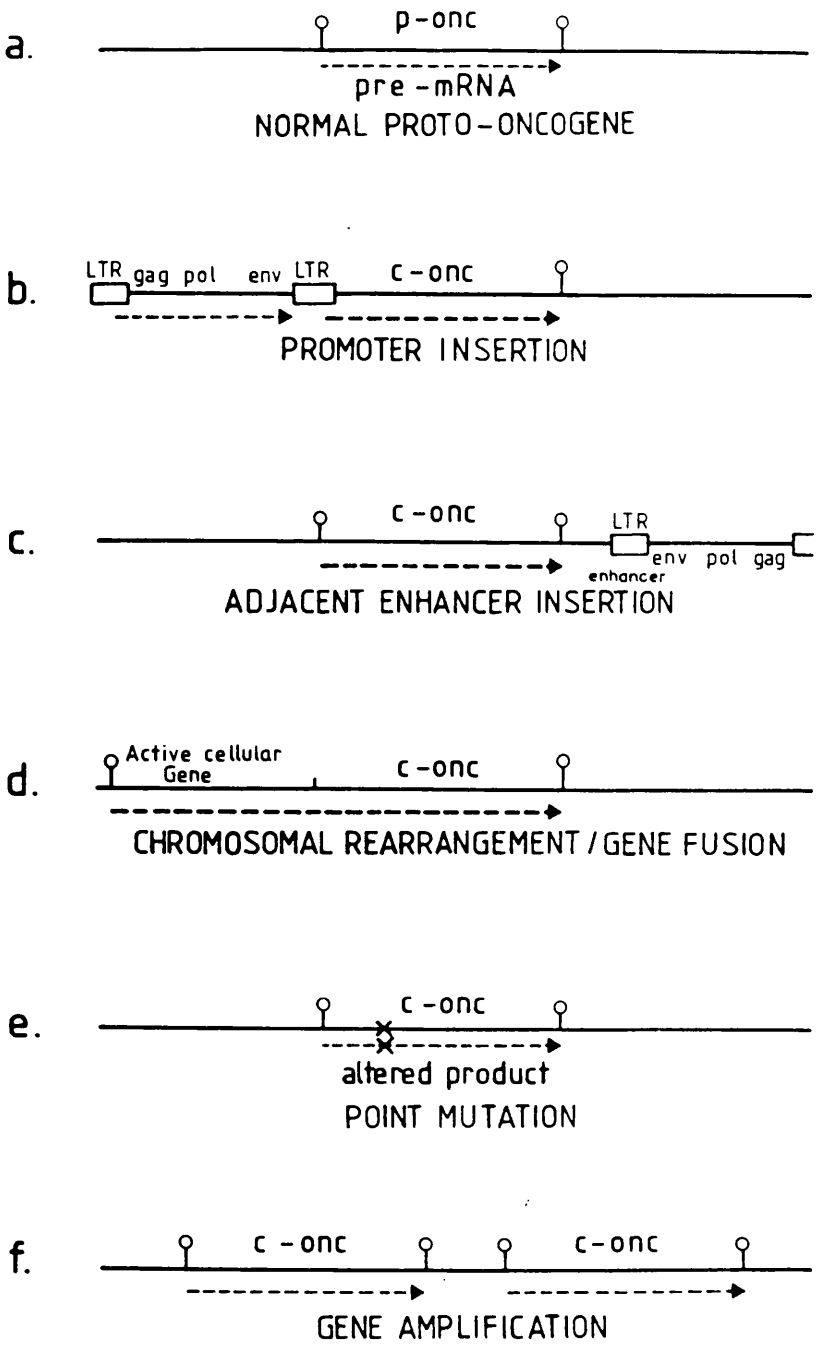


Diagram representing a normal proto-oncogene (a), and mechanisms by which such a proto-oncogene could be activated following tumour virus infection or exposure to carcinogens (b-f). Reproduced from Paul, (1987).

as ras and p53 (eg. in hepatocellular carcinoma) indicating that such regions of the proteins are functionally important.

GENE AMPLIFICATION is often seen in advanced and more aggressive tumours. The exact mechanism of such oncogene activation is uncertain but is thought to involve end-to-end replication of the gene followed by reinsertion.

4. PROTEIN-PROTEIN INACTIVATION OF TUMOUR SUPPRESSOR GENES

Discussion has so far centred on one class of oncogenes which result in tumourigenesis following activation. A second class of oncogenes exist called recessive oncogenes, anti-oncogenes or tumour suppressor genes, which as a consequence of inactivation, result in tumourigenesis (reviewed by Marshall, 1991). I shall adopt the term tumour suppressor gene as this appears to be currently more widely used in the literature.

DNA tumour virus oncoproteins frequently interact with host cell proteins, some of which are now known to be tumour suppressor genes. Interaction with p53 and the retinoblastoma susceptibility gene product (Rb) are the two best documented cases and will be discussed in this section.

The tumour suppressor p53 is thought to either regulate the assembly or function of the DNA replication-initiation complex (Braithwaite et al, 1987, Gannon & Lane, 1987) or alternatively p53 may act as a transactivator of gene transcription, either promoting or repressing mRNA synthesis (Raycroft et al, 1990; Fields & Jang, 1990).

Viral oncogenes complex with p53 (Levine, 1990; Levine et al, 1991) including SV40 T antigen, adenovirus E1b-55K and HPV E6. The formation of such viral oncogene-tumour suppressor protein complexes may alter either the function of p53 or the viral oncogene by protein-protein interactions. In SV40, T antigen mutants which fail to bind p53 also fail to transform cells (Peden et al, 1989). Complexing of p53 to the T antigen blocks the DNA polymerase binding to T antigen (Gannon & Lane, 1987) and prevents the initiation and replication of SV40 DNA (Braithwaite et al, 1987, Struzbecher et al, 1988, Wang et al, 1989). In HPV, only with those viruses which are associated with a high risk of anogenital cancers, has it been shown that E6 binds to p53 (Werness et al, 1990) correlating the ability of the virus proteins to complex with p53 with their association with cancer. The complexing of E6 to p53 is proposed to stimulate the

degradation of p53 by a ubiquitin dependent protease system (Scheffner et al, 1990). In Ad5 and Ad7 transformed cells p53 is complexed with E1B p55 (discussed by Boulanger & Blair, 1991), however, in Ad12 transformed cells, no stable E1B p55/p53 complexes can be detected (Zantema et al, 1985; Mak et al, 1988; Blair-Zajdel & Blair, 1988). The reason for this is uncertain as the E1B sequences of Ad5 and 12 are identical (reviewed by Boulanger & Blair, 1991).

The Rb protein is thought to inhibit proliferation within the cell with control of this function being determined by the level of Rb phosphorylation (Cooper & Whyte, 1989). It is proposed that the phosphorylation status of Rb alters its ability to form tight nuclear interactions which are essential for growth regulation and that the binding of viral oncoproteins to Rb polypeptide pre-empt this interaction (Mittnacht & Weinberg, 1991). SV40 and polyoma large T antigens, adenovirus type 5 E1A and HPV types 16 and 18 E7, all bind Rb (De Caprio et al, 1988; Dyson et al, 1989a, 1990; Whyte et al, 1988).

HSV has also been shown to interact with both p53 and Rb. This interaction is seen to colocalize with HSV replication complexes in infected cells (Wilcock & Lane, 1991). The relevance of this observation is as yet undetermined and no HSV coded proteins have been detected binding to p53 or Rb (Lane personal communication).

In addition to p53 and Rb, other cellular proteins are found to interact with viral oncogenes. One such polypeptide is a 107Kd protein (Whyte et al, 1989) which may be related to Rb, but is clearly distinct (Dyson et al, 1989b). This polypeptide interacts with the large T antigens of both SV40 and JC virus in addition to E1A of adenovirus (Dyson et al, 1989c). The functions of this and other such proteins are as yet unknown.

5. HSV TRANSFORMATION : POSSIBLE MECHANISMS

Both HSV-1 and HSV-2 have been shown to induce morphological transformation of rodent embryo cells (Duff & Rapp, 1971, 1973; Darai & Munk, 1973, 1976; Macnab, 1974, 1975, 1979; Wilkie et al, 1974; Boyd et al, 1975; Cameron, 1982). One region in HSV-1 capable of inducing morphological transformation, called the morphological transforming region MTRI, maps within the *Bgl* IIi fragment (0.311-0.415mu), (Camacho & Spear, 1978; Reyes et al, 1979). This is adjacent to the two transforming fragments of HSV-2, MTRII, mapping in the

Bgl IIn fragment (0.58-0.62mu), (Reyes et al, 1979; Macnab & McDougall, 1980; Galloway & McDougall, 1981; Cameron et al, 1985), and MTRIII, in the *Bgl* IIc fragment of HSV-2 (0.54-0.58mu), (Jariwalla et al, 1983). The MTRII activity was later localized to 737bp in the left hand region of *Bgl* IIn (Galloway et al, 1984). The positions of these regions are outlined in Figure 4. In a model system in mice, HSV has also been shown to induce uterine cancer, with prior immunization of these mice with HSV preventing the development of tumours (Wentz et al, 1983b).

HSV-2 DNA sequences have been detected in a small proportion (10%) of cervical carcinomas (Galloway & McDougall, 1983; Park et al, 1983; Macnab et al, 1985b, 1986; Prakash et al, 1985). However, despite extensive studies no transforming DNA sequences of HSV are found to be retained at the level of one copy in the transformed cell (Cameron et al, 1985) nor has any HSV encoded transforming protein been found to be essential for the maintenance of the transformed state (reviewed by Macnab, 1987). This has led to the proposal that HSV induced transformation occurs via a "hit and run" mechanism (Galloway & McDougall, 1983). Many different possibilities exist as to how HSV could transform cells via a "hit and run" mechanism, some of which are briefly described.

(1) The DNA stem loop structure in the *Bgl*II n fragment has been proposed to act as either an enhancer, a mutagen or to activate a cellular oncogene (Galloway et al, 1984).

(2) Ribonucleotide reductase (RR) has been speculated to be involved in transformation by Huszar & Bacchetti (1983) as this is encoded by the region of the virus which can transform cells (Reyes et al, 1979; Jariwalla et al, 1980; Galloway & McDougall, 1981; Dutia, 1983). Prior to this RR had previously been suggested to be involved in transformation by destabilizing dNTP pools in infected cells resulting in mutagenesis (Jamieson & Bjursell, 1976). Recent experiments using transient gene expression of RR in mammalian cells did not increase mutagenesis in cells, nor did its expression in transgenic mice affect the mice. However, in both cases inefficiencies of the experimental systems used could not be ruled out as the cause of the negative results (Clarke, 1990). Further experiments have shown that MTRIII which encodes the N terminal half of the large RR (amino acids 71-502) induces neoplastic transformation of

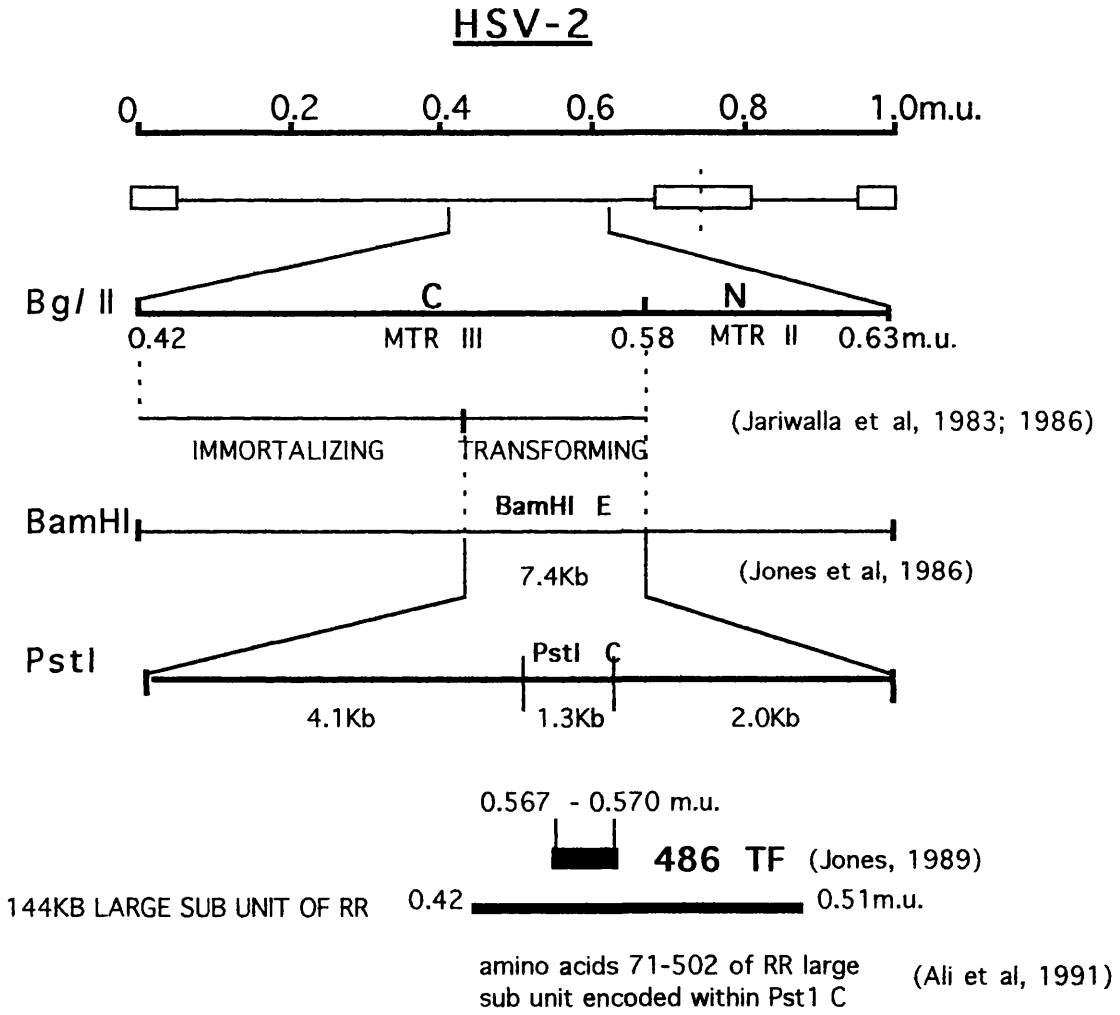


FIGURE 4 : Diagrammatic representation of the HSV genome and the positions (in map units) of the morphological transformation regions (MTR) of HSV- 2

immortalized cell lines. By transient expression of this region a distinct protein domain (of 65Kd) was observed in these constructs as was a protein kinase autophosphorylation potential (Ali et al, 1991). However, it remains to be established whether this protein kinase activity is necessary or sufficient for MTRIII induced transformation.

(3) HSV may transform cells by acting as a biological carcinogen or as a mutagen (Pilon et al, 1985) by inducing gene amplification. Jones et al, (1986) and Jariwalla et al, (1986) proposed that the MTRs of HSV contain repetitive sequences which could be implicated in gene rearrangement or activation. It was then later reported that MTRIII could in fact function as a complex promoter (Jones, 1989). HSV infection has also been demonstrated to inhibit host cell DNA methylation (Macnab et al, 1988), a mechanism which may also alter host gene expression resulting in an important step in transformation by HSV.

(4) HSV has also been shown to activate endogenous C-type viruses which could act as co-carcinogens. In addition to this HSV has also been proposed to act as a co-carcinogen with human papilloma viruses (Di Paolo et al, 1990), acting as one of the multiple steps required to result in a transformed phenotype.

(5) Finally HSV may transform cells by a mechanism which results in the altered expression of host cell polypeptides by cellular gene activation. A series of experiments (Macnab, 1974, 1975, 1979; Buultjens & Macnab 1981) was previously thought to indicate that HSV functions were being expressed in the HSV transformed cell, however Cameron et al, (1985) reported that no virus DNA at the level of 0.01 copies/cell, nor functional RR encoded protein was expressed in HSV transformed cells when tested by Southern blot analysis. Further analysis with a range of antibodies against HSV-coded proteins, showed no HSV polypeptides could be consistently detected in HSV transformed cells (Macnab, 1987). These polypeptides previously thought to be virus encoded, were found to be cellular in origin and found in all virus transformed and immortalized cells tested (Macnab et al, 1985). This was the first report of specific cellular gene products which were induced by HSV infection and where the expression of these products continued in the transformed cell.

Filion & Suh (personal communication, 11th International Herpesvirus Workshop; Filion et al, 1988) have identified cDNAs which are increased following HSV-2 transformation. Several groups have reported a similar

increased expression of cellular genes following HSV-2 infection (LaThangue et al, 1984; Kemp et al, 1986a, Kemp et al, 1986b; Patel et al, 1986; LaThangue & Latchman 1988; Kemp & Latchman, 1988b; Jang et al, 1991). The stimulation of mRNA levels of the estrogen receptor by HSV infection has been observed (Offord et al, 1989) and Goswami (1987) has reported transcriptional induction of c-fos in the host cell following HSV-2 infection. One cellular polypeptide, U90, increased by HSV infection and also present in transformed cells will be investigated in the results section of this thesis.

SECTION 2 : HERPES SIMPLEX VIRUS

1. CLASSIFICATION OF HERPESVIRUSES

The Herpesviridae is a large family of double stranded DNA viruses consisting of over eighty members which lytically and/or latently infect a wide range of hosts spanning many species (reviewed by Roizman, 1982). These viruses have been identified by means of base composition, genome structure, immunological cross reactivity and size (Nahmias, 1972).

The herpesvirus particle which can range from 150-200nm in diameter has four distinct morphological features which characterize the virus (Fenner, 1976). These features are detailed below:

The core was previously reported to be an electron dense fibrillar spindle surrounded by genomic DNA (Epstein, 1962). Furlong et al, (1972) proposed that the DNA was wound in a toroidal manner around a cylindrical structure and Schrag et al, (1989) hypothesized that the viral DNA was contained within an inner icosahedral shell. It has recently been established that the viral DNA is not in fact wound around any structure but is packaged in a similar manner to phage DNA (Booy et al, 1991).

The capsid is composed of 162 capsomers (Wildy et al, 1960), 150 of which are hexavalent and 12 are pentavalent capsomers (Schrag et al, 1989). These capsomers which are roughly cylindrical create a 5nm thick shell around the core.

The tegument which surrounds the capsid is described as a layer of amorphous material lying between the capsid and the viral membrane (Morgan et al, 1968; Schwartz & Roizman, 1969; Roizman & Furlong, 1974).

The viral membrane is derived from the host cell's nuclear membrane (Wildy et al, 1960) as the virus buds from the cell, but contains numerous viral encoded glycoprotein spikes (Morgan et al, 1959; Wildy et al, 1960; Asher et al, 1969; Spear & Roizman, 1972).

The Herpesviridae can be further divided into three groups based on the biological properties of cytopathology, host range, the reproductive cycle and the characteristics of the latent infection of the virus (Mathews, 1982; Roizman, 1982).

Alpha herpesviruses include HSV-1, HSV-2, VZV and pseudorabiesvirus. Members of this group can have either a narrow or a wide host range in tissue culture and a replicative cycle of less than 24 hours. Infection results in cell destruction, however, latent infection is frequently established in neural cells.

Beta herpesviruses include HCMV. These viruses have a narrow host range in tissue culture, a long replicative cycle and slow lytic infection resulting in the enlargement of infected cells (called cytomegalia). These viruses can establish latent infections but usually only in kidney cells, lymphoreticular cells and secretory glands.

Gamma herpesviruses include EBV. These also have a limited host range with replication only occurring in the host T or B-lymphocytes. Such viruses have a variable replicative cycle and often result in persistent infection (as opposed to lytic infection) in lymphoid tissue.

2. PATHOGENICITY OF HUMAN HERPESVIRUSES

Herpesvirus infections can occur as a result of a primary infection or by reactivation of a latent infection. HSV-1 and HSV-2 infection can result in a number of clinical syndromes (reviewed by Nahmias et al, 1991). Mild to moderately severe disease includes urogenital infection in both males and females, predominantly, but not exclusively by HSV-2. However, studies show a 50% incidence of HSV-1 in Glasgow and 80% HSV-1 in Edinburgh (Macnab, personal communication). Gingivostomatitis of the mouth, herpes labialis of the lip, keratitis and conjunctivitis of the cornea and/or the conjunctiva are also predominantly a consequence of HSV-1 infections. Latent infection by HSV-1 usually occurs in the trigeminal or thoracic ganglia whereas HSV-2 latent infection usually occurs in the sacral ganglia.

Severe to fatal diseases caused by HSV infection include meningoencephalitis of the brain and the spinal cord, and localized or generalized infection of the skin, eyes, brain or visceral organs in newborn children. HSV-2 infection is also associated with cervical cancer, where it is thought to act as a cofactor (discussed in section A 1e).

Few people world-wide escape infection by HCMV, normally a self-limiting disease. Symptoms are generally only apparent in the

immunodeficient, resulting in cytomegalo mononucleosis, post transfusion and post transplantation syndromes. In neonates infection can be very serious resulting in severe neurological deficiencies. Like HSV-2, HCMV has also been associated with malignant disease (section 1e).

VZV manifests itself as two diseases, the first varicella (or chickenpox), occurs at primary infection, resulting in fever and a characteristic rash. The second, zoster (or shingles), is a consequence of reactivation of virus from the dorsal root ganglia or the cranial nerve resulting in painful unilateral vesicles.

EBV infection is the cause of infectious mononucleosis - an acute febrile illness involving children and young adults causing lymphadenopathy and sore throats. EBV is also associated with neoplastic disease as outlined in section 1e. The more recently discovered human herpes virus type 6 (HHV- 6) which was isolated from T cells in vitro (Salahuddin et al, 1986) is thought to be the causative agent of exanthem subitum, a prevalent childhood disease characterized by high fever and a rash (Yamanishi et al, 1988). HHV-6 has been associated with AIDS (Downing et al, 1987; Tedder et al, 1987; Agut et al, 1988; Becker et al, 1988; Lopez et al, 1988; Pietrobani et al, 1988) but HHV-6 has also been isolated from the saliva of normal adults (Harnett et al, 1990; Levy et al, 1990). Infection with HHV-6 appears to occur early in childhood as sero conversion rates approach 100% in the first few years of life (HHV-6 reviewed by Cone, 1991).

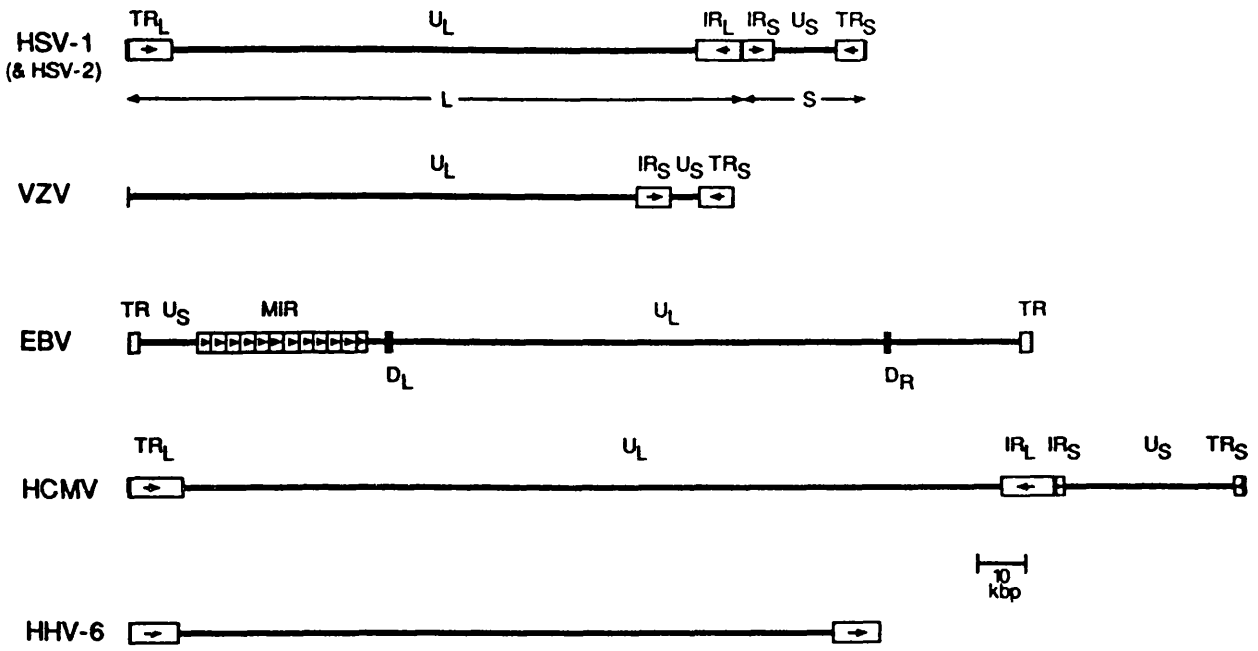
A seventh herpes virus has now been isolated from $CD4^+$ T cells (Frenkel et al, 1990). Human herpes virus type 7 (HHV-7) is immunologically distinct from HHV-6 (Wyatt et al, 1991). Unlike HHV-6 and other herpesviruses sero-conversion occurs later in childhood but no disease as yet has been associated with HHV-7.

3. THE GENOMES OF HUMAN HERPESVIRUSES

The genomes of human herpesviruses vary in size, base composition and the pattern of repeated DNA sequences (Honess, 1984) as illustrated in Figure 5. McGeoch et al, (1988) published the complete HSV-1 DNA sequence showing 152,260 nucleotides on each strand, with one overhanging nucleotide at each end and a G+C base composition of 68.3%. The genome is composed of two unique regions, U_L and U_S each flanked at either end by oppositely orientated repeat

FIGURE 5

COMPARISON OF THE HUMAN HERPESVIRUSES GENOMES



Each linear genomic DNA is shown with unique sequences as heavy lines and repeat elements as boxes with relative orientations indicated by arrows. Reproduced from McGeoch, (1989).

elements termed R_L or R_S. Relative inversion of these unique regions results in the production of four isomers which can be isolated in equimolar amounts from viral DNA preparations (reviewed by McGeoch, 1989). Directly repeated sequences of 400bp, called the a sequences exist at the termini of the genome and at least one copy of this sequence is found in inverted orientation at the junctions of the two unique regions. The genomes of HSV type 1 and type 2 are reported to be co-linear as shown by intertypic recombinant studies carried out by Davison & Wilkie (1983). Sequencing of the HSV-2 genome is incomplete as yet, however no evidence suggesting that HSV-1 and HSV-2 are not co-linear has been found (McGeoch, personal communication). The open reading frames corresponding to the proposed HSV-1 genes are shown in Figure 6.

The genomes of VZV and HSV are very similar, however VZV is unique in herpesviruses so far in that isolated VZV DNA has unequal proportions of isomers present (Davison, 1984). HCMV is larger than both VZV and HSV, but again is very similar in genomic structure. HCMV contains an "a" sequence similar to HSV and also exists in four isomeric forms present in equimolar amounts (Weststrate et al, 1983).

EBV was the first herpesvirus to be sequenced (Baer et al, 1984) and has a marked difference in its repeat elements compared to other herpesviruses. The EBV genome carries an internal set of large directly repeated elements which together with the terminal repeats can be present in varying copy numbers.

Preliminary investigations of HHV-6 suggests it has a genome of 166Kbp consisting of a single unique region flanked by direct repeats, closely related to HCMV (Lawrence et al, 1990). The structure of HHV-7 has yet to be determined.

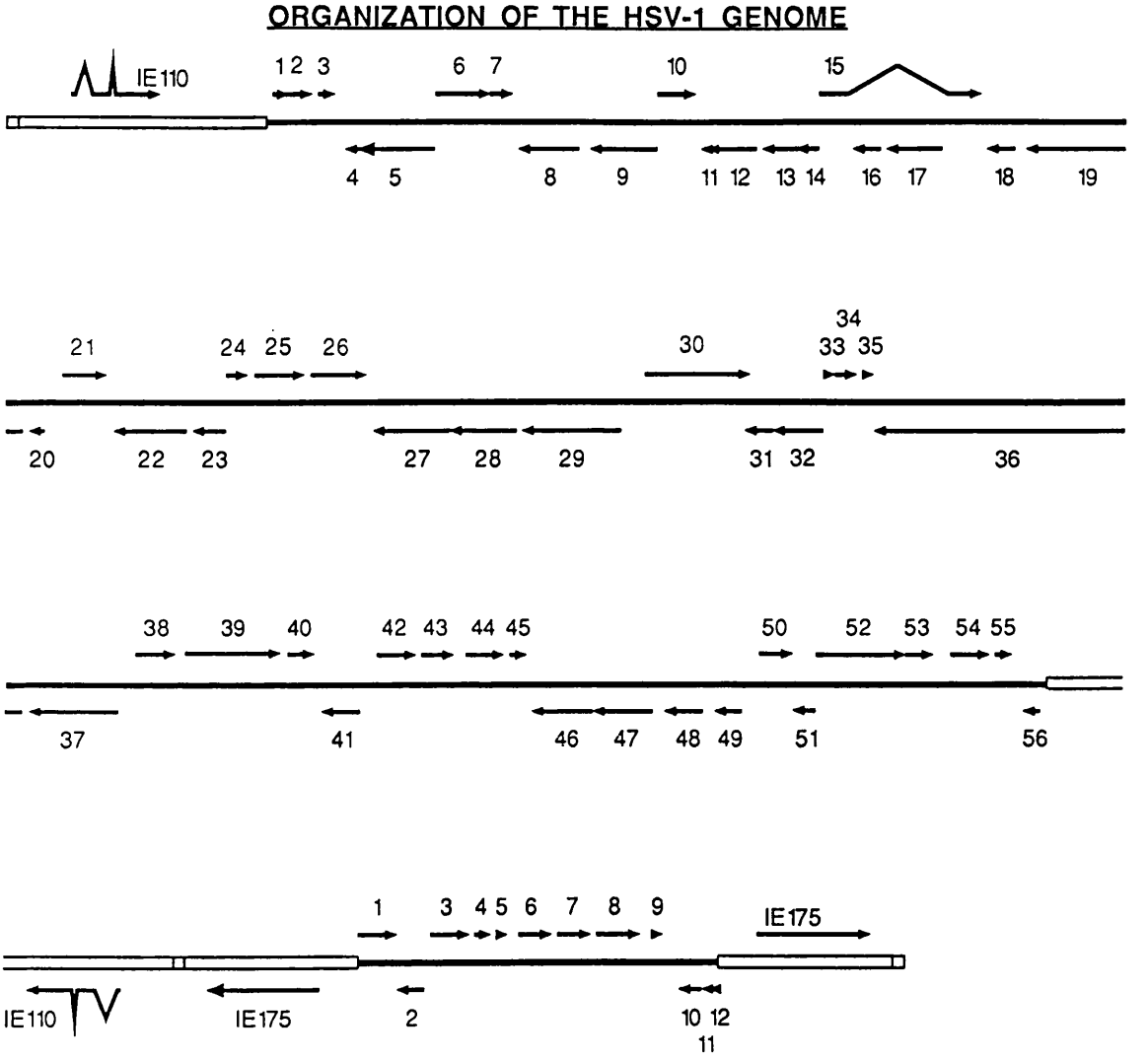
4. HSV LYTIC INFECTION : AN OVERVIEW

This section will briefly outline herpes virus lytic infection with emphasis on HSV-1.

4.(a) ADSORPTION AND PENETRATION

HSV adsorption to the host cell membrane is proposed to occur via heparin sulphate on the host cell surface and is mediated via viral glycoproteins (WuDunn & Spear, 1989) in particular gB, gC, gD and gH (Kuhn et al, 1990; Forrester et al, 1991). Another putative receptor has been proposed to

FIGURE 6



Organization of the HSV-1 genome, represented on four successive lines of 40KB each. Locations of reading frames are shown by arrows, with splicing within the coding regions indicated. The genes UL1-UL56 are shown as 1-56 above the solid line representing HSV DNA, with genes US1-US12 shown as 1-12 below the solid line. The genomes of HSV-1 and HSV-2 are colinear as reported by Davison & Wilkie (1983) and suggested by HSV-2 sequence data (McGeoch, personal communication). Reproduced from McGeoch, 1989.

be essential as a portal of cellular entry for HSV-1, the basic fibroblast growth factor (bFGF) receptor (Baird et al 1990; Kanner et al 1990). However, this has been questioned (Herold et al, 1991). Recently Muggeridge et al, (1992) have demonstrated that HSV infection can occur without the involvement of the bFGF receptor. However Muggeridge et al, (1992), state that HSV binding via bFGF receptor cannot be excluded as Sears et al, (1991) have provided evidence to support the existence of two independent receptor pathways on the one cell type. For this reason it may still be possible that the bFGF receptor plays a role in HSV entry in certain cell types. Following adsorption, the viral envelope fuses with the host cell plasma membrane (Morgan, 1968) releasing the viral capsid into the cell cytoplasm (Fuller & Spear, 1985), which then delivers the viral DNA to the host cell nucleus (Knipe et al, 1981; Batterson & Roizman, 1981).

4(b). VIRAL GENE EXPRESSION

Three groups of viral polypeptides exist in herpes simplex virus which are coordinately regulated and sequentially expressed. These are the immediate early (IE) polypeptides, which are mainly viral regulatory functions and detected one hour post infection, early (E) polypeptides, which are implicated in viral replication and maximally synthesized between 2-7 hours post infection, and finally the late (L) polypeptides which are first detected after the onset of viral replication at around 2 hours post infection and are mainly viral structural polypeptides (Honess & Roizman, 1974). The L genes can be divided into two classes - true late and leaky late genes, with the distinction that only the true late genes are completely inhibited in the absence of viral replication.

The IE genes are transcribed by an unmodified host cell RNA polymerase, and are essential to enable the transition from IE to E gene expression (Watson & Clements, 1980). Both the IE and E polypeptides are required for L gene expression.

The viral polypeptide Vmw65, an essential structural polypeptide (Ace et al, 1988) which is a component of the tegument (Campbell et al, 1984) is responsible for the induction of IE gene expression by binding to TAATGARAT regulatory sequences in the upstream control regions of IE genes. Transactivation by Vmw65 occurs independently of de novo protein synthesis (Post et al, 1981) with Vmw65 interacting with the viral DNA via an IE complex

on TAATGARAT in the presence of host cell factors (McKnight et al, 1987; O'Hare & Goding, 1988; Preston et al, 1988).

A cellular factor named Oct-1 has been shown to be required for complex formation (O'Hare & Goding, 1988; Gerster & Roeder, 1988) and an additional cellular factor has been proposed to interact with the GARAT region of TAATGARAT (Gerster & Roeder, 1988; Kristie et al, 1989). The formation of this IE complex is essential but not sufficient for transactivation by Vmw65. In addition to the above a GA element and two GC boxes (SP1 binding sites) are also proposed to be involved with immediate early gene induction by functioning as enhancer elements.

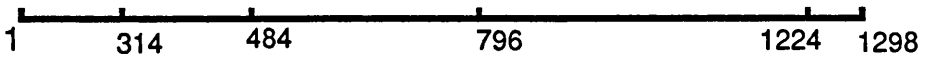
Following IE polypeptide synthesis the IE gene expression is autoregulated by Vmw175, a phosphorylated IE polypeptide, which is purified as a homodimer and found to localize in the nucleus (Pereira et al, 1977). Functional Vmw175 is required for early gene expression, for IE gene repression (Watson & Clements, 1980; Dixon & Schaffer, 1980) and is also thought to possess a distinct function necessary for late gene expression (DeLuca et al, 1984). Most of the IE polypeptides bind DNA in vitro (Hay & Hay, 1980), but Vmw175 specifically binds DNA with the element ATCGTC being considered to be the most important motif (Beard et al, 1986; Faber & Wilcox, 1986, 1988; Muller, 1987; Roberts et al, 1988; Kristie & Roizman, 1986a, 1986b). Vmw175 however, will also bind DNA sequences lacking the above element (Kristie & Roizman, 1986b; Micheal et al, 1988).

Mutants in Vmw175 fail to synthesize DNA or produce early or late polypeptides (Benyesh-Melnick et al, 1974; Marsden et al, 1976; Watson & Clements, 1978, 1980; Preston, 1979; Dixon & Schaffer, 1980) and overproduce IE transcripts (Preston, 1979; Dixon & Schaffer, 1980). Vmw175, which binds its own promoter resulting in repression was previously thought to act similarly on the Vmw110 promoter (O'Hare & Hayward, 1985). Recent work however, indicates that under normal HSV infection Vmw175 has no repressive effect on Vmw110 gene expression (Everett & Orr, 1991). Figure 7 illustrates the functionally important regions of Vmw175 with regards to transcriptional transactivation and repression as reported by Paterson & Everett, (1988a, 1988b) and Deluca & Schaffer (1988).

FIGURE 7

FUNCTIONAL DOMAINS OF Vmw175

VMW 175



AMINO ACID	POTENTIAL FUNCTIONS
275-490	DNA binding
	Repression
	Transactivation
682-774	Nuclear localization
840-1100	Transactivation
797-1224	Essential for growth

The Vmw 175 sequence is divided into structural domains 1-5 (McGeoch et al, 1986). The functional domains and their potential roles are illustrated above (DeLuca & Schaffer, 1988; Paterson & Everett, 1988a, 1988b).

Vmw175 acts as a transactivator of E gene expression with the resulting E polypeptides acting in conjunction with the IE polypeptides permitting viral gene replication to occur. This is then followed by L gene expression. Viral replication has been proposed to alleviate some restraint which prevents L viral gene expression such as altering viral DNA conformation (Workman et al, 1988).

4(c). HSV Replication

On entering the host cell nucleus it is proposed that the viral genome circularizes and that viral replication occurs via a rolling circle mechanism generating linear concatemers of repeating viral genomes (Jacob et al, 1979; reviewed by Chalberg & Kelly, 1989; Rabkin & Hanlon, 1990). Other possibilities have also been proposed including Cairns type replication, (where the separation of the daughter molecules is slow), or replication followed by the rapid joining of daughter molecules by recombination (reviewed by Chalberg & Kelly, 1989). Seven HSV genes are required for origin dependent DNA replication, although other additional genes are likely to be involved, mutations in these genes can be complemented by the dividing host cell. Some of these polypeptides have been characterized including the viral DNA polymerase, (distinguishable from the host cell polymerase), single stranded DNA binding protein, double stranded DNA binding protein, origin binding protein and helicase/primase activity.

4(d). VIRION ASSEMBLY AND RELEASE

Following replication newly synthesized viral DNA concatemers accumulate in the host cell nucleus where site specific cleavage of the DNA occurs. A cleavage signal is located within the a sequence (Vlazny & Frenkel, 1981; Mocarski & Roizman, 1982; Stow & McMonagle, 1983; Spaete & Mocarski, 1985), in addition to two cis-acting maturation and packaging signals (Varmuza & Smiley, 1985; Deiss et al, 1986). The cleavage and packaging of the viral DNA has been proposed to be linked (Deiss et al, 1986), and together with capsid formation, is dependent on several viral gene products (Matz et al, 1983; Preston et al, 1983; Sacks & Schaffer, 1987; Sherman & Bachenheimer, 1987, 1988; Rixon et al, 1988; Addison et al, 1990).

Site specific cleavage of viral DNA generates unit length genomic molecules and packaging of viral DNA into virions takes place. Nucleocapsids containing an approximate genome length are then enveloped at the inner lamella of the nuclear membrane and transported to the cytoplasm (Roizman & Furlong, 1974; Vlanzy et al, 1982; Stow et al, 1986a). The mature virions are proposed to leave the cell via the golgi apparatus in a similar manner to secreted soluble proteins (Johnson & Spear, 1982).

5. HSV LATENCY

Latency involves the maintenance of the viral genome in specific tissues without evidence of replication, virus gene expression or clinical disease while retaining the capacity to reactivate and yield infectious progeny (Stevens & Cook, 1971; McLennan & Darby, 1980). HSV like other herpesviruses has this ability and was first postulated to establish latent infections in nervous tissue in 1929 by Goodpasture. It is now widely documented that HSV establishes latent infections: HSV-1 primarily in the sensory dorsal root, trigeminal and thoracic ganglia and HSV-2 primarily in the sacral ganglia (Klein, 1982; Knox et al, 1982; Hill, 1985). In addition virus can be harboured latently at the site of infection (Hill et al, 1980; Al-Saadi et al, 1983; Cook et al, 1987), but the significance of this is unclear.

Two different models have been used to further the understanding of HSV latency. These are the animal models (using mice, guinea pigs and rabbits) and tissue culture models which have lead to the elucidation of the following points. This work has been reviewed elsewhere (Roizman & Sears, 1987; Stevens, 1989) and therefore will only be briefly discussed.

Latency can be established within 24 hours of infection (Steiner et al, 1990), and is thought to spread by retrograde axonal transport within the axon (Cook & Stevens, 1973). In the latent state the viral DNA is thought to be endless (Rock & Fraser, 1983; Efstathio et al, 1986) primarily in an extrachromosomal circularized or concatemerized form (Mellerick & Fraser, 1987). The viral DNA is present at between 0.1-0.01 copies per neuron (Harris & Preston, 1991) and is associated with nucleosomes in a chromatin structure (Deshmane & Fraser, 1989).

Viral replication does not appear to be necessary for the establishment of latency in the mouse eye model (Steiner et al, 1990) but is claimed to be required at the periphery (Fields et al, 1979) with very little or no viral gene expression being required for the establishment of latency (Russell et al, 1987a; Steiner et al, 1990; Wilcox et al, 1990).

In the latently infected cell two major viral gene transcripts termed LAT of around 2.2Kb and 1.5Kb are seen (Spivack & Fraser, 1987; Wagner et al, 1988), however a longer 8.3Kb transcript has also been detected (Wagner et al, 1988). Virus mutants lacking these LATs can establish and maintain latent infections normally and reactivate (Lieb et al, 1989; Sedarati et al, 1989; Steiner et al, 1989; Stevens, 1989) suggesting that the LATs are not essential for these functions. However, in some studies LAT mutants reactivate with normal kinetics (Block et al, 1990), but in others the frequency and reactivation is delayed (Leib et al, 1989; Trousdale et al, 1991). The significance of LATs is therefore still not clear.

6. HSV NEUROVIRULENCE

The neurovirulent nature of HSV has been investigated using a range of HSV mutants and variants. This has allowed the identification of regions of the HSV genome involved with neurovirulence, and subsequently the gene and its product implicated with neurovirulence.

In attempts to identify the viral gene function associated with neurovirulence Efstathiou et al (1989) showed that a thymidine kinase negative variant of HSV which failed to replicate in the mouse neural tissue established latency poorly and reactivated only following superinfection with wt virus. Using HSV-1 ts mutants Cameron et al, (1988) implicated both sub-units of the viral ribonucleotide reductase in neuro-pathogenicity. A further HSV-1 variant with a mutation in Vmw65, a transactivating factor, has its ability to grow in tissue culture severely impaired and is avirulent following intracranial inoculation (Ace et al, 1989). In contrast however, this Vmw 65 mutant in the mouse trigeminal ganglia system can establish latency and will reactivate as normal following explantation (Steiner et al, 1990).

A neurovirulent locus has now been isolated by Brown and co-workers using the variant JH2604 of HSV-2 strain HG52 (Harland & Brown, 1985). This virus has an LD_{50} of $>10^7$ pfu/mouse (Taha et al, 1988) and is mutated in the long repeat region (TR_L/IR_L) of the genome (Taha et al, 1989a). JH2604 has a 1488bp deletion in the TR_L and IR_L regions (Taha et al, 1989b) and fails to replicate in the mouse brain (Taha et al, 1990), indicating that this region has a specific role in neural replication of HSV.

The deletion of the homologous region in HSV-1 has also recently been shown to abolish neurovirulence (Chou et al, 1990; MacLean et al, 1991). A gene encoding a 43K polypeptide has been identified within this region and designated ICP34.5 (Ackermann et al, 1986; Chou & Roizman, 1986, 1990). This is located in the inverted repeats flanking the long unique region and is therefore present at two copies/genome.

Using recombinant viruses encoding ICP34.5 Chou et al (1990), have shown that the ICP34.5 gene product extends the host range of the virus and enables it to replicate and destroy brain cells. This gene product in both HSV type 1 and 2 has been analyzed by McGeoch & Barnett (1991), who have found the ICP34.5 equivalent in the two viruses to be 83% identical at the residue level, and have identified a polypeptide in mouse, MyD116, which contains a homologue of a 63 residue domain present in the viral polypeptide. This polypeptide has been suggested to act as a transcription factor or modifier of cytoskeletal structure (McGeoch & Barnett, 1991).

SECTION 3. VIRUS/HOST CELL INTERACTIONS

1. HOST IMMUNOLOGICAL RESPONSE

HSV infection induces both the acquired immunity and innate immunity of the host (reviewed by Nash & Wildy, 1983). Acquired immunity is specific for a given antigen and consists of the T and B cell mediated responses. Innate immunity is non-specific and is comprised of a variety of factors, e g macrophages, natural killer (NK) cells and interferon (reviewed by Benjamini & Leskowitz, 1991). The two arms of the immune system act to give maximum immunological protection of the host, with the outcome of HSV infection being a result of complex interactions between both acquired and innate immunity.

The first line of defence in the host following primary HSV infection is generally provided by the innate immune response. However, in animal models the relative contribution of the two arms can vary depending on the route of inoculation.

Viral invasion of the host stimulates cells of the monocyte/macrophage cell lineage can act in a non-specific manner, engulfing and digesting virus- an action which may prevent viral replication (discussed by Whitley, 1990).

Natural killer cells induced by HSV infection have been shown to play an important role in resistance to HSV infection (Lopez, 1981; Rager-Zisman et al, 1987). The NK cells recognise the Fc domain of antibodies and components of the complement system which are bound to virus proteins. The NK cells then destroy antibody or complement coated virus-infected cells by secretion of a variety of cytokines such as interleukin 1, interleukin 2, interferon and B cell growth factor, rather than by phagocytosis.

Experiments by Habu et al, (1984) and Bukowski & Welsh, (1986) showed that resistance to HSV-1 can correlate with early interferon production. This work agrees with that of Gresser et al (1976), where the neutralization of interferon with anti-interferon antiserum in vivo resulted in increased viral replication and cell death.

HSV stimulation of the B cell mediated response of the acquired immune system results in the secretion of virus-specific antibodies (Martin & Rouse, 1987). These antibodies react with viral proteins either on the virus itself or on virus infected cells with a number of consequences: for example, by coating the surface of the virus particle which then allows the non-specific phagocytes to attack more efficiently and destroy it. The antibodies which are secreted initially are IgM antibodies. Two to six weeks following the primary infection,

production of IgA and the more specific IgG antibodies occurs and the IgM secretion decreases. Such antibodies are found to be directed against a number of surface HSV glycoproteins including gB, gC, gD, gE, gG or gH. These antibodies play an important role in defence against HSV infection as they are neutralizing antibodies. Other antibodies have also been found to be directed against the non-surface polypeptides RR and Vmw175 (or ICP4).

Four different forms of T cell response exist and are summarized below (Benjamini & Leskowitz, 1991). All T cells express specific surface receptors which can interact with the major histocompatibility complexes (MHC) and processed antigen on target cells. Cytotoxic T cells come in contact with such cells resulting in their death. T helper cells act to increase the expression of soluble antibody from B cells by secreting lymphokines. The third class of T cells are delayed type hypersensitivity (DTH) T cells. These produce an inflammatory response by secreting lymphokines which in turn activate monocytes and macrophages. The final form of T cell, the suppressor T cell, acts to modulate the immune response so preventing excess tissue damage.

Secondary infection by HSV is in general unlikely to succeed. This is due to the high anti-HSV antibody and T cell levels in the host induced by the primary infection.

Latent HSV infection, as discussed in Section 5, enables the virus to lie dormant in the sensory nerve ganglia of the host, where no viral expression is thought to occur. The immune system is therefore unable to detect the presence of the virus. If however the virus reactivates, it travels down the nerve and replicates. The immune system is then able to attack the virus. Some virus may escape the immune surveillance, the consequence of which, is manifested in the formation of lesions at the primary site of infection.

In the immunosuppressed host such as AIDS, cancer or transplant patients, HSV infections are much more severe due to the impaired ability of the host cell immune response (Bustamante & Wade, 1991). Reactivation of the virus can occur at multiple sites in such patients with the healing process taking over six weeks.

2 HSV INDUCED HOST CELL SHUTOFF

HSV-1 and HSV-2 infection of culture cells results in the rapid inhibition of both host cell DNA synthesis (Roizman & Roane, 1964) and the majority of RNA and protein synthesis (Roizman et al, 1965). This phenomenon has been named host shutoff and is one of the earliest events in lytic infection (Nishioka & Silverstein, 1977, 1978; Fenwick & Walker, 1978; Fenwick & Clark, 1982; Hill et

al, 1983; Read & Frenkel, 1983; Fenwick & McMenamin, 1984; Schele & Bachenheimer, 1985). This can be divided into two categories, primary or virion associated host shutoff and delayed or secondary expression-dependent host shutoff (Fenwick & Clark, 1982). Virion associated shutoff results in the disaggregation of cellular polyribosomes (Sydiskis & Roizman 1966, 1967) and degradation of host mRNA (Schele & Bachenheimer, 1985). A mutant virus in virion associated host shutoff, *vhs*1, (Read & Frenkel, 1983) allowed the host shutoff function to be mapped to the UL41 gene (Kwong et al, 1988). The UL41 gene product is predicted to encode a 489 amino acid protein of 58K (Frink et al, 1981; McGeoch et al, 1988) which is a non-essential structural component of the virus (Fenwick & Everett, 1990) and acts in the absence of viral gene expression (Fenwick & Walker, 1978; Nishioka & Silverstein, 1978). The host shutoff function has been proposed to cause a structural change in mRNA making it non-functional (Fenwick & McMenamin, 1984). This function has also been proposed to be important in the regulation of viral mRNA in addition to host mRNA (Oroskar & Read, 1989).

HSV-2 is generally more efficient at shutting off host cell synthesis than HSV-1 (Schele & Bachenheimer, 1985) and degrades RNA more quickly than HSV-1. The efficiency of host shutoff of the virus is also seen to vary between strains of HSV. Comparison of the UL41 gene of different viruses reveal sequence differences, with the HSV-2 strain HG52 being reported to be deficient in host shutoff (Everett & Fenwick, 1990).

Not all cellular genes are shutoff, but some continue to be expressed at their normal levels or are even upregulated. This will be discussed in the next section.

3 HSV ACTIVATION OF CELLULAR GENE EXPRESSION

Despite the well documented ability of HSV lytic infection to result in the shutoff of host protein synthesis, some cellular proteins escape this fate. A similar activation of a small number of cellular genes is seen to occur with several other viruses including adenovirus, Newcastle disease virus, polyoma and SV40. Induction of stress response proteins (or heat shock) has been reported for HSV (Notarianni & Preston, 1982; La Thangue et al, 1984; Kennedy et al, 1985; Yura et al, 1987) although in some situations as elevated temperatures were used in the experimental systems, it is difficult to establish if the induction of stress proteins is due to HSV infection or the elevated temperatures (Yura et al, 1987). Stress proteins have also been reported to be induced following

infection with Newcastle disease virus (Collins & Hightower, 1982), adenovirus (Nevins, 1982), SV40 and polyoma virus (Khandijian & Turler, 1983).

Stress proteins are not the only cellular proteins which escape the shutoff function of HSV lytic infection. Histone H3 mRNA transcript levels remain unchanged following infection (Mayman & Nishioka, 1985) as do the small nuclear RNAs, U-snRNA, which remain at a constant level for up to 36 hours post infection (Bachmann et al, 1986). In both these cases however, the presence of mRNA following infection is due to the stability of the mRNA as opposed to transcriptional activation. Deadenylation of cellular mRNA occurs in the vast majority of transcripts resulting in destabilization of the mRNA (Silverstein et al, 1973), however, the above RNAs appear to avoid this fate.

Investigation of cellular gene induction by differential screening analysis of RNA levels has lead to the isolation of cDNA clones, whose expression is either maintained at the same level or increased after HSV infection (Patel et al, 1986; Kemp et al, 1986a, 1986b). Increased levels of c-fos transcripts have also been reported one hour post infection with HSV-2 (Goswami, 1987).

Some cellular proteins are seen to be increased by up to 15 fold following HSV infection, including the small RNA binding proteins, Sm, RNP, Ro and La (Bachmann et al, 1986). Studies on increased ubiquitin B gene expression indicate that HSV induction of cellular genes occurs in a specific manner, as neither the ubiquitin A nor C genes are expressed at increased levels following HSV infection (Kemp & Latchman, 1988b). Other studies include co-transfection experiments involving HSV IE genes and the rabbit beta-globin gene. These resulted in the increased expression of the beta globin in transfection studies, however when the beta-globin gene was in its normal chromosomal location, the increase in gene expression could not be repeated (Everett & Dunlop, 1984). Despite this, these experiments suggested a role for IE proteins Vmw110 and Vmw175 in the transactivation of cellular genes.

Following the identification of a group of polypeptides which were increased after HSV infection, similar proteins were seen to be present in HSV transformed cells (Suh et al, 1980; Suh, 1982; Macnab et al, 1985a; La Thangue & Latchman, 1988; Hewitt, 1988; Hewitt et al, 1991). This group includes polypeptides of molecular weights 90,000 (a doublet) 40,000 and 32,000 which can be recognised by antiserum raised against HSV infected cells, by a monoclonal antibody TG7A, raised against HSV infected cell DNA binding proteins and by serum taken from tumour bearing animals named TBS (Macnab et al, 1985a). The 90,000 molecular weight polypeptide named U90 (Hewitt, 1988; Hewitt et al, 1991) and the 40,000 molecular weight polypeptides have been

studied in more detail (Hewitt, 1988; Hewitt et al, 1991; Grassie et al, 1993 and this thesis). The studies on the U90 and its induction form the basis of this thesis and shall be described in the results and discussion sections.

Transfection experiments have been used to investigate if Vmw110 and Vmw175 were involved in such cellular gene induction (Everett & Dunlop, 1984). Vmw175 has also been implicated in the induction of ubiquitin B (Kemp & Latchman, 1988b) and is either directly or indirectly involved with the transcriptional activation of a number of mRNAs (Kemp & Latchman, 1988a). AP-1 DNA binding activity has been reported to be induced by HSV infection, with Vmw110 being essential for this induction (Jang et al, 1991). It is claimed that HSV infection induces c-jun transcription and AP-1 dependent promoters (Jang et al, 1991), however it is interesting to note that the induction of c-fos which was previously reported by Goswami in 1987 was not investigated in the study of Jang and co-workers.

Another study indicated that a minority of cellular genes induced by HSV infection could occur without any viral protein synthesis (Kemp et al, 1986a). Later, Vmw65 the virion protein and transducing factor, was implicated with U3 Sn-RNA transactivation by specifically interacting with the octamer binding motif (Kemp & Latchman, 1988c). Vmw63 has also been shown to be required for the accumulation of a 40,000 molecular weight polypeptide (Estridge et al, 1989), Vmw68 required for repression of some transcripts (Kemp & Latchman, 1988a) and as indicated previously Vmw110 has been reported to be required for the upregulation of an AP-1 binding activity (Jang et al, 1991).

No one virus encoded protein has been consistently seen to be implicated with cellular gene induction, however, Vmw110 and Vmw175 have been reported on more than one occasion to be required for cellular gene induction. The properties of Vmw175 and its effect on cellular polypeptides are outlined in this chapter, Section 2:4(b) and the induction of U90 in particular will be discussed further in this thesis.

SECTION 4 : TUMOUR ANTIGENS

This section will discuss tumour antigens, their presence, the potential mechanisms by which they become immunogenic to the host and their possible function. Although tumour antigens have been given a number of names including tumour specific polypeptides, tumour specific transplantation antigens and tumour rejection antigens, I shall use the term tumour antigen, as this term encompasses all of the above forms. Generally these other names have been acquired as a consequence of the circumstances under which a particular tumour antigen was first identified.

Tumour antigens may be molecules with a causative role in oncogenesis or may represent a difference in the metabolism of the normal and the tumour cell. Such tumour antigens have been defined as "chemical moieties associated with a tumour in an animal that can elicit an immune response in the host" (Levine, 1982). The tumour antigen may reside on the tumour cell surface and as such, can be associated with tumour rejection. Alternatively, it may be located within the cell where it can be processed to generate peptides fragments which may then be presented to the immune response, the result of which can be cell death or tissue necrosis.

By characterizing the structure and the immunogenicity of such tumour antigens, progress in the field of tumour immunology may be made. To do this the immunological relationship between the tumour and the host must be understood. Using this knowledge, the host immune response to tumours could be utilized for the diagnosis, prophylaxis and immunotherapy of tumours.

1 THE HISTORY OF TUMOUR ANTIGENS

The immunogenic nature of tumours was first reported by Gros (1943) who transplanted a sarcoma into a genetically identical, histocompatible mouse. The transplanted tumour cells produced nodules which subsequently regressed. However, transplantation of a second sarcoma to the same mouse resulted in no tumour formation. From these experiments it was concluded that this was a result of the mouse becoming immunologically resistant to the transplanted tumour cells. A series of studies of this nature have been carried out using chemically induced tumours (Foley, 1953; Baldwin, 1955; Prehn & Main, 1957; Klein et al, 1960; Old et al, 1962). From these studies it became clear that the mice were only immune to the tumour to which they had been exposed, and not to any other tumour, even if the tumour was induced by the same chemical carcinogen (Prehn & Main, 1957).

As indicated above, chemically induced tumours have little or no cross reactivity. This is most probably explained by the random nature of the mutations which arise from treatment with carcinogens. Conversely tumours induced by oncogenic viruses do exhibit cross-reactivity. This is most likely because infection with a tumour virus induces the same antigen(s), eg a virus encoded protein, on each tumour regardless of tissue type or host species (Benjamini & Leskowitz, 1991).

2. A DESCRIPTION OF SOME TUMOUR ANTIGENS

A number of tumour antigens have been identified which can be classified into two basic groups. These are essentially tumour antigens which are host cell encoded but have been altered in some manner such that they become immunogenic to the host, or alternatively tumour antigens coded by an infectious agent which have been introduced into the host, for example, by virus infection.

A number of virus-encoded tumour antigens have been identified including SV40 large and small tumour antigens, which generate antibodies in hamsters bearing tumours induced by SV40 (Tevethia et al, 1980) and E7 of papillomavirus (Chen et al, 1991). In the case of SV40 large T antigen, about 10% of the protein is expressed at the cell surface where it plays a role in host tumour rejection. The SV40 large T antigen is therefore termed a tumour specific transplantation antigen or TSTA. Esptein-Barr virus also expresses tumour antigens known as Epstein Barr virus nuclear antigens (EBNAs), of which there are six. Such tumour antigens expressed as a consequence of infection with a tumour virus, show extensive cross-reactivity with tumours induced in other hosts by the same virus.

The second group of tumour antigens include a large number of host polypeptides and glycoproteins. These can generate an antigenic response in the host for any one of a number of reasons which are outlined below. Foetal tumour antigens (Old, 1981) such as the carcinoembryonic antigen (CEA) (reviewed by Thomas et al, 1990) and alpha foetoprotein (AFP), (reviewed by Deutsch, 1991), are normally only expressed in foetal tissue. If however, these proteins are expressed in the adult cell they are recognised as foreign, as they have not previously been presented to the immune system, and consequently the immune response generates antibodies against them. AFP was first seen in foetal sera of humans, then found in animals with graft-induced and spontaneous hepatomas, and later in human patients with hepatomas and various other malignancies (Deutsch, 1991). CEA was first described in 1965 and

subsequently used as a tumour marker for detecting and monitoring patients with colorectal cancer (reviewed by Thomas et al, 1990). However, as more sensitive techniques of detection were developed, it was discovered that CEA was in fact expressed in low levels in normal tissues and was expressed at elevated levels in patients with benign disease. As a result of this discovery Coggins (1986) redefined these foetal antigens as differentiation antigens.

Despite the presence of such differentiation antigens in normal cells, CEA is still considered to be the best tumour marker for monitoring colorectal cancer. The discovery that CEA was not purely tumour specific initiated renewed efforts to identify highly specific tumour markers.

Another group of tumour antigens is thought to become immunogenic to the host by the alteration of normal cell surface molecules such that a new antigenic determinant is presented to the host. An example of such a tumour antigen can be seen in some adenocarcinomas, where glycolipids can lack the terminal glucosamine residue which is present in normal ganglioside cells (discussed in Levine, 1982). The resulting glycolipid is presented to the host in a different manner in an adeno-carcinoma cell compared to the normal cell. Alteration of the glycosylation of the surface glycoprotein has also been well documented as a method of generating tumour antigens. A number of altered normal cell glycoproteins have been identified in gangliosides, melanomas and neuroblastomas, for example GD2, GD3, GM2 and GM3, (reviewed by Hakamori, 1989).

Other mechanisms by which normal cell proteins can become tumour antigens include mutation, an increase in the protein concentration or by complexing with foreign antigens, resulting in a "self" protein being presented to the immune system in a different form. The tumour suppressor protein p53 has been documented to act in all of the ways described above: antibodies targeted against p53 have been seen where tumour cells have increased levels of p53 and the p53 protein has been seen to complex with viral tumour antigens resulting in its presentation to the immune system in an altered form (Levine, 1990; Werness et al, 1990; Levine et al, 1991).

A single point mutation is seen to generate cytotoxic T cell specificity in the tum$^-$ tumour antigen, P91A, described in a mouse tumour model (Lurquin et al, 1989). A single mutation in exon 4 of P91A (DePlean et al, 1988) in mouse mastocytoma cells results in tumour rejection when these cells are injected into mice. It has also been proposed that the tumour antigen FD and a homologous GP95/97 tumour antigen which are present in human melanoma cells may become antigenic to the host as a consequence of point mutations (reviewed by

Thurin, 1990). This however, has still to be clarified by sequence comparison of the normal and tumour cell proteins.

3. THE POSSIBLE FUNCTION OF TUMOUR ANTIGENS

The tumour antigen GP96 has been studied in some detail and its potential function investigated (reviewed by Srivastava & Maki, 1991). GP96 is expressed on methylcholanthrene induced sarcomas (Meth A, CMS5 and CMS13), (Srivastava et al, 1986; Palladino et al, 1987; Srivastava & Old, 1988) and induces T cell mediated immunity. Adoptive transfer of T lymphocytes from GP96 immunized mice to immunologicaly naive mice can result in the regression of pre-existing Meth A tumours in a tumour specific manner (Palladino et al, 1987). This suggests that GP96 acts as a tumour rejection antigen protecting the host from tumour development. GP96-like proteins can be detected in a number of normal tissues. These however, appear to be immunologically distinct from the GP96 isolated from tumours because they do not confer Meth A tumour immunity (Srivastava & Maki, 1991).

GP96 has considerable homology with stress proteins, in particular with the GRP94 family (Maki et al, 1990). Stress proteins have been proposed to be associated with transformation (Nevins, 1982; Khandijian & Turler, 1983) and have now been proposed to be tumour specific antigens (Kaufmann, 1990; Srivastava & Maki, 1991). Work by Bensuade & Morange (1983), agrees with this proposal as they have shown the presence of heat shock proteins in embryonal carcinoma cells in the absence of stress normally required for the induction of such stress proteins (reviewed by Pelham, 1986; Tanaka et al, 1988).

It has also been proposed that instead of acting as tumour rejection antigens (by the normal stress protein becoming altered), stress proteins may participate in the processing and presentation of tumour specific epitopes from other sources (Kaufmann, 1990; Srivastava & Maki, 1991). If this were the case this would account for the limited number of stress proteins, but the great antigenic polymorphism present on chemically induced tumours. As a consequence of these properties, stress protein have been likened to MHC molecules because of their ability to bind to a number of different molecules and small peptides with specificity and high affinity (reviewed by Srivastava & Maki, 1991).

4. U90, A TUMOUR ANTIGEN

The U90 polypeptide which will be investigated in this thesis fulfils the criteria of a tumour antigen, as it could not be detected in normal cells, but was

seen to be expressed on the surface of tumour cells and elicits an immune response in the host (Macnab, 1979). The U90 has also been shown to be expressed in primary rat embryo cells generated from embryos of up to 14 days (Macnab, unpublished results), suggesting it could be a foetal or differentiation tumour antigen. The characterization of the U90 may therefore help to elucidate how a normal cell polypeptide could become a tumour antigen.

CHAPTER 2 : MATERIALS

1. CELLS

(a) RE fibroblast cells were prepared from 16-17 days sibling embryos of Hooded Lister rats taken from an inbred colony of rats maintained within the Institute. These were used mainly as secondary cultures.

(b) Bn5T cell line was derived from an in vitro culture of a tumour induced by injection of Bn5 cells, which are RE cells transformed by the cloned *Bgl* IIn fragment of HSV-2, strain HG52 (Cameron et al, 1985; Macnab et al, 1985).

(c) BHK-21 clone 13, a fibroblastic cell line derived from baby hamster kidney cells (Macpherson & Stoker, 1962) was used to grow and titrate virus.

(d) 12-2, a quasi-epithelial mouse cell line was transformed by HPV 16 and c-fos (Crook et al, 1988). These cells were kindly donated by Dr Lionel Crawford (ICRF, London).

(e) Human embryonic lung cells (Helu) were maintained within the Institute but were intially prepared by Dr JCM Macnab.

(f) Human Jurkat cells, a lymphoid cell line grown in suspension was kindly donated by Dr John Moore. (Glasgow Vet School).

(g) MCF-7 cells, a human breast carcinoma cell line derived from pleural effusion of a breast tumour (Soule et al, 1973), originally obtained from ICRF, London and the Department of Biochemistry, University of Liverpool.

(h) PC12, a rat pheochromocytoma cell line (Sheng et al, 1988), was obtained from Dr T McKee, Institute of Virology, Glasgow.

2. CELL CULTURE MEDIUM AND SOLUTIONS

Unless otherwise stated cells were grown and maintained in Dulbecco's minimal essential medium, supplemented with 1% v/v non-essential amino acids, 100units/ml penicillin and 100ug/ml streptomycin, known hereafter as supplemented DMEM, plus 5% newborn calf serum (NCS). BHK C13 cells were grown in EC10, which is Eagles minimal essential medium supplemented with 100units/ml penicillin, 100ug/ml streptomycin, 7.5% Na bicarbonate, 5% tryptose phosphate (TP) broth and 10% NCS. PC12 cells were grown in supplemented DMEM plus 10% heat inactivated horse serum + 5% NCS, 12-2

cells grown in supplemeted DMEM plus 1μM dexamethasone and 5% NCS and Helu cells grown in supplemented DMEM plus 5% foetal calf serum. All components were supplied by Gibco Ltd, Scotland with the exception of TP which was supplied by Difco Laboratories (England) and was prepared in the Media department within the Institute of Virology.

Eagles medium without methionine, and Eagles lacking methionine and glucose were also prepared in the media department in addition to the following:

Versene - 0.6mM EDTA in PBS containing 0.002% (w/v) phenol red.

Trypsin - 0.25% (w/v) solution of trypsin (supplied by Difco Laboratories, England) in tris-saline.

3. VIRUS

Virus stocks were supplied by Mrs Mary Murphy (Institute of Virology, Glasgow). Virus strains used included HSV-2 strain HG52 (Timbury, 1971), HSV-1 strain 17^+ (Brown et al , 1973), HSV-1 tsD, HSV-1 tsK and HSV-1 tsT, temperature sensitive mutants of HSV-1 strain 17, the mutations being within the gene encoding Vmw175. Further HSV mutants in the IE genes were used including virus mutated in IE12, named HG52X163X12 or 2607, (Brown & Harland, 1987), IE63, named 1703 or XD2 (MacLean & Brown, 1987), IE110, named dl1405 (Stow & Stow, 1986), IE136, named ts1207 (Preston et al, 1984) and IE175, named in1411 (Russell et al, 1987b).

4. CHEMICALS

Analytical grade reagents were obtained from BDH Chemicals Ltd, England with the exception of; 3 cyclohexylamino-1-propanesulphonic acid (CAPS), (Aldrich Chemical Company Inc., England), Problott membrane, trifluoroacetic acid, (TFA), (Applied Biosystems, California), ammonium persulphate (APS), N,N'-diallyltartardiamide (DATD), Coomassie brilliant blue R-250, gelatin (E1A grade), horse radish peroxidase colour development reagent, N,N,N',N'-tetramethylethylenediamine (TEMED), FPLC grade acrylamide, glycine, SDS, tricine and tris (BioRad Laboratories, California), complete Freund's adjuvant, tryptose phosphate (Difco Laboratories, USA), acetic acid (glacial), chloroform, hydrochloric acid, methanol, (May & Baker Ltd., England), Immobilon-P transfer membrane, (Millipore Coporation, USA),

Ecoscint A scintillation fluid (National Diagnostics, New Jersey), PCR grade ultra pure set of dNTP, (Pharmacia LKB Biotechnology, England), triton X-100, 10% solution (Pierce, Illinois, USA), HPLC grade acetonitrile (Rathburn Chemicals Ltd., Scotland), nitrocellulose (Schleicher & Schuell, West Germany), actinomycin D, cycloheximide, calcium ionophore A23187, pepsin, phenylmethylsulphonyl fluoride (PMSF), tunicamycin, (Sigma Chemical Company, England).

5. ENZYMES

Enzymes were obtained from the following sources:
Ampli Taq DNA polymerase 5units/ul, (Perkin Elmer Cetus, USA) AMV reverse transcriptase 20 units/ul, SA 21,600 units/mg (Biolabs, New England), proteinase K (Boehringer Mannheim) ribonuclease inhibitor (human placenta) 35 units/ul (Amersham International), *Staph. aureus* V8 protease 500units/mg (Sigma), T4 DNA ligase 3 units/ul and T4 polynucleotide kinase 2 units/ul (Boehringer Mannheim).

6. RADIOACTIVE COMPOUNDS

[^{35}S] L-methionine SA 800 Ci/mmol, [γ-32P] adenosine triphosphate 5,000Ci/mmol, [C^{14}] methylated proteins and Rainbow protein markers both 14,300-200,000 and 2,350-46,000 mol wt, each 5μCi/ml, all Amersham International Plc., England.

7. IMMUNOLOGICAL REAGENTS

Pansorbin (Calbiochem, California), Mab PR-6, a rat monoclonal antibody against GRP94 (Estes et al, 1987) - a gift from Prof D Edwards (Department of Pathology, University of Colorado, Colorado), anti-GRP94 raised against the first 20 amino acids of the amino terminus of GRP94 - a gift from Drs Ullrich and Appella (NIH, Bethesda, Maryland), anti-endoplasm antibody, a mono-specific rabbit antiserum against GRP94 (Koch et al, 1986; Booth & Koch, 1989) - a gift from Dr Gordon Koch (MRC Laboratories of Molecular Biology, Cambridge).

CHAPTER 3 : METHODS

1. CELL CULTURE

1(a) PREPARATION OF PRIMARY RAT EMBRYO CELLS

Primary rat embryo cells were prepared from 16-17 day old embryos from an inbred colony of Hooded Lister rats which were maintained by brother sister mating over 20 years in the Institute. The colony has no history of spontaneous tumours (Macnab, 1979). Embryos from a single litter were removed from the uterus of a pregnant rat, washed with versene, eviserated, washed again with versene and finely minced. A single cell suspension was generated by treating the cells with 0.25% trypsin for 30 minutes (37°C), the supernatant removed and the remaining cell clumps treated with trypsin for a further 30 minutes. Newborn calf serum (NCS) was added (to 5% v/v) to stop the action of the trypsin, and the supernatants centrifuged at 1,000rpm for 10 minutes (4°C) in order to collect the cells. The resulting cell pellet was resuspended in Dulbecco's + 5% NCS and seeded at $2x10^8$ cells per $850cm^2$ burrler. Once sub-confluent the cells were maintained in Dulbecco's + 2% NCS at 31° and fed weekly for up to 4 weeks.

1(b) CELL LINE MAINTENANCE

Bn5T cells and all other cells used (except those stated below) were maintained in $850cm^2$ plastic burrlers at 37°C in Dulbecco's + 5% NCS + 5% CO_2. Once confluent these cells were split 1:5. BHK C13 cells were maintained in EC10 + 5% CO_2 in the same manner as Bn5T cells, but were split 1:10. Helu cells were treated as the Bn5T cells, but were grown in DMEM + 10% FCS in $175cm^2$ flasks + 5% CO_2 and split 1:2 when confluent. 12-2 cells were grown in DMEM + 5% NCS + 1μM dexamethasone + 5% CO_2 in $175cm^2$ flasks, and split. 1:2. PC12 cells were grown in DMEM + 10% heat inactivated horse serum + 5% NCS + 5% CO_2.

In all cases the cells were fed every 3 days and cells were split by washing twice with versene then once with trypsin/versene (1:4).

1(c) CELL HARVESTING

Cells were harvested after removing the culture medium by washing with PBS, then scraping with a rubber policeman. The cells were pelleted at 2,000rpm for 10 minutes (4°C), the supernatant removed and the pellet stored.

1(d) CELL STORAGE

For storage, actively growing cells were trypsinized and centrifuged (1,000rpm for 10 minutes at 4°C), then the medium decanted. The cells were then resuspended in 1ml of medium containing twice the normal serum content + 10% DMSO (v/v). The cells were frozen slowly overnight at -70°C, then transferred to liqiud nitrogen (-140°C). To recover the cells they were thawed quickly and put in $25cm^2$ flasks with the appropriate medium. The medium was changed the following day to remove any dead cells and DMSO.

2. VIRUS

2(a) VIRUS STOCK PRODUCTION

Stocks of HSV-1 and HSV-2 were produced by infection of 10 and 20 burrlers respectively of sub-confluent BHK C13 cells at a multiplicity of 1pfu/300 cells. This was done in a total volume of 40mls and 20mls respectively at 31°C for 4-7 days until the cells were rounded and falling off the plastic.

HSV-1 was harvested by shaking the cells off the plastic, collecting the cells and medium, centrifuging at 2,000rpm for 10 minutes (4°C) in 250ml MSE glass bottles. The pellet was resuspended in 5ml of supernatant to generate the cell associated stock. This was checked for sterility and stored at -70°C (stage 1). The supernatant was then centrifuged at 12,000rpm for 2 hours (4°C) in 250ml plastic Sorvall bottles following which the pellet was resuspended in 5ml of the supernatant to generate the supernatant stock. This was then checked for sterility and frozen.

Sterility checks were done by streaking virus on blood agar plates which were incubated inverted, at 37°C for 5 days. The supernatant stock was sonicated once before use, whereas the cell associated stock was sonicated, freeze/ thawed, centrifuged at 2,000rpm then resuspended in 5ml of medium. This procedure was completed twice before use or aliquoting and freezing at -70°C until required.

HSV-2 stocks were treated as HSV-1 to stage 1, then sonicated, centrifuged at 2,000rpm for 10 minutes (4°C) and resuspended in 5ml of medium. This extraction was repeated twice before aliquoting the virus and freezing at -70°C.

HSV-1 mutant stocks of tsK, tsD and tsT were prepared in the same manner as HSV-1.

2(b) TITRATION OF VIRUS STOCKS

Serial ten fold dilutions of virus stocks were carried out in EC10 with 200ul of the required dilutions being absorbed onto 50mm plates of sub-confluent BHK C13 cells for 1 hour at 37°C (31°C for ts mutants), overlaid with 50% EC10/50% methyl cellulose to prevent secondary virus plaques forming, and incubated at 37°C or 31°C for 2 or 3 days respectively until plaques were visible. The overlay medium was removed and the cells stained with Giemsa stain for 15 minutes. This was gently washed off with water, the plaques on each plate counted and the virus titre calculated.

3. ANTISERA

3(a) PREPARATION OF TUMOUR BEARING ANTISERA (TBS)

TBS was produced by subcutaneous injection of 8 week old Hooded Lister rats with $1x10^7$ Bn5T cells. Tumours formed within 1-2 months at which stage the rats were anaesthetized and blood taken by cardiac puncture. The blood was kept at 4°C for 24 hours to allow it to clot, centrifuged at 2,000rpm for 10 minutes (4°C) and the serum supernatant decanted, aliquoted and frozen at -20°C.

3(b) PREPARATION OF MONOSPECIFIC ANTIBODY AGAINST U90

Using the purification strategy for the U90 polypeptide which I devised for this project (which is described in chapter 4 section 4) the U90 was purified from HSV-2 infected Bn5T cells by Mr David McNab, electroeluted from polyacrylamide gel slices and approximately 2ug of U90 was emulsified in Freund's complete adjuvant and injected into a rabbit on three occasions at 14 day intervals. Following the second boost, blood from the rabbit was tested by Dr Joan Macnab by immunoprecipitation reactions and by western

blotting. The antiserum was found to be positive in western blots for activity against the U90.

4. METABOLIC RADIOLABELLING OF POLYPEPTIDES

4(a) RADIOLABELLING WITH [^{35}S] L-METHIONINE

Cellular polypeptides were metabolically radiolabelled with [^{35}S] L-methionine in vitro by growing the cells in medium lacking methionine for at least 1 hour prior to the addition of 50uCi of [^{35}S] L-methionine/ml of medium. Radiolabelling was carried out for 17 hours unless otherwise stated, after which the cells were harvested as described previously (section 1c).

4(b) PULSE RADIOLABELLING

Pulse radiolabelling was carried out in PBS +5% NCS for either 2 hours (50uCi/ml of medium) or 5 minutes (2.5mCi/ml of medium). Cells in both cases were starved of methionine for at least 1 hour prior to the addition of radiolabel and harvested as described (section 1c).

4(c) PULSE/CHASE RADIOLABELLING

Pulse chase labelling was carried out as described previously for 2 hours (section 4b), however after 1 hour (unless otherwise stated) the label was removed, the cells washed with PBS A and total medium including cold methionine was added for the required chase time before cell harvesting.

5. INDUCTION AND INHIBITION OF CELLULAR POLYPEPTIDES

5(a) HSV INDUCTION OF CELLULAR POLYPEPTIDES

The medium was removed from sub-confluent monolayers of cells and the cells infected with virus at a multiplicity of 5-10pfu/cell, the virus being absorbed for 1 hour at 37°C in the minimum volume possible (200ul for a 50mm plate, 10ml for a 850cm^2 burrler). The inoculum was removed and replaced with either 2ml or 10ml of medium respectively. If the polypeptides were being radiolabelled, [^{35}S] L-methionine was added at this stage in methionine-free medium. Such infections were carried out for 17 hours unless otherwise stated. The cells were harvested by scraping with a rubber policeman, the

medium and cells pelleted at 2,000rpm for 10 minutes (4°C), the supernatant discarded and the infected cell pellet either used immediately or stored -20°C.

5(b) INDUCTION OF CELLULAR POLYPEPTIDES BY TUNICAMYCIN

Tunicamycin was added to methionine starved sub-confluent cells at a final concentration of 2ug/ml in methionine minus medium together with 50uCi/ml [^{35}S] L-methionine for 17 hours. Following this treatment the cells were harvested as normal.

5(c) INDUCTION OF CELLULAR POLYPEPTIDES BY CALCIUM IONOPHORE

Calcium ionophore A23187 was added to methionine starved, sub-confluent cells at a final concentration of $6x10^{-7}$M in the presence of 50uCi/ml of [^{35}S] L-methionine for 17 hours. Following this treatment the cells were harvested as normal.

5(d) INDUCTION OF CELLULAR POLYPEPTIDES BY GLUCOSE STARVATION

Glucose regulated proteins were induced by starving sub-confluent cells of glucose by growing in medium lacking both glucose and methionine. Following the starvation period the cells were radiolabelled for 17 hours and harvested as described previously.

5(e) CYCLOHEXIMIDE AND ACTINOMYCIN D TREATMENT OF CELLS

Cellular protein synthesis was inhibited at the level of translation by treating cells with 50ug of cycloheximide/ml of culture medium. Treatment was maintained for 17 hours, after which the cells were washed five times with cycloheximide free medium to remove any remaining traces of cycloheximide. Radiolabelling with [^{35}S] L-methionine was performed over a period of 2 hours in the presence of 5ug/ml actinomycin D in PBS plus 1% serum.

6. PROTEIN EXTRACTION

Cellular polypeptides were extracted from cell pellets using the following methods and buffers.

6(a) RIPA EXTRACTION

Radioimmunoprecipitation analysis buffer, RIPA (1% triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150mM NaCl, 10mM tris pH 7.4) modified from Docherty et al, 1981, was used to prepare total cell extracts for immunoprecipitation reactions. The harvested cell pellet was resuspended in RIPA plus protease inhibitors (0.034mg/ml PMSF, 0.03mg/ml benzamidine + 0.1mg/ml phenanthroline) and the cells sonicated until clear then left on ice for 30 minutes. The samples were then centrifuged at 12,000rpm for 10 minutes (4°C) to remove cell debris, and the supernatant stored at -70°C.

6(b) CELL FRACTIONATION

Cell fractionation was carried out with three separate buffers to generate the cytoplasmic, membrane and nuclear fractions. Freshly prepared cell pellets were resuspended in hypotonic lysis buffer (25mM tris pH 7.5, 5mM KCl, 0.5mM $MgCl_2$) plus 0.5mM PMSF + 1mM DTT, left to swell on ice for 30 minutes and dounce homogenized with 20 strokes using the B pestle (Wheaton, USA) which by the manufacturers definition shears only the cell membrane. Centrifugation at 2,500rpm for 10 minutes (4°C) generated the cytoplasmic fraction as the supernatant (Burnette & Till, 1971) and a pellet which was then treated with nuclear extraction buffer (20% glycerol, 25mM tris pH 7.5, 0.6mM KCl) plus 1mM PMSF and 1mM DTT for 30 minutes at 4°C with rotation. The sample was centrifuged at 2,500rpm for 10 min (4°C) and the supernatant stored as the nuclear fraction. The resulting pellet was then resuspended in membrane extraction buffer (1% triton X-100 in PBS), sonicated until clear, left on ice for 30 minutes, centrifuged at 12,000rpm for 10 minutes (4°C) and the supernatant retained as the membrane associated fraction.

6(c) LARGE SCALE PROTEIN EXTRACTION

The cell pellets were resuspended in hypotonic buffer plus protease inhibitors (as described in 6b) using 1ml of buffer for every $2x10^8$ cells, the cells left to swell on ice for 30 minutes, Dounce homogenized with pestle B for 20 strokes as above, centrifuged at 2,500rpm for 10 minutes (4°C) to give a pellet containing the membrane fraction and supernatant containing the cytoplasmic fraction. The membrane pellet was then resuspended in 1ml of

membrane extraction buffer (1% triton X-100, 0.5mM PMSF and 1mM DTT in PBS) per $2x10^8$ cells, sonicated until clear and left on ice for 30 minutes. This sample was centrifuged at 12,000rpm for 10 minutes (4°C) and the supernatant kept as the membrane associated fraction.

6(d) QUANTITATION OF INCORPORATED [^{35}S] L-METHIONINE

The amount of radiolabel incorporated in each sample was measured by TCA precipitation of 1ul of each sample onto a Whatman filter disc. To do this, the filters were washed twice in 5% TCA, once in acetone and dried under a heat lamp. The discs were then added to 3ml of Ecoscint A scintillation fluid and the [^{35}S] counted in a scintillation counter.

7. PROTEIN ANALYSIS

7(a) IMMUNOPRECIPITATION REACTIONS

Immunoprecipitation (IP) reactions were carried out based on the technique described by Macnab et al, (1985). A specific amount of incorporated [^{35}S] L-methionine per sample (from $4x10^6$-$4X10^7$ cpm) was used in each IP reaction, with the total volume being made up to a defined amount (either 100ul or 200ul) with extraction buffer. An appropriate volume of antibody was added to the samples for one hour with rotation (4°C), then incubated for 1 hour with 60ul of protein A sepharose (4°C). The immune complexes were pelleted by centrifugation at 12,000rpm for 2 minutes, the supernatant discarded and the pellets washed twice with the extraction buffer, then once with PBS. This was done by resuspending the pellet in the required buffer by pipetting up and down, followed by centrifugation and then discarding the supernatant. The final pellet was resuspended in boiling mix (see Table 1) and heated to 70°C for 10 minutes to release the antibody/antigen complexes from the protein A sepharose. The protein A sepharose was then remove by centrifugation at 12,000rpm for 5 minutes, and the IP reaction (supernatant) either analyzed by SDS page (section 7b) or stored at -70°C.

7(b) SDS PAGE

Sodium dodecasulphate polyacrylamide gel electrophoresis (SDS PAGE) (Laemmli, 1970) was used to separate polypeptides for analysis, using 7.5% and 18% discontinuous SDS PAGE. The lower resolving gel was crosslinked with 2.5% N-N methylene bisacrylamide and polymerized with 0.04% (w/v) APS and 0.04% (v/v) TEMED then overlaid with butanol. After polymerization the butanol was washed off the gel with water, the stacker gel added (12.5% acrylamide cross-linked with 0.76% N N diallyl-tartar-diamide DATD) and polymerized as before with a teflon comb inserted to form wells. All solutions and the relative proportions used for SDS PAGE are outlined in Table 1. Samples were loaded on the gel in boiling mix (Table 1) and gels of 220x155mm run at 70mA, 188x155mm at 35mA (with constant voltage) and the BioRad mini-gel kit (102x74) at 200V. The gels were run until the dye front reached the bottom of the gel, the gel removed from the glass plates, fixed and/or stained for 1 hour (50% methanol, 7% acetic acid +/- 0.2% Coomassie blue R-250), destained for 1 hour or as long as required in 10% methanol + 7% acetic acid, then dried under vacuum onto 3mm Whatman filter paper. If the gels contained radiolabelled proteins they were exposed to Kodak X-omat S film at -70°C for varying lengths of time.

7(c) PEPTIDE MAPPING ANALYSIS

Using a modified version of Cleveland et al, (1977) the relationship of one polypeptide to another was compared by the size and number of peptides generated following total enzymatic digestion by *Staph. aureus* V8 protease of radiolabelled polypeptides. The protein of interest was excised from a dried gel by aligning the autorad film with the gel, (by means of radioactive ink spotted around the gel), and the slice placed in rehydration buffer (0.5M tris pH 6.8, 5% SDS, 100mM EDTA + bromophenol blue to colour) for 30 minutes. The gel slice was next placed in the well of an 18% gel containing a stacking gel of double the normal height and was overlaid with rehydration buffer plus 20% glycerol. The tank buffer was added, then 5ug/track of proteolytic enzyme (in rehydration buffer + 10% glycerol) and the gel run until the dye front had reached 5mm before the resolving and stacker gel interface. At this point, the power is turned off for 1 hour to allow digestion to occur and subsequently restored to complete SDS page as normal. When the dye front reached the

TABLE 1 : GEL SOLUTIONS

TABLE 1a

	Acrylamide	DATD	Bis-acrylamide	Water
Stacking gel mix (30%)	58.44g	1.56g	-	200ml
Resolving gel mix (30%)	55g	-	5.0g	200ml

TABLE 1b

	Tris	SDS	Water	pH
Resolving gel buffer	181.5g	4.0g	1,000ml	8.9
Stacker gel buffer	59g	4.0g	1,000ml	6.7

TABLE 1c

	Tris	Glycine	Tricine	Water
Tank buffer	6.32g	4.0g	-	1,000ml
Tris-tricine buffer	6.32g	-	4.0g	1,000ml

TABLE 1d

	Acrylamide mix	Buffer	APS	TEMED	Water
Stacker gel	6ml	9ml	300ul	30ul	21ml
Resolving gel	24ml	24ml	600ul	40ul	48ml

Boiling mix : 30% stacker buffer, 30% glycerol, 12.5% SDS, 15% β mercapto-ethanol (all v/v)

Fix : 50% methanol, 7% acetic acid, (+/-) 0.2% Coomassie Blue stain in H_2O

Destain: 10% methanol,, 7% acetic acid in H_2O

bottom of the gel, the gel was treated as described previously (section 7b), but after destaining the gel, it was soaked in EN^{3}HANCE for 30 minutes, washed with several changes of water over 30 minutes, dried down as described previously and then exposed to film, initially for 2 weeks then for up to 3 months.

7(d) WESTERN BLOTTING

Following SDS-PAGE, polypeptides were eluted from gels onto various supports for different purposes. Western blotting was carried out using a modified version of Burnette (1981) and a BioRad mini transfer kit. The gel was removed from the glass plates after SDS PAGE and soaked in transfer buffer (10mM CAPS pH11, 10% methanol). The transfer sandwich was assembled in the order - sponge, 2 sheets of 3mm Whatman filter paper, nitrocellulose, gel, nitrocellulose, 2 sheets of filter paper, then sponge. The apparatus was filled with buffer and assembled so that the protein was transferred towards the anode using a constant current of 120mA for 80 minutes. Rainbow marker proteins (Amersham, England), were loaded on one track of each blotted gel, enabling the efficiency of protein transfer to be observed.

After transfer, the nitrocellulose was blocked by incubating with 3% gelatin in TBS buffer (20mM tris pH 7.5, 0.5M NaCl) for 1 hour at 37°C, washed with TBS buffer to remove the gelatin, reacted with the appropriate dilution of the 1st antibody (in TBS buffer, 0.05% tween 20 + 0.05% Na azide) for a minimum of 1 hour at 37°C, washed with TBS and incubated with the 2nd antibody (1:1000 dilution of horse radish peroxidase (HRP) conjugated to protein A for rabbit antibodies and 1:300 dilution of horse radish peroxidase conjugated to rat IgG for rat antibodies), both made up in 0.05% tween 20 in TBS buffer. The nitrocellulose plus antibody was brought to room temperature before removing the 2nd antibody and washed thoroughly with PBS with at least 4 changes of buffer in order to remove the tween 20 and Na azide (which can interfere with the colour reaction). The protein antibody complexes were then visualized by adding a colour developing reagent (60mg of HRP colour developer reagent added to 20ml of ice cold methanol, and 60µl of H_2O_2 added to 100ml of TBS buffer. These solutions were mixed just prior to use). The colour reagent was added to the nitrocellulose and shaken at room temperature until

bands could be visualized. The reaction was stopped at the required stage by removing the colour reagent with several changes of water and then the filters were air dried.

7(e) ELECTROBLOTTING PRIOR TO PROTEIN SEQUENCING

The electroblotting technique was used prior to protein sequencing to transfer protein and peptides as purified and well separated bands onto an appropriate support for N-terminal protein sequence analysis. This also allowed an approximate quantitation of the total amount of protein present on the support by eye following Coomassie blue staining (Matsudaira, 1987).

The protocol for western blotting (section 7d) was followed with the exception that Problott, a PVDF membrane was used, and only the highest grade of reagents and chemicals were used to avoid the introduction of contaminants. Before use, Problott was soaked in methanol but thereafter treated as described for nitrocellulose. After transfer the membrane was washed with water, saturated with methanol, then stained with 0.1% Coomassie blue in 40% methanol + 1% acetic acid for 30 seconds. The membrane was then destained until as much Coomassie blue as possible had been removed and the protein bands were clearly visible. The membrane was then air dried and the bands of interest carefully excised and sent for sequencing.

7(f) ELECTROELUTION

Protein was electrophoretically eluted from Coomassie blue stained gel slices onto an anisotropic membrane contained within an centricon microconcentrator tube (Amicon, UK). This procedure allows the concentration of protein and exchange of buffers in which the protein is contained (LeGendre & Matsudaira, 1990). Using electroelution apparatus, gel slices are placed in epindorf tubes with holes pierced in the top and bottom to allow contact with the transfer buffer (10mM CAPS pH11, 10% methanol). These were placed in the centricon tubes, the apparatus filled with transfer buffer and a current of 30mA applied for a minimum of 3 hours or overnight at 5mA. Care must be taken to avoid bubbles forming in the epindorf tube or below the membrane. After transfer the centricon tube was carefully removed and centrifuged in the RC5B SS34 rotor at 5,000rpm (4°C) until the dead stop volume is reached (about 30 minutes). The use of higher speeds may

result in the damage of the centricon membrane. The buffer may be changed by repeating the above procedure, and the protein isolated by inverting the tube and the electroeluted protein centrifuged from the membrane into the collecting vessel.

The centricon tube may also be used to concentrate protein in a sample by reducing the volume of the sample buffer to protein ratio. However, in doing so, some losses must be expected due to adsorption.

7(g) PROTEIN CONCENTRATION

Protein samples were concentrated using centricon-30 microconcentrator tubes (Amicon, UK) by adding the sample to the tube (maximum volume of 2ml) and spinning the centricon tube in an RC5B SS34 rotor at 5,000rpm (4°C) until the dead stop volume of approximately 200µl is reached (about 30 minutes). The use of higher speeds may result in the damage of the centricon membrane so should be avoided. Addition of a second solvent to the tube can be used to exchange buffers in which a sample is maintained in. This requires at least three washes of 2mls with the second buffer to be assured that 99.9% of the first buffer has been removed.

7(h) PROTEIN QUANTITATION

The protein content of samples was estimated using the BioRad Bradford's protein quantitation kit, which is based on the method described by Bradford, (1976). The manufacturer's instructions were followed for the standard assay procedure using bovine serum albumin as a protein standard.

7(i) DENSITOMETER SCANNING

Densitometer scanning of autoradiographs and Coomassie blue stained gels was carried out using a Shimadzu CS-9000 dual-wavelength flying spot scanning densitometer. Autoradiographs were scanned in "transmission photo mode" using a wavelength of 485nm and photographs were scanned in "reflection mode" at a wavelength of 535nm. In both circumstances zig-zag scanning was performed to enable analysis of the entire band in question using a "smoothing" value of 11. This results in 11 values for each peak being taken, with the mean of these values being given as the final figure. The

optimum wavelength to use for each condition was determined by performing a spectrum scan.

8 PROTEIN PURIFICATION

8(a) AMMONIUM SULPHATE PRECIPITATION OF PROTEIN

Saturation of a protein solution to different degrees with ammonium sulphate results in the selective precipitation or salting out of different proteins (Scopes 1987). Ammonium sulphate (0.176g/ml of sample) was added slowly over a defined period of time, which was 20 minutes, to give a 30% saturated solution, and the solution was left to stir on ice for 1 hour. During this time a proportion of the proteins precipitated out of solution and were subsequently collected by centrifugation at 10,000rpm for 15 minutes with the remaining soluble polypeptides being found in the supernatant. The protein pellets generated by centrifugation of the 30% saturated sample were collected and resuspended in the appropriate buffer (section 8b).

8(b) FPLC ANALYSIS

A Pharmacia FPLC system was used with a mono Q HR515 column (a 1ml anion exchange column) and a "superloop" which enabled up to 50ml to be loaded onto the column at once. The mono Q column has a maximum capacity of 20-50mg of protein and care must be taken not to exceed this limit (by quantitating the protein content of the sample). Prior to addition of a sample, the 20% ethanol storage buffer was removed from the column by washing with 5ml of low ionic strength buffer A, (see below), followed by 10ml of high ionic strength buffer B, (see below) then equilibrated with buffer A. To clean the column it was incubated with 1mg/ml of pepsin, 0.1M acetic acid, 0.5M NaCl at 37°C for a minimum of 1 hour. It was then washed at least twice with 2M NaCl, 2M NaOH, 75% acetic acid until the UV absorption trace (detecting protein coming off the column) gave a steady base line.

3 sets of buffers were used :

A.1 (50mM tris pH8)	B.1 (50mM tris pH8 + 1M NaCl)
A.2 (buffer A.1 + 1% triton)	B.2 (buffer B.1 + 1% triton)

A.3 (buffer A.1 + 0.1% triton) B.3 (buffer B.1 + 0.1% triton)

Prior to loading samples on the column the samples were sonicated until clear, the sample was desalted by passing it through a PD10 desalting column (Pharmacia, England), which was equilibrated with 30ml of the relevant buffer A. Each column had 2.5ml of sample passed over it, which was then eluted with 3.5ml of buffer A and centrifuged at 12,000rpm for 5 minutes to remove any particulate material which could clog the pre-column filter. The sample was then loaded into the superloop, the required programme used and 1ml samples collected.

8(c) MICROBORE HPLC ANALYSIS

Protein separation carried out at picomole levels, was facilitated by the use of a reverse phase column on a Applied Biosystems microbore HPLC system. The reverse phase column, an aquapore RP300 column (2.1mm in diameter and 30mm in length) allowed 50ul to be automatically loaded onto the column, however further sample could be added by multiple manual loadings. Two buffers were routinely used, buffer A (0.1% TFA in H_2O) and buffer B (0.1% TFA in 80% acetonitrile in H_2O). Samples were collected manually either by collecting protein peaks as indicated by the UV absorbance of a fraction coming off the column at any one time, or by collecting samples according to the percentage composition of the buffer, i.e 0-100% buffer B.

9. SYNTHETIC OLIGONUCLEOTIDES

9(a) OLIGONUCLEOTIDE PURIFICATION

Oligonucleotides were kindly synthesized by Dr John Maclauchlin on a Biosearch 8600 DNA synthesizer using the microscale synthesis process. The DNA was eluted from the column by resuspending the beads in 1ml of ammonia, the solution heated to 55°C for 5 hours, the ammonia removed by lyophilization and the oligonucleotide resuspended in 100ul of water. One quarter of this solution was used at a time to obtain purified oligonucleotide by running the samples on a sequencing gel, i.e. 15% polyacrylamide containing 7M urea in half strength TBE (90mM tris, 89mM boric acid, 1mM EDTA) and polymerized with 0.08% APS (w/v) and 0.08% TEMED (v/v). The gels were pre-

run in 0.5 xTBE running gel buffer for 15 minutes before addition of the samples in 50% formamide. A dye mix (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol + 20mM EDTA) was run on a separate track to act as a marker (no dye was added to the DNA samples as this would prevent the DNA being visualized by UV light if the dye and DNA co-migrated). The gel was run at 250V until the dye front was two thirds down the gel.

To visualize the DNA, the gel was wrapped in cling film, placed on a thin layer chromatography plate and held under short wave UV light. DNA absorbs the UV light producing a dark band against the chromatography plate which uniformly fluoresces. The DNA band of correct molecular weight was excised (so avoiding partially synthesized oligos in the purified sample) and eluted by gentle shaking overnight at 4°C in a small volume of TE buffer (10mM tris pH8.0 + 1mM EDTA). The DNA was then extracted once with phenol/chloroform (1:1), once with chloroform, then precipitated in 3 volumes of ethanol + 0.3M Na acetate on dry ice for 30 minutes. The DNA was then pelleted by centrifugation at high speed in a microfuge for 10 minutes, the pellet washed with 100% ethanol, lyophilized and resuspended in 50ul of water.

9(b) OLIGONUCLEOTIDE QUANTITATION

To quantitate the oligonucleotide sample, the optical density (OD) was measured at 260 and 280nm. Using the conversion factor of 1 OD (at 260nm) = 20ug/ml the concentration of the oligonucleotide sample was calculated. To calculate the molarity of each oligonucleotide solution the following equation was used:

$$E_m = A(16{,}000) + G(12{,}000) + C(7{,}000) + T(9{,}600)$$

$$\text{where molarity} = \frac{OD_{260}}{E_M}$$

where A, G C and T are the number of A, G, C and T residues respectively in the oligonucleotide.

9(c) 5' END LABELLING OF OLIGONUCLEOTIDES

Oligonucleotide (200ng) was resuspended in 50ul of kinase buffer (50mM tris, 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA) containing 10 units of bacteriophage T4 polynucleotide kinase + 50uCi of [γ-^{32}P] ATP and

incubated at 37°C for 30 minutes (Sambrook et al, 1989). This end labelled DNA was used immediately without further purification.

10. cDNA LIBRARY SCREENING USING DEGENERATE OLIGONUCLEOTIDES

10(a) PREPARATION OF AMPICILLIN PLATES

L-broth agar (1% tryptone, 0.5% yeast extract, 1% NaCl - all w/v, pH 7 plus 1.5% bacto agar) was melted and allowed to cool until hand hot. Ampicillin was added at a concentration of 50ug/ml, the agar poured onto sterile 90mm diameter or 20cm^2 petri dishes and allowed to set. The plates were inverted and dried overnight before being stored at 4°C until required.

10(b) SCREENING LIBRARY

cDNA libraries were plated out on ampicillin plates at approximately 20,000 plaques per 20x20cm plate. These were incubated inverted at 42°C until plaques were visible (after 4-6 hours). The plates were then chilled to harden the agarose before asymeterically marked nitrocellulose filters were placed in contact with the surface of plate. This was done twice to create duplicate filters, the second filter being left on the plate for twice as long compared to the first. The filters were then placed in denaturing solution (1.5M NaCl, 0.5M NaOH) for 60 seconds, in neutralizing solution (1.5M NaCl, 0.5M tris pH8) for 5 minutes and finally in 2xSSC (0.3M NaCl, 0.3M trisodium citrate pH7) for 5 minutes, air dried and then baked at 80°C in a vacuum oven for 2 hours.

Before the addition of the end labelled probe the filters were washed in 50mM tris pH8, 1M NaCl, 1mM EDTA + 0.1% SDS for 2 hours at 42°C followed by 2-4 hours at 37°C in hybridization buffer (6xSSC, 1xDenhardts, 0.5% SDS+ 20ug/ml denatured salmon sperm DNA), where Denhardts solution is 0.02% polyvinylpyrolidone, 0.02% BSA + 0.02% ficoll. The probe was then added in as small a volume as possible of fresh hybridization buffer at 37°C for 16-24 hours with shaking. Any unbound DNA probe was removed by washing the filters 3-4 times in 6xSSC + 0.05% Na pyrophosphate. This was then repeated once at 37°C for 1 hour, then finally at the required temperature for 10 minutes. The filters were air dried and exposed to Kodak X-Omat S film using an intensifying screen.

Plaques which gave a positive signal on both duplicate filters were picked, replated and the entire process of screening repeated until all the plaques on any one plate were positive.

11. EXTRACTION OF CELLULAR DNA FROM BN5T CELLS

Cells were lysed in a small volume of TE buffer (50mM tris pH 8.0, 10mM EDTA, 100mM NaCl, 0.4% SDS) plus 200ug/ml of proteinase K, and rotated overnight at room temperature. The lysed cells were extracted twice with an equal volume of TE saturated phenol, then once with chloroform. The chloroform extracted DNA was added to boiled dialysis tubing and dialysed against a large volume of TE buffer with frequent changes of buffer during the initial stages of the overnight dialysis.

The DNA solution was adjusted to 0.25M Na acetate and 2 volumes of ethanol was added and the DNA slowly precipitated overnight at -20°C. The DNA was pelleted at 3,000rpm for 10 minutes, the supernatant removed, the pellet dried under vacuum and finally resuspended in as small a volume of water as possible.

To remove RNA, ribonuclease A (previously boiled for 10 minutes) was added to a final concentration of 100ug/ml and incubated at 37°C for 3 hours. The DNA was then re-extracted in phenol and chloroform as before to remove the ribonuclease A, dialysed and quantitated (OD_{260} of 1 = 50ug/ml for DNA).

12. EXTRACTION OF CELLULAR RNA

12(a) CYTOPLASMIC RNA EXTRACTION

Cells were harvested as normal and resuspended in RNA extraction buffer A (0.15M NaCL, 0.01M tris pH7.8, 1.5mM $MgCl_2$, 0.65% NP40), using 250ul of buffer for cells harvested from one 80cm^2 flask, incubated on ice for 3 minutes and then centrifuged at 3,000rpm for 5 minutes to remove the nuclei and cell debris. An equal volume of buffer B (7M urea, 0.35M NaCl, 0.01M EDTA, 0.01M tris pH7.8 1% (w/v) SDS) was added to the supernatant, and the RNA extracted three times with phenol/chloroform. A final extraction with chloroform was carried out before the RNA was precipitated with 3 volumes of ethanol + 0.25M NaCl at -70°C and the RNA pelleted at 12,000rpm for 10 minutes.

The pellet was resuspended in sterile H_2O, quantitated (OD_{260} of 1 = 40ug/ml RNA), and stored at -70°C.

12(b) EXTRACTION OF POLY A RNA USING DYNABEADS OLIGO $D(T)_{25}$.

Dynabeads oligo $d(T)_{25}$ (Dynal UK Ltd., Ireland), were used to isolate poly A RNA from total cell extracts. The cells were harvested as described in the Dynabeads protocol, using PBS. The cells were quickly centrifuged out of solution at 12,000rpm, then using $12X10^6$ cells, 400ul of the manufacturer's lysis buffer (10mM tris HCl pH7.5, 0.14M NaCl, 5mM KCl, 1% triton-X-100) was added for 1 minute on ice. This was then centrifuged at high speed for 30 seconds and the supernatant transferred to an epindorf containing oligo $d(T)$ beads in 100ul of 2X binding buffer (supplied with kit). This was mixed gently for 3-5 minutes to allow hybridization. The beads were separated from the remainder of the sample using the supplied magnet, the beads washed twice with 200ul of washing buffer (supplied), 100ul of elution buffer (supplied) added, then the sample was heated to 65°C for two minutes. The beads were then separated from the polyA RNA (using the supplied magnet) and the supernatant containing the purified polyA RNA either used immediately or stored at -70°C.

13. POLYMERASE CHAIN REACTION

13(a) cDNA PRODUCTION AND AMPLIFICATION

Based on the method described in Steinthordottir & Mautner (1991), cDNA was generated by using an oligonucleotide primer complementary to the message in question in a reverse transcription reaction. PCR was then used to amplify this cDNA. Each reaction was carried out in 10ul of 1x PCR buffer (10mM tris pH8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin) containing 1mM of each dNTP, 100 units RNasin, 10 units AMV reverse transcriptase, 1-10ug of cytoplasmic RNA (prepared as described in section 9a and quantified as described in section 9b) and 1uM of the first primer (complementary to the mRNA). The sample was then treated at room temperature for 10 minutes, 42°C for 15 minutes (variable depending on the size of the cDNA being generated), 100°C for 5 minutes then quickly chilled on ice. The cDNA produced by this reaction was then amplified by PCR in 50ul of 1x PCR buffer, 0.2uM of each

primer, 1-2.5 units of Taq polymerase, and overlaid with mineral oil to avoid evaporation. This was carried out on a Cambio thermal cycler under varying conditions and for different numbers of cycles. Aliquots of the resulting samples were run on an agarose gel, and the DNA generated visualized with UV light.

13(b) PREPARATION OF AGAROSE MINIGELS

Minigels were prepared to visualize DNA products generated in PCR reactions. These were prepared using either a 1-2% solution of agarose in TBE buffer (0.89mM boric acid, 0.03mM EDTA, 0.89mM tris-HCl pH8.3), or 3% Nusieve agarose + 1% agarose in TBE buffer. The agarose solution was heated to allow the agarose to dissolve, then ethidium bromide (2ul of a 10mg/ml stock solution) was added to 30ml of the agarose solution after it had cooled. The ethidium bromide allows the DNA to be visualized under UV illumination.

DNA samples were loaded in 5ul of sample buffer (5X TBE buffer, 10% w/v ficoll, 0.2% w/v bromophenol blue + 100mM EDTA) and the gels were run with TBE as electrophoresis buffer at 10V/cm until the sample dye front had travelled the appropriate distance through the gel.

13(b) DNA AS A TEMPLATE FOR PCR

In addition to RNA, genomic DNA was also used as a template for PCR. The DNA was isolated as described in section 11 and treated in the same manner as the synthesized cDNA in section 13(a).

13(c) CLONING OF PCR PRODUCTS

PCR products were run on a 3% NuSieve agarose gel, visualized by UV light and the band of interest excised. The gel slice was then melted at 68°C, and the DNA extracted twice with phenol, once with chloroform and precipitated in 3 volumes of ethanol + 0.3M Na acetate. The DNA was next ligated into the Sma1 site of M13mp8.

The precipitated DNA was resuspended in 10ul of water and added to 50ul of kinase buffer (0.5M tris pH7.6, 0.1M $MgCl_2$ 50mM DTT, 1mM spermidine, 1mM EDTA) containing 2 units of kinase, 5 units of ATP and incubated at 37°C for 30 minutes. This was then put on dry ice to inactivate the kinase enzyme, and the samples loaded on a 3% NuSieve agarose gel to repurify the DNA. The DNA was

visualized using long wave UV light as before, the DNA band excised, the gel melted and the DNA extracted twice with TE saturated phenol. The DNA was then precipitated as before and resuspended in 10ul water.

The kinased DNA (20% of the total sample), was next ligated into M13. The reaction included 200ng of phosphatased vector + 2 units of ligase in 10ul of ligase buffer (50mM tris pH7.5, 10mM $MgCl_2$, 10mM DTT) which was incubated for 4 hours at room temperature. The ligated DNA and M13 was then transfected into competent bacteria.

13(d) PREPARATION OF COMPETENT BACTERIA

Based on the method of Sambrook et al, (1989), a 0.3ml aliquot of an overnight standing culture of bacteria was used to inoculate 30ml of 2YT buffer. The culture was grown to an OD of 0.4 -0.6 at 630nm at 37°C. A 30ml sample of these bacteria were centrifuged at 2,000rpm for 10 minutes then resuspended in 2.5ml of ice cold TFB (10mM MES, 10mM rubidium chloride, 45mM manganese chloride, 10mM CaCl + 3mM hexamine-cobaltic chloride) and left on ice for 15 minutes. Keeping the sample on ice, the following additions were made; 100ul of DMSO, incubated for 5 minutes, followed by 100ul of 2.25mM DDT + 40mM potassium chloride pH 6 left for 10 minutes, then addition of 100ul of DMSO for 5 minutes. This resulted in the production of competent bacteria.

A 200ul aliquot of competent bacteria was added to a 3ml tube to which 20ul of the relevant DNA fragment/vector ligation was added and incubated at 42°C for 3 minutes. To 3ml of top agar (1% bacto tryptone, 0.8% NaCl +0.8% bacto agar) 25ul of 2% Xgal in DMF plus 25ul of 2.5% IPTG in water was added and heated to 42°C. This top agar mixture was added to the competent bacteria, mixed well then poured onto TYE plates (1.5% agar in 2YT). These were left to set for 15 minutes then incubated inverted overnight at 37°C.

A white plaque on the bacterial lawn represented a successful transformation event, with the blue plaques lacking any cloned DNA insert.

CHAPTER 4 : RESULTS

AIMS AND BACKGROUND

A set of transformation specific polypeptides have been identified which react with sera from tumour bearing rats (TBS), and antisera raised against HSV-2 infected cells (Macnab et al, 1985a, Hewitt et al, 1991). These polypeptides were also recognised by the monoclonal antibody TG7A, which was raised against cellular polypeptides accumulating on HSV-2 infection (La Thangue & Latchman, 1988). The transformation specific polypeptides are of molecular weights 200,000, 90,000 (a doublet consisting of the upper and lower 90, named U90 and L90), 40,000 and 32,000 (Macnab et al, 1985a; Hewitt, 1988). These have been shown to accumulate on HSV infection and to be present in the transformed cell (Macnab et al, 1985a).

Initial immunofluorescence studies with tumour cells using antisera raised against HSV-2 infected cells, showed fluorescence to be located at the cell surface and also within the cell cytoplasm and perinuclear area (Macnab, 1979). Immunoprecipitation reactions of cellular fractions using TBS subsequently confirmed that the U90 was predominantly membrane associated but was also located in the cytoplasm (Hewitt et al, 1991).

Analysis of transformed, immortalized and control primary cells by immunoprecipitation of radiolabelled cells, indicated that U90 was present in the immortalized and transformed cell lines tested, but not in the control cells (Macnab et al, 1985a). In addition to the above, the U90 shows oncofoetal expression in up to 14 day old rat embryos (Macnab, unpublished results) and so it is possible that the U90 may have a role in differentiation or proliferation in the embryo. The aberrant expression of such a protein could therefore be strongly implicated in transformation and tumourigenesis.

Previous attempts to characterize the U90 by purification and amino acid sequencing resulted in the sequencing of a contaminant protein (Hewitt, 1988). This was almost certainly due to the presence of insufficient purified U90. In addition, other workers have suggested that the U90 is related to the heat shock protein hsp90 (La Thangue & Latchman, 1988). The aims of this thesis were therefore to investigate the U90 polypeptide and its expression, in particular following HSV infection. After the further characterization of the U90 (to aid the purification process), the subsequent objective was to purify and obtain amino acid sequence of the U90 in order to identify the polypeptide and to allow molecular studies to be initiated. The effect of HSV infection on a second cellular polypeptide was also investigated.

The initial observations that a set of transformation specific polypeptides existed in tumour cells was confirmed by repeating the immunoprecipitation (IP) reactions with TBS and cells extracted from [^{35}S] L-methionine radiolabelled Bn5T cells using RIPA buffer. These IP reactions show the presence of the transformation associated polypeptides, U90 and L90 in the tumour cell line Bn5T, but their absence in the control RE cells (Figure 1). The lower molecular weight polypeptides of 40K and 32K cannot be seen under these 7.5% SDS page conditions.

Unless otherwise stated all IP reactions reproduced in this thesis were analysed by 7.5% SDS page, the gels dried under vacuum and exposed to Kodak X-omat S film. Each experiment was carried out on a minimum of three separate occasions unless indicated otherwise.

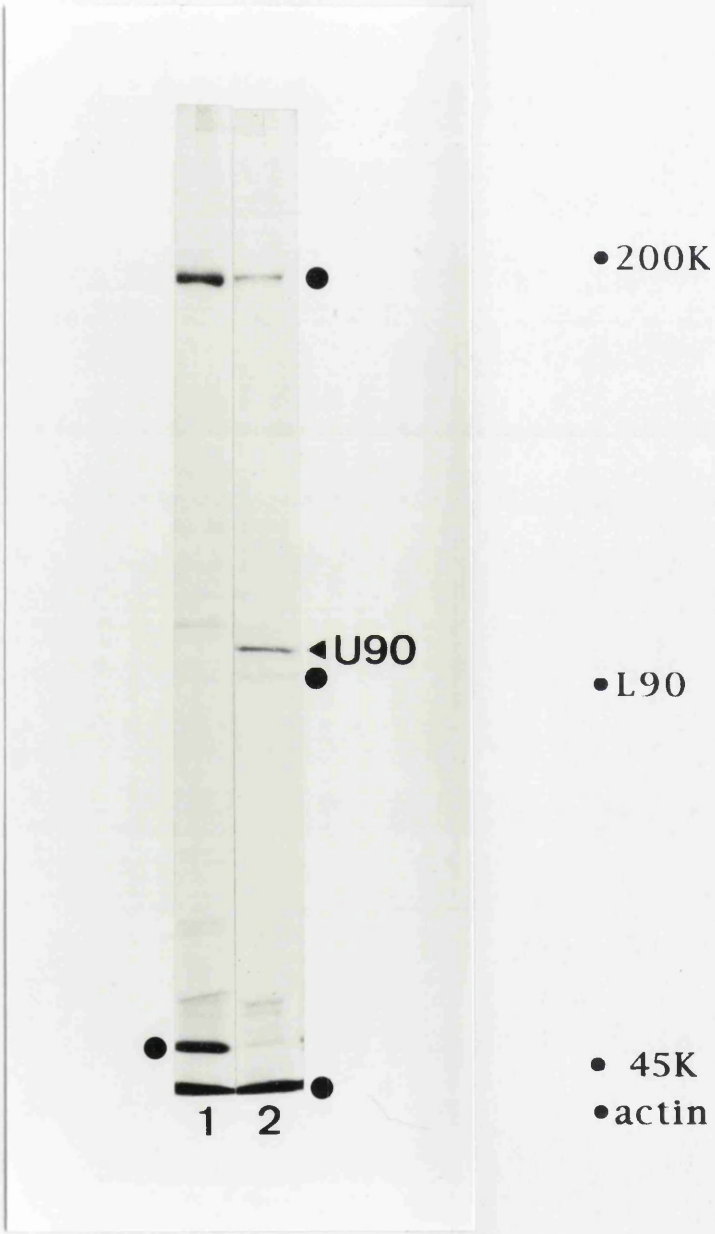


FIGURE 1 : Autoradiograph of IP reactions with TBS of $4X10^6$ cpm each of [^{35}S] radiolabelled polypeptides extracted in RIPA from RE cells (track 1) and Bn5T cells (track 2). The U90 and L90 are precipitated from Bn5T cells as indicated, but not from RE cells. A 200K polypeptide and a polypeptide believed to be actin can be seen in both tracks 1 & 2. A further band (of about 45K) migrating close to actin is seen to be preferentially precipitated from RE cells.

SECTION 1 : CHARACTERIZATION OF U90

1. U90 Can Be Detected By Immunoprecipitation Reactions In Cells Of Different Lineages And Species

In addition to the tumour cell line Bn5T, cells of different lineages and species were radiolabelled with [^{35}S] L-methionine and IP reactions with TBS performed to visualize the tumour specific U90. These studies were carried out to establish if any one cell line expressed elevated levels of the U90 polypeptide in order to simplify the purification of the U90.

Immunoprecipitation of both the human fibroblastic cell line human embryonic lung cells (Helu), and the transformed mouse quasi-epithelial cell line 12-2 (transformed by HPV-16 and c-fos, Crook et al, 1988) resulted in a polypeptide highly similar to the U90 being visualized (Figure 2a). Comparison of the peptide maps of the 90K polypeptides and the U90 also showed them to be indistinguishable (Figure 2b), indicating that the U90 was expressed in epithelial and fibroblastic cells, transformed and established cells, in addition to cells from different species - human, mouse and rat.

Analysis by IP reactions of a number of additional cell lines, including human cell lines, showed that a similar 90K polypeptide was also present. These included a human lymphoid cell line, Jurkat, a human breast carcinoma cell line MCF-7, a rat adrenal pheochromocytoma line, PC12, and the hamster fibroblastic line BHK C13. Although the 90K polypeptide migrated slightly differently in some of these cells (Figure 3), peptide mapping analysis of the 90K polypeptide isolated from the Jurkat cell line confirmed that these differences were most likely species or cell type specific as a highly similar peptide pattern to the U90 was generated. The digestion pattern of the 90K polypeptide isolated from Jurkat cells could not be photographically reproduced due to the low amounts of radioactivity incorporated in this polypeptide. All peptides generated following digestion of both U90 and the Jurkat 90K were of 14K or less and migrated at similar rates.

These results indicated that the U90 could not only be detected in immortalized, transformed and tumour cells of rodents (Macnab et al, 1985a), but also in human cells and cells of different lineages such as adrenal, epithelial, fibroblastic and lymphoid cells. This indicated that the U90 was highly conserved and as such was likely to have an important role in the cell. As a consequence of these studies the U90 can no longer be termed tumour specific as previously thought, (Macnab et al, 1985a) but rather tumour associated.

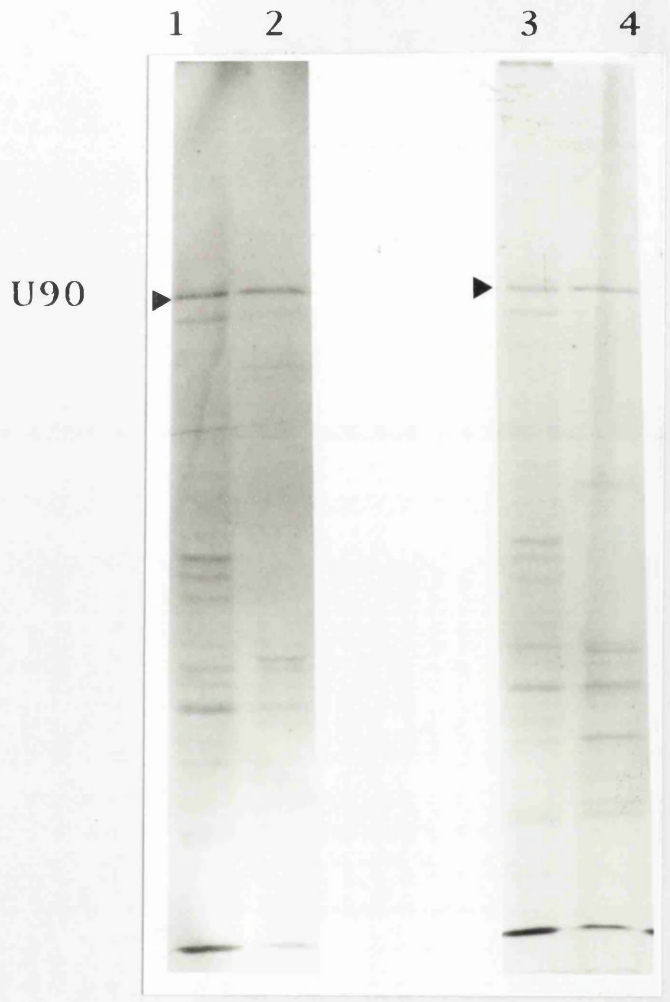


FIGURE 2a : Autoradiograph of IP reactions with TBS, using $4X10^6$ cpm each of [^{35}S] L-methionine radiolabelled polypeptides extracted from Bn5T cells using RIPA buffer (tracks 1 & 3), 12-2 cells (track 2) and Helu cells (track 4). This illustrates the presence of U90 in the Bn5T cells and a similar molecular weight polypeptide to the U90 in both 12-2 and Helu cells.

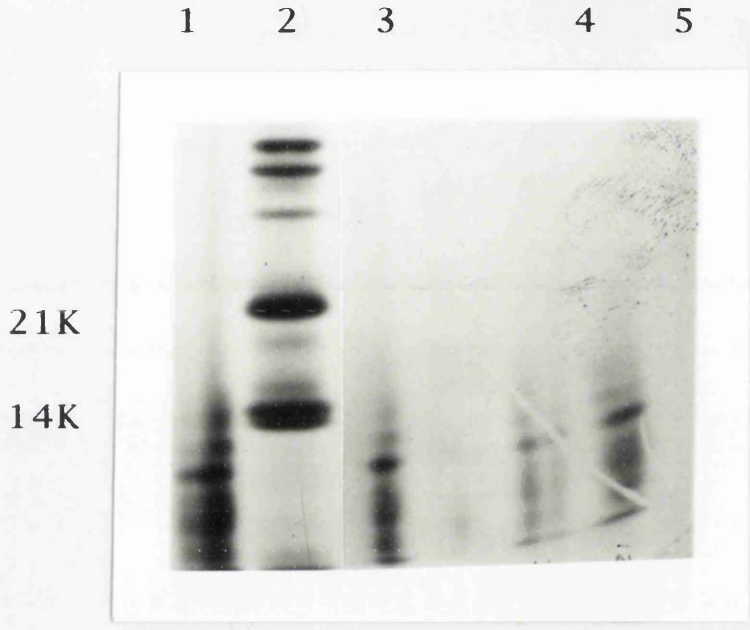


FIGURE 2b : Autoradiograph of a comparison of the peptide digestion patterns of the 90K polypeptide immunoprecipitated by TBS from 12-2 cells (tracks 3, 4 & 5) and U90 from Bn5T cells (track 1) by peptide mapping analysis. Digestion was carried out using 5μg of V8 protease/track in a 18% polyacrylamide gel. Tracks 3 & 5 show the 90K band isolated from 12-2 cells following HSV-1 and HSV-2 infection respectively with track 4 showing a 90K band isolated from uninfected 12-2 cells. Molecular weight markers are shown in track 2 and their values in kilodaltons indicated to the left of the photograph.

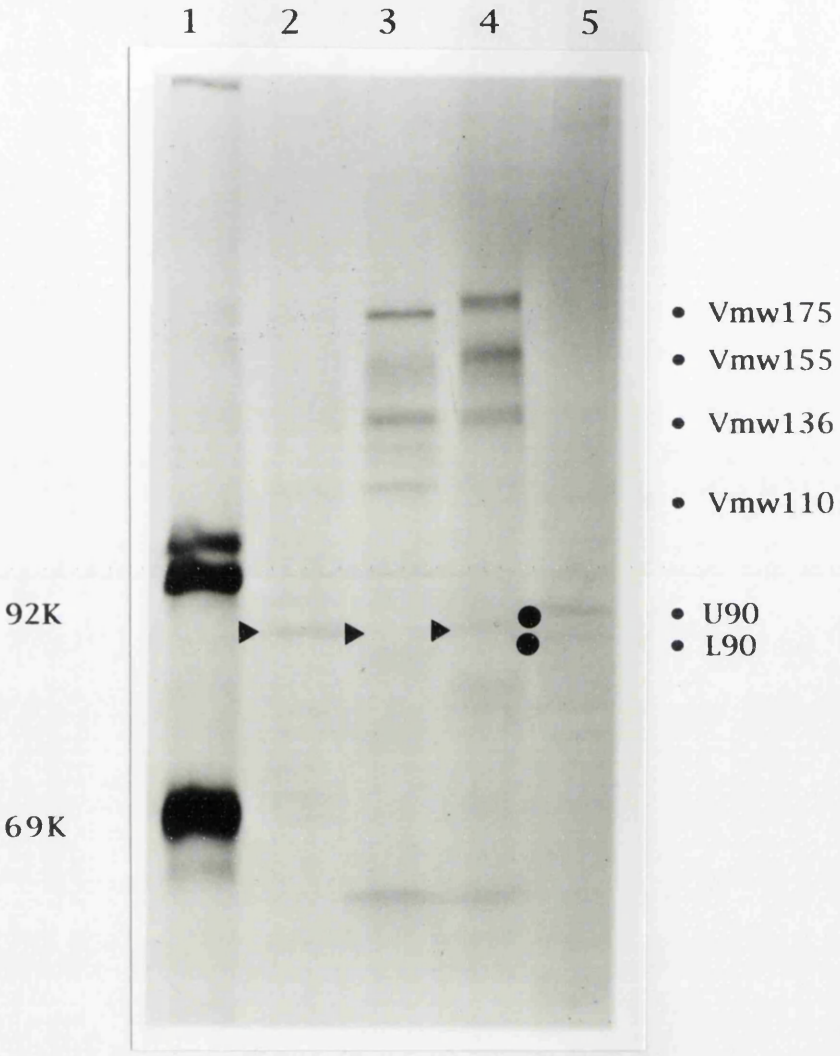


FIGURE 3 : Autoradiograph of IP reactions with TBS using $4X10^6$ cpm each of [^{35}S] L-methionine radiolabelled polypeptides extracted using RIPA buffer from MCF-7 cells (tracks 2, 3 & 4) and Bn5T cells (track 5), illustrating a 90K polypeptide in MCF-7 cells of slightly lower molecular weight than U90. Tracks 3 and 4 show MCF-7 cells following HSV-1 and HSV-2 infection respectively. In track 4 precipitation of HSV-1 viral polypeptides of 175K, 155K, 136K and 110K can be seen and are indicated (•) to the right. This is a feature common in IP reactions carried out in the presence of viral polypeptides. The corresponding HSV-2 values (track 3) are 182K, 157K and 140K (not shown) respectively. Track 1 shows molecular weight markers with values indicated to the left of the photograph.

2(a) HSV Infection Increases U90 Expression In Bn5T Cells

The original studies of Macnab et al (1985), showed that after HSV-2 infection, U90 was increased as detected by a monoclonal antibody (MAB) TG7A. This MAB however was unable to detect an increase in U90 following HSV-1 infection. The assumption was therefore made that the U90 was probably more efficiently induced by HSV-2 than HSV-1 infection. I firstly addressed this problem by investigating both HSV-1 and HSV-2 infection in Bn5T cells using TBS. It was not possible to repeat the studies using the TG7A antibody as this antibody was not made available. TBS was therefore used as a substitute.

HSV-1 and HSV-2 infection of Bn5T cells radiolabelled with [^{35}S] L-methionine was carried out for a period of 17 hours at a multiplicity of 5-10pfu/cell and analyzed by IP reactions using TBS, followed by separation using SDS page. Exposure of the dried gel to Kodak X-omat S film showed that more radiolabelled U90 was immunoprecipitated in both the HSV-1 and HSV-2 infected samples compared to the uninfected samples. Figure 4a shows a representative autoradiograph of an IP of HSV-2 infected and uninfected control Bn5T cells and the data from the corresponding densitometer scan can be seen in Figure 4b. Densitometer scanning at a later stage showed the U90 to be increased by 3.1 fold following HSV-2 infection and 5.3 fold following HSV-1 infection after 17 hours. The induction of U90 by HSV-2 was initially believed to be of the same order as that of the induction of U90 by HSV-1, however the subsequent availability of the Shimadzu scanning densitometer (Department of Biochemistry, Glasgow University) indicated that this was not the case in the gels subjected to quantitative analysis. However, in general HSV-2 does increase U90 efficiently.

To ensure that the 90K polypeptide immunoprecipitated following HSV infection was indistinguishable from the U90 as seen in the uninfected cell, peptide mapping analysis was carried out using *Staph. aureus* V8 protease (Figure 5). Enzymatic digestion under these conditions was carried out to the limit of possible digestion and shall therefore described as total digestion in this thesis. Comparison of the resulting total digestion patterns of the U90 isolated from infected and uninfected Bn5T cells showed the two polypeptides to have indistinguishable digestion patterns.

2(b) HSV Infection Increases U90 In Human and Mouse Cells

Infection of the 12-2 and Helu cell lines with either HSV-1 or HSV-2 (5-10pfu/cell for 17 hours at 37^{o}C), also resulted in increased amounts of U90 being detected by immunoprecipitation of [^{35}S] L-methionine radiolabelled cell polypeptides by TBS (Figure 6a), unfortunately the control Helu track was

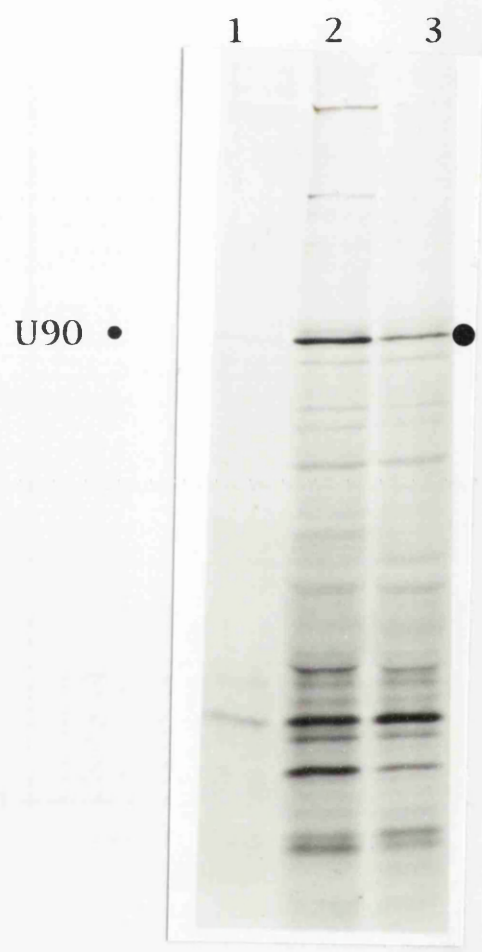


FIGURE 4a : Autoradiograph of IP reactions with TBS using $4X10^6$ cpm each of [^{35}S] L-methionine radiolabelled polypeptides extracted using RIPA buffer from Bn5T control cells (track 1), Bn5T cells infected with 10pfu/cell HSV-1 (track 2) and Bn5T cells infected with 10pfu/cell HSV-2 (track 3). Cells were infected at 10pfu/cell for 17 hours at 37°C. Increased background polypeptides in tracks 2 and 3 are usually seen and are most probably as a result of co-precipitation of viral polypeptides with the immune complexes as discussed previously.

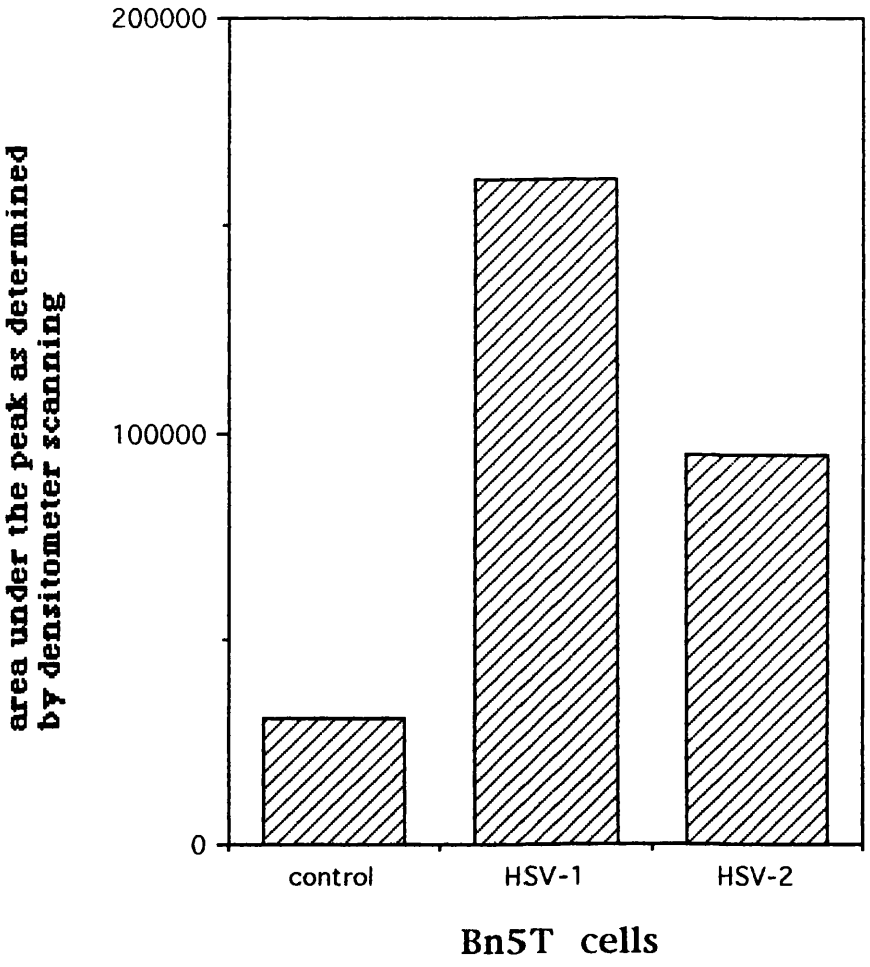


FIGURE 4b Quantitation of U90 visualized following IP reaction with TBS in Figure 4a. The above data was generated by scanning the resulting U90 bands on the autoradiograph using a Shimadzu CS-9000 dual-wavelength flying spot scanning densitometer and zig-zag scanning with the resulting data being the mean of 11 scans, as detailed in Method 7(i). The resulting error generated was insignificant (and too small to be visualized on the above graph). HSV-1 infection in this experiment resulted in a 5.3 fold increase and HSV-2 infection resulted in a 3.1 fold increase. Both HSV types 1 and 2 infection were found to result in an increase of U90 on a minimum of 5 occasions, ranging from a 2.5 - 5.3 fold increase (Hewitt et al, 1991).

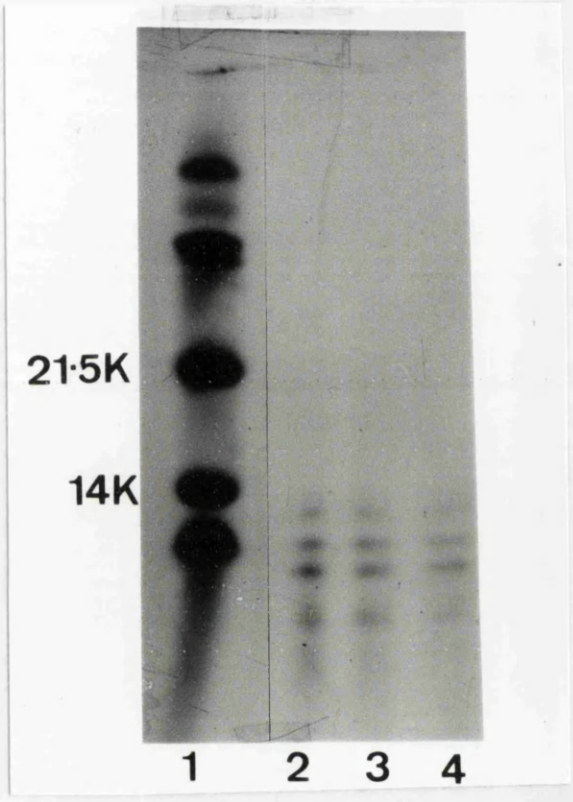


FIGURE 5 : Autoradiograph of a comparison of the peptide digestion patterns of U90 bands immunoprecipitated by TBS and cut from dried gels. U90 bands were isolated from control Bn5T cells (track 4), HSV-1 infected Bn5T cells (track 3) and HSV-2 infected Bn5T cells (track 2) and compared by peptide mapping analysis. Digestion was achieved using 5μg/track of V8 protease in an 18% SDS-page system. Molecular weight markers are present in track 1 and their values indicated in kilodaltons to the left of the gel.

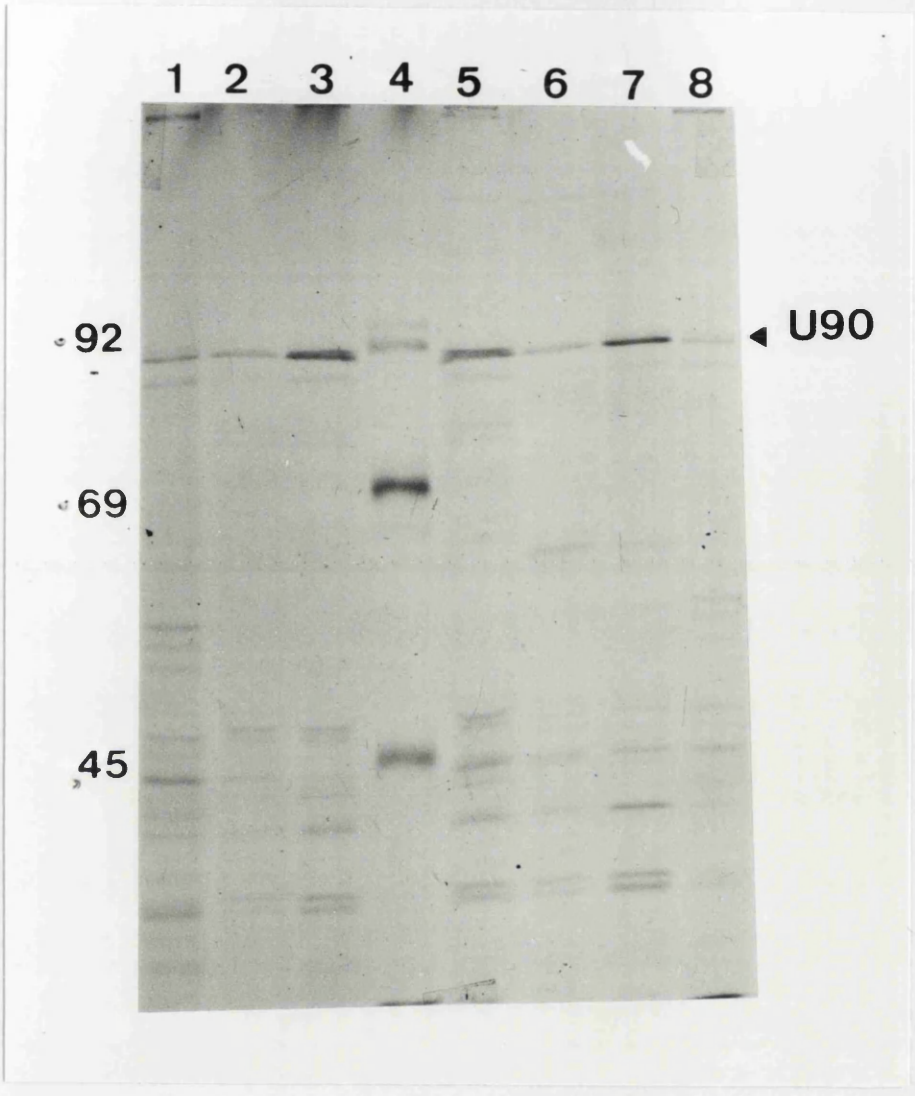


FIGURE 6a : Autoradiograph of IP reactions with TBS using $4X10^6$ cpm each of [^{35}S] L-methionine radiolabelled polypeptides extracted using RIPA buffer from 12-2 cells (tracks 2, 3 & 5) and Helu cells (tracks 6 & 7), where U90 is indicated by a filled triangle. Tracks 3 & 6 show cells following HSV-1 infection and tracks 5 & 7 cells following HSV-2 infection. Bn5T cells IP with TBS are shown in tracks 1 & 8 with molecular weight markers in track 4 (whose values are indicated in kilodaltons to the left of the gel). All viral infections were carried out at 10pfu/cell for 17 hours at 37°C. Unfortunately the control Helu track was removed but a comparison of Bn5T and Helu cells can be seen in Figure 2a.

removed by error, but can be compared with the uninfected Helu cells shown in Figure 2a. Densitometer scanning of the resulting autoradiograph of the 12-2 cells IP reaction confirmed this induction (Figure 6b). It could therefore be concluded that HSV infection of not only Bn5T cells, but of other human and rodent cells from which the U90 could be immunoprecipitated, resulted in the increase of the U90 polypeptide. No cell type however was found which expressed greater quantities of U90 than the Bn5T cell line.

3. HSV Infection Increases Total U90 Content In The Cell

The increase in U90 following HSV infection, as seen by immunoprecipitation of radiolabelled cell polypeptides could occur by at least two distinct mechanisms. The increase could either be a consequence of larger quantities of the U90 polypeptide being present in the infected cell, or alternatively the U90 in the infected cell could incorporate more radiolabel, thus generating a stronger U90 band - as seen by autoradiography.

To determine which of these two mechanisms was correct, gels with radiolabelled IP reactions of HSV-2 infected and uninfected Bn5T cell polypeptides, were stained with Coomassie blue to quantitatively as well as qualitatively analyze the amount of U90 in each sample. Coomassie blue staining indicated that an order of 8 fold more U90 was present in the infected cell compared to the uninfected cell as determined by densitometer scanning (Figure 7a), and that the increase in U90 following infection was at least as much as was seen by autoradiography. Technical difficulties which occur when scanning a Coomassie blue stained gel meant that a photographic reproduction of the gel had to be scanned. This might possibly result in a less accurate quantitation as compared to scanning an autoradiograph (which was not possible), but was not subsequently proven. To illustrate this, the stained gel has also been reproduced in Figure 7b.

It was therefore concluded that the increase in the U90 as seen by radiolabel incorporation was in fact due to an increase in the total amount of U90 in the cell

4. An Early Event In HSV Infection Increases U90

To investigate the stage of HSV infection at which the U90 was induced, Bn5T cells infected with HSV-2, at 5-10pfu/cell at 37^{o}C, were radiolabelled with [^{35}S] L-methionine, harvested at regular intervals between 2 and 16 hours post adsorption, and immunoprecipitated with TBS. The result indicated that the increase in U90 was clearly visible by 3 hours post adsorption and was therefore a consequence of an early event after HSV infection.

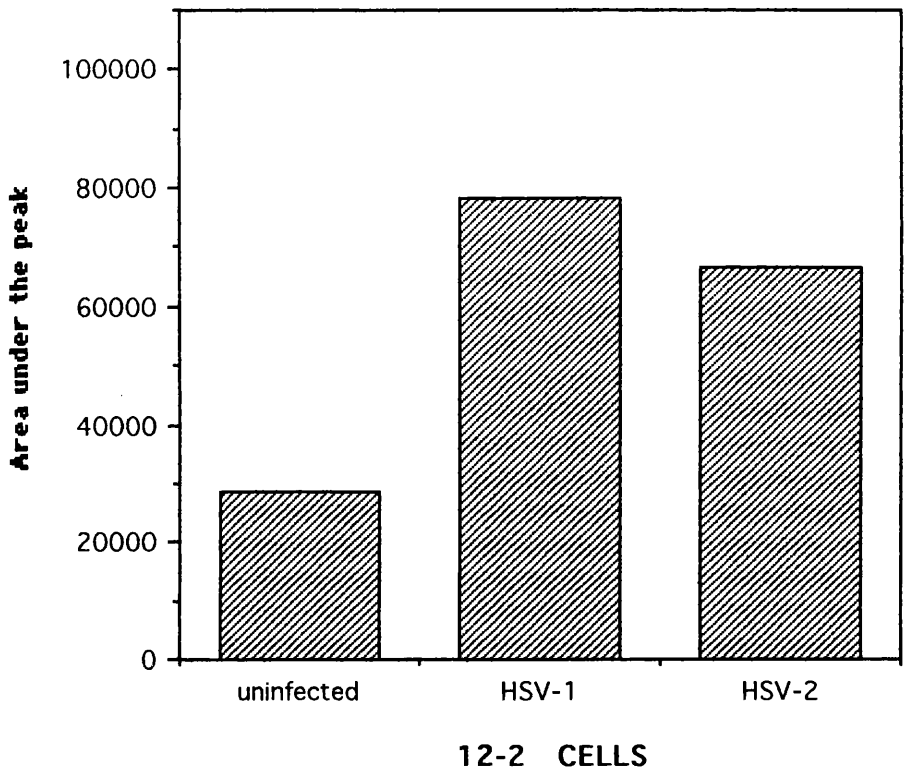


FIGURE 6b : Densitometer scan of U90 in control (uninfected), HSV-1 and HSV-2 infected 12-2 cells following IP reactions with TBS and visualization by autoradiography. In a similar manner as seen with Bn5T cells, HSV-1 induced slightly greater amounts of U90 in the 12-2 cells than HSV-2. The above data was generated by scanning the resulting U90 bands on the autoradiograph using a Shimadzu CS-9000 dual-wavelength flying spot scanning densitometer and zig-zag scanning with the resulting data being the mean of 11 scans, as detailed in Method 7(i). Error bars cannot be seen due to the very small error produced by the scanner.

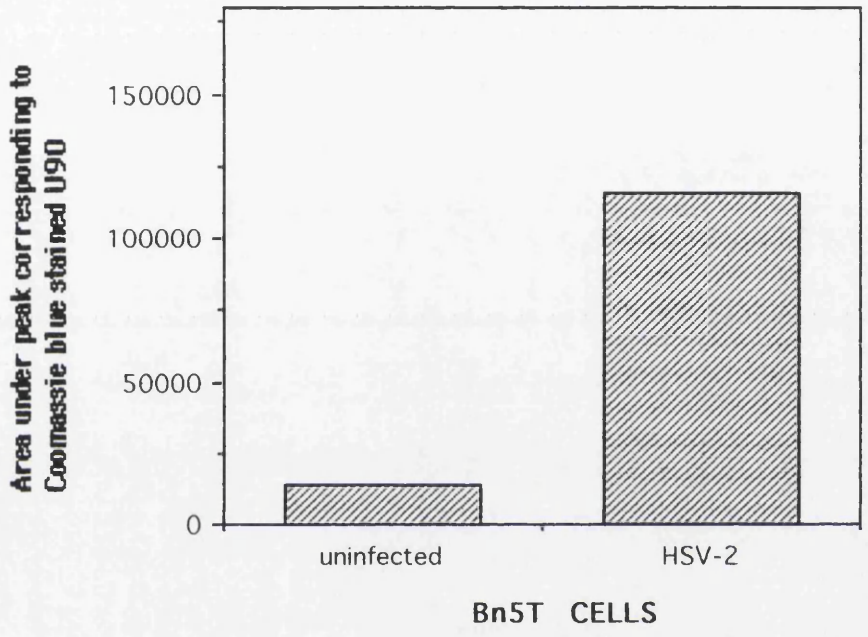


FIGURE 7a : Densitometer scan of U90 in control (uninfected) and HSV-2 infected Bn5T cells after IP with TBS as visualized by Coomassie blue staining. The above data was generated by scanning the resulting U90 bands on a photographic reproduction of the stained gel as seen in Figure 7b. A Shimadzu CS-9000 dual-wavelength flying spot scanning densitometer was used as described previously with the resulting data being the mean of 11 scans, as detailed in Method 7(i). Error bars cannot be seen due to the insignificant error produced by the scanner.

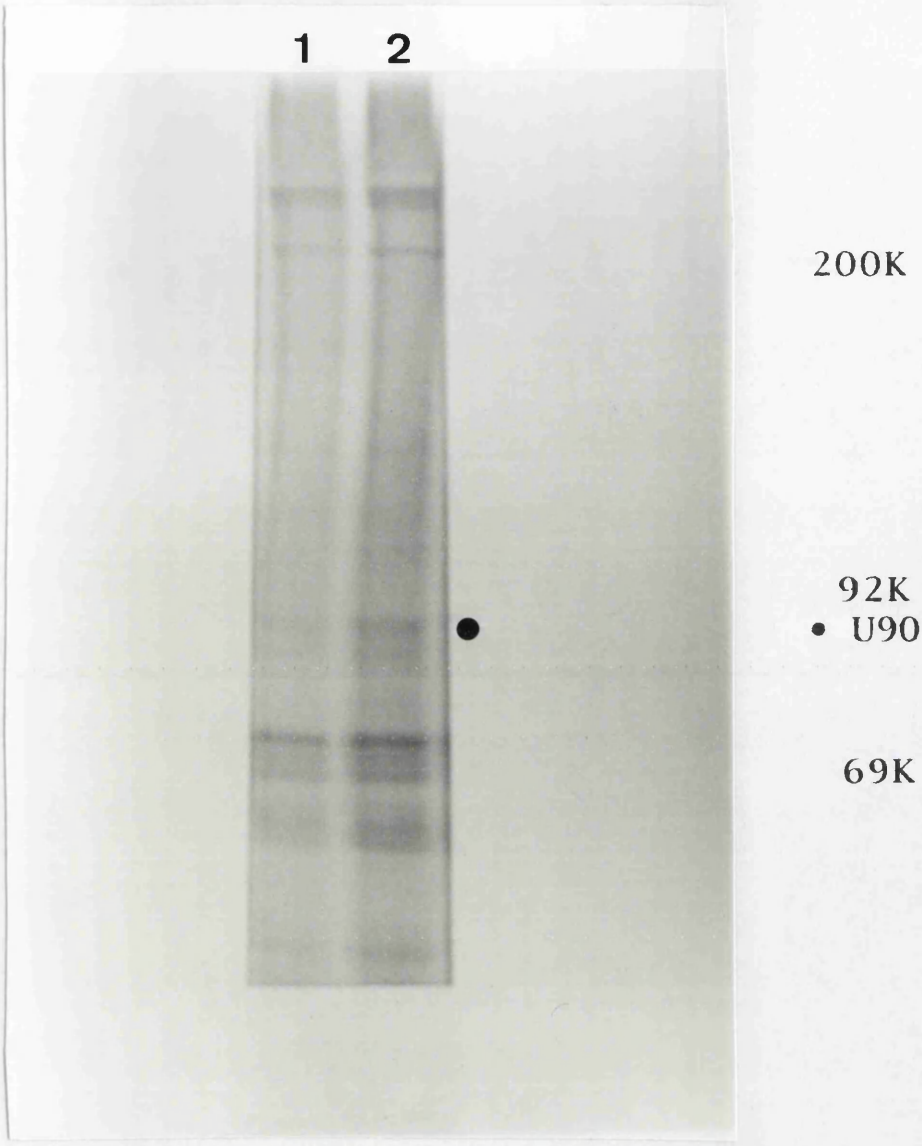


FIGURE 7b : IP reactions with TBS using 500ug each of polypeptides extracted using RIPA buffer from control Bn5T cells (track 1) and HSV-2 infected (10pfu/cell at 37°C for 17 hours) Bn5T cells (track 2) visualized by Coomassie blue staining. The U90 band indicated above (•) was identified by overlaying the stained gel with the corresponding autoradiograph of the gel which clearly showed the immunoprecipitated U90. The positions of molecular weight markers (shown in kilodaltons) are indicated to the right of the figure. The additional stained bands are bacterial polypeptides generated by the use of Pansorbin [(Calbiochem), a comercial preparation of a suspension of *S.aureus* Cowan 1 strain, which expresses a coat of protein A), as a alternative to protein A sepharose.

This was further investigated by pulse-labelling HSV-2 infected Bn5T cells for 2 hours at 1, 3, 5 and 7 hours post-absorption. The amount of [^{35}S] L-methionine incorporated in the U90 in the infected and uninfected cells was quantitated by densitometer scanning of the IP reactions of cells labelled and harvested at these time points. Comparison of newly synthesized U90 in infected and uninfected Bn5T cells shows that the greatest increase in U90 as a consequence of HSV infection, which was 720% of the control cells, occurs within 1-3 hours post adsorption (Figure 8). By 7-9 hours post absorption the increase in U90 as a consequence of HSV infection has fallen from 720% of the uninfected U90 to only 300%. These results confirmed that an event prior to the onset of viral DNA synthesis was involved in the induction of U90, ie either an immediate early function of HSV, or an earlier event (ie viral adsorption or entry).

5. Identification of HSV Function Involved In U90 Induction

A selection of viruses mutated in immediate early (IE) genes were used to investigate the possibility that an IE event in infection increased the U90. Intially tsK (Preston 1981), a temperature sensitive mutant of Vmw175 which over-expresses Vmw175 and the other IE genes at its non-permissive temperature (38.5^{o}C) was used. Infection of Bn5T cells with tsK at 5-10pfu/cell at both the PT of 31^{o}C for 17 hours and the NPT of 38.5^{o}C for 8 hours, was seen to increase U90 (Figure 9a). This suggested as before, that an early event in infection increased U90 - ie, either an IE function or an event prior to this, such as viral adsorption or entry to the cell. Infection with tsK at the NPT was carried out for 8 hours (as opposed to 17 hours) as previous work indicated that infection for longer periods of time resulted in a breakdown product of 90K being generated (McDonald, 1980).

Experiments carried out by two undergraduate students Helen Ritchie and John Yates using various HSV mutants investigated this aspect further. Their work confirmed that neither viral adsorption nor entry into the cell resulted in the induction of U90. This was done by infecting cells with either UV irradiated virus or HSV in the presence of actinomycin D (to inhibit transcription). Neither of these conditions resulted in an increase of U90 expression as seen by immunoprecipitation.

In order to expand the experiment investigating U90 induction following early infection, in addition to tsK infection at the non-permissive temperature (NPT) which resulted in the over-expression of IE genes, the viruses tsT and tsD were also investigated. Both viruses which contain mutations in Vmw175

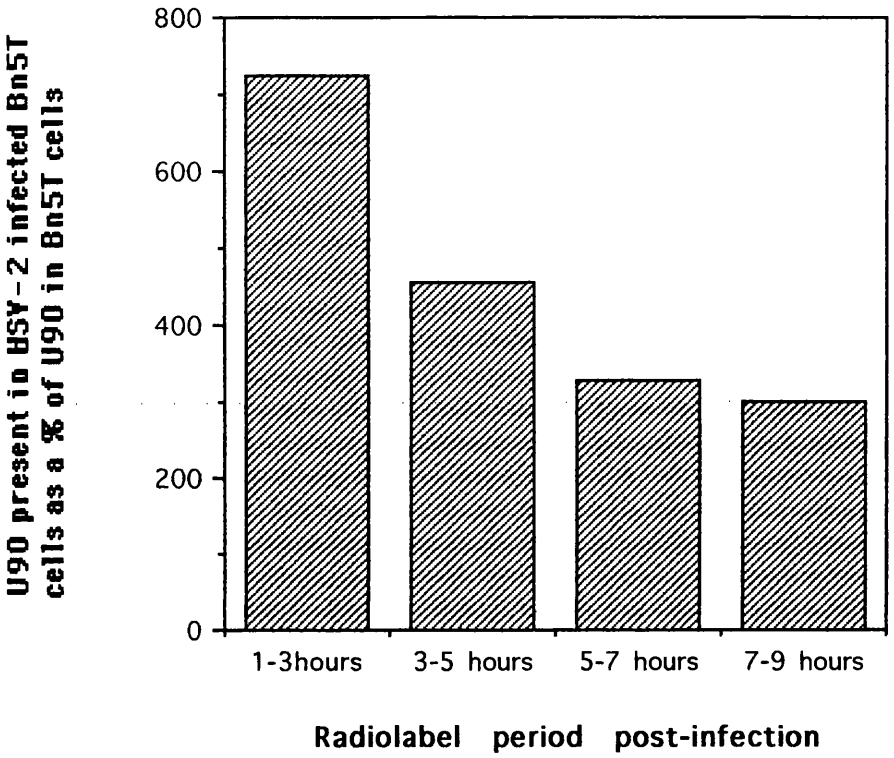


FIGURE 8 : The amount of U90 expressed in HSV-2 infected cells at 1-3, 3-5, 5-7 and 7-9 hours post-adsorption is expressed in this graph as a % of the amount of U90 in control cells at corresponding time points. Cells were radiolabelled with [^{35}S] L-methionine for a period of 2 hours, polypeptides analyzed by IP with TBS, the U90 visualized by autoradiography and quantitated in both control and HSV-2 infected Bn5T cells by scanning the resulting U90 bands on the autoradiograph using a Shimadzu CS-9000 dual-wavelength flying spot scanning densitometer and zig-zag scanning with the resulting data being the mean of 11 scans, as detailed in Method 7(i). Error bars cannot be seen due to the insignificant error produced by the scanner.

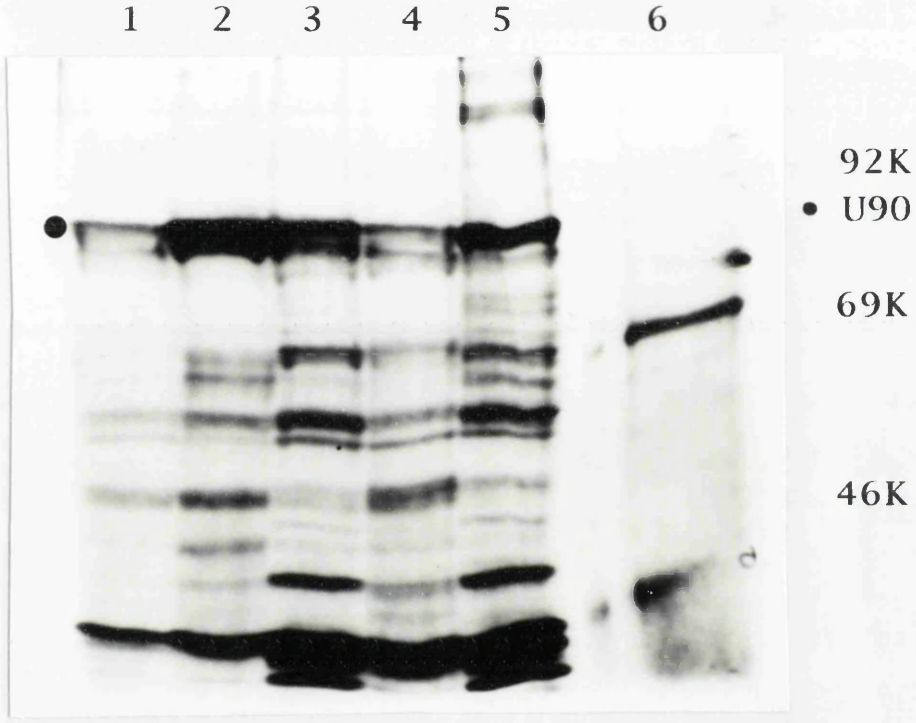


FIGURE 9a : Autoradiograph of IP reactions with TBS of [35S] L-methionine labelled polypeptides extracted using RIPA buffer from control Bn5T cells at 37°C (track 1), Bn5T cells infected with tsK at 38.5°C for 8 hours (track 2), infected with tsK at 31°C for 17 hours (track 3), control cells at 31°C (track 4) and cells infected with HSV-2 at 37°C for 17 hours (track 5). To the right of the gel U90 is indicated (•) and molecular weight markers are shown in track 6 and their values shown in kilodaltons. All infections were carried out at 10pfu/cell.

(Preston, 1981) over-express the IE genes at the NPT and were found to induce U90.

Bn5T cells were infected with a mutant in1411, which expresses a small truncated non-functional Vmw175 due to the introduction of a stop codon between codons 83 and 84 (Russell et al, 1987). Similar levels of U90 were seen in the in1411 infected cells as compared to the control Bn5T cells. However, the tsK mutant which over-expresses a non-functional Vmw175 greatly increases U90 at the NPT. Figure 9b shows the induction of U90 following tsK infection at the NPT, compared to basal levels of U90 in cells infected with in1411 and control Bn5T cells. (The virus in1411 is not a temperature sensitive mutant and as such, the control for this virus is infection of Bn5T cells with HSV-1 at 37°C, which has been clearly demonstrated previously to induce U90). Infection of Bn5T cells with tsK at the PT as indicated in Figure 9a, resulted in an increased U90 as would be expected, since tsK at 31°C behaves as wild type. This is similar to the induction of U90 seen following infection with tsD and tsT, which also express non-functional Vmw175. This confirms that the induction of U90 by HSV infection is independent of the expression of a Vmw175 polypeptide functional for transactivation, which is required under normal circumstances for viral E polypeptide induction, but the expression of the first 83 amino acids of Vmw175, as seen in in1411, is insufficient for U90 induction.

It could therefore be concluded that certain sequences of Vmw175 were essential for the increase in expression of the U90 following HSV infection, but virus with a mutation rendering Vmw175 non-functional for lytic infection was nevertheless capable of inducing U90. How Vmw175 induces U90 is not known. It could act in an indirect manner by stimulating the other IE genes (which was previously thought to be the case in studies on HSV replication, Wu et al, 1988), or Vmw175 could act directly on U90 induction.

6. Does HSV Infection Alter The Half-life Of U90?

The increase in the total amount of U90 present in the cell following HSV infection could occur at a number of stages during the synthesis of the polypeptide. At this point only the possibility that the half-life (or turnover) of U90 polypeptide was altered by HSV infection could be investigated as no DNA sequence data was available to investigate the levels of U90 mRNA in the cell. The experiments to investigate the U90 half life were done by pulse chase experiments, as described below.

Pulse chase experiments were carried out on infected and uninfected Bn5T cells by radiolabelling, then varying the chase period between 3 and 24 hours. IP reactions of each of these conditions indicated that very little difference

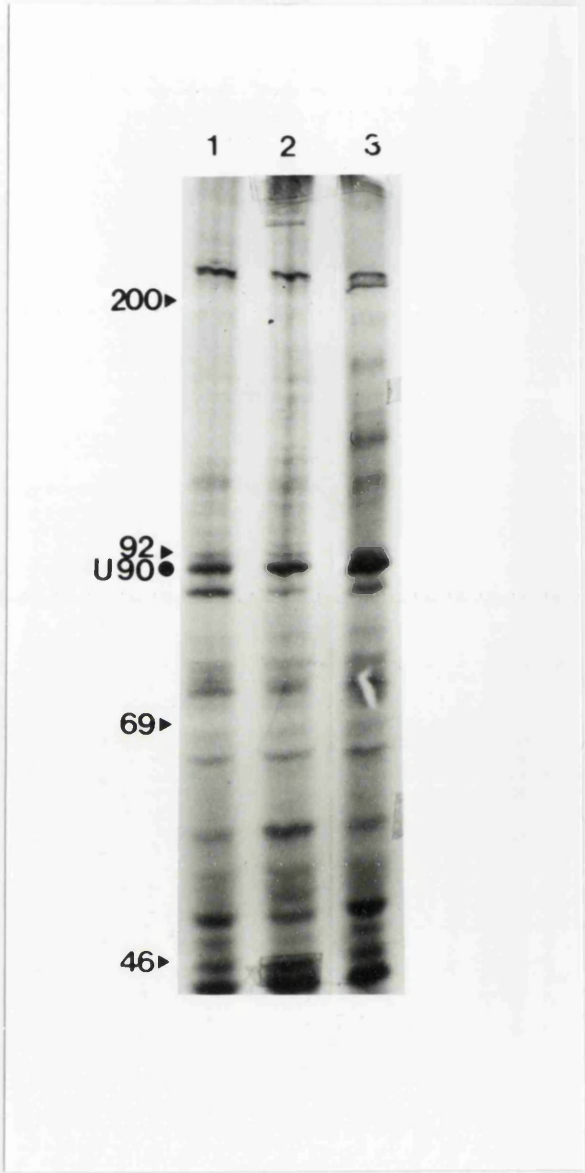


FIGURE 9b : Autoradiograph of IP reactions with TBS of [^{35}S] L-methionine labelled polypeptides extracted using RIPA buffer from Bn5T control cells (track 1), Bn5T cells infected with in1411 at 10pfu/cells for 17 hours at 37°C (track 2) and Bn5T cells infected by tsK at the NPT of 38.5°C for 8 hours using 10pfu/cell (track 3). tsK infection at the PT of 31°C resulted in a characteristic increase in U90 as expected of an HSV-1 infection (Figure 9a). Markers in kilodaltons are indicated to the left of the gel. This experiment was kindly carried out by Helen Ritchie.

could be seen between the half life of the infected and uninfected U90 (Figure 10). Due to technical difficulties in achieving incorporation of sufficient radiolabel in the U90, densitometer scanning of the resulting autoradiographs was very difficult. The data for this experiment was therefore limited. The data available however do indicate that the half life of the U90 in the infected and control Bn5T cells are fundamentally the same with the half life of the U90 in control Bn5T cells being estimated 10 hours and 4 minutes and 12 hours 54 minutes in the infected cells. These figures can can only be taken as approximate values due to the limited nature of the data. It should be noted however that a similar result for the U90 half life was later obtained by Macnab & McNab (personal communication). It was concluded that this difference in the half life of the U90 was most probably due to experimental error as a consequence of the limited data which could not account for the 8 fold increase in U90 seen at an early stage in infection. Therefore, the increase in U90 in the infected cells was not due to a stabilization of the U90 by post-translational modification.

7. Alteration Of U90 Location Following HSV Infection

Studies by Macnab & McNab had previously shown that the U90 was predominantly located in the membrane associated fraction of uninfected Bn5T cells. However, following HSV-2 infection of Bn5T cells, (5-10pfu/cell for 17 hours at 37^{o}C), quantitative analysis of the membrane and cytoplasmic fractions (generated by cell fractionation) was carried out by western blotting using TBS and the monospecific U90 antibody (Methods section 3b). This indicated that only one band could be seen in the western blot, the U90, which accumulated in the cytoplasmic fraction of the cell following HSV infection (Figures 11a & 11b). All western blots in this thesis were reproduced by a combination of laser printing and photocopying which neither introduced nor removed any background material. The western blot in Figure 11b was kindly carried out by Dr JCM Macnab. Cell fractionation was obtained as described in Methods, Section 6b, by swelling cells in a hypotonic buffer, dounce homogenization to release the cytoplasmic proteins followed by a detergent extraction of the membrane proteins.

Purification of the U90 from the membrane fraction had previously been found to be very difficult as a consequence of the U90 being membrane associated and also only being present in small amounts (Macnab & McNab, unpublished results). The fact that the U90 was present in a soluble form in the cytoplasm of infected cells greatly improved the possibility of purifying the

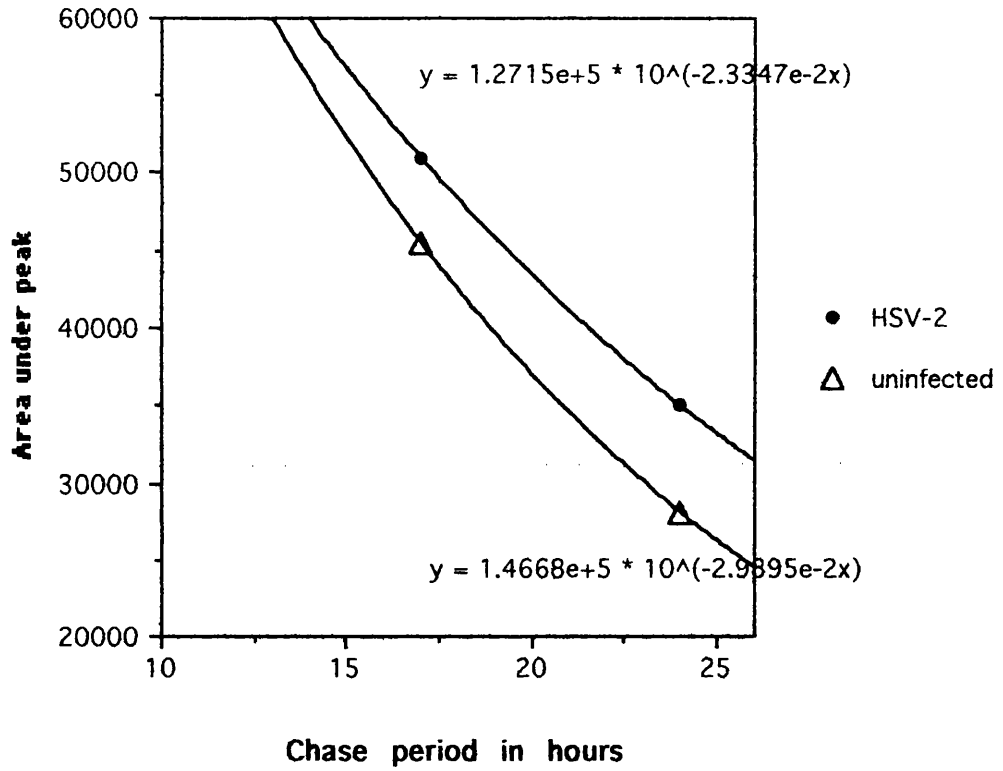


FIGURE 10 : Densitometer scan of U90 in control (uninfected) Δ and HSV-2 infected Bn5T cells (•) after 7 hours [^{35}S] L-methionine radiolabel, followed by a chase period of either 17 or 24 hours with excess cold methionine. Cells were then harvested, extracted with RIPA buffer, IP with TBS, analysed by SDS-page and visualized by autoradiography. Data correspond to the area under the peak when the U90 band on the autoradiograph was quantitated by scanning densitometery, as described previously.

The equations of the curves (as determined assuming exponential decay using the Apple Macintosh programme Cricket graph version 1.3.2) are illustrated adjacent to the respective curves, which allows the half life of the U90 in control Bn5T cells and HSV-2 infected Bn5T cells to be calculated. The half life of U90 was found to be 12 hours 54 minutes in HSV-2 infected cells and 10 hours 4 minutes in control Bn5T cells. This difference is thought to be due to experimental error as a consequence of the limited data which was readable by the scanner.

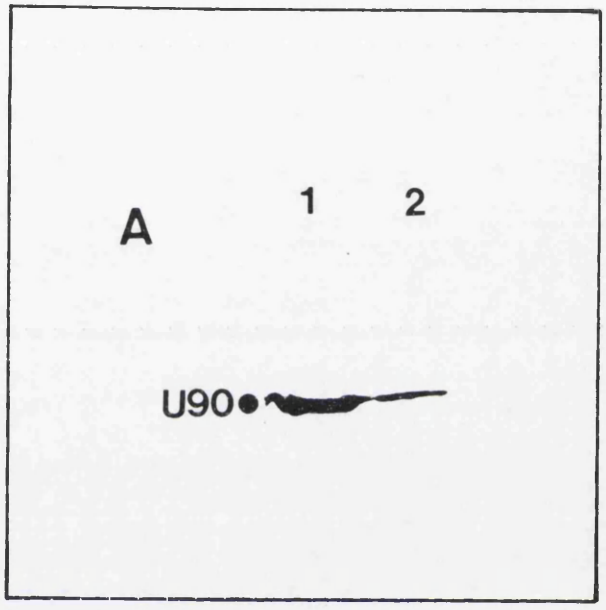


FIGURE 11a : Western blot with TBS of Bn5T cells infected by HSV-2 (10pfu/cell for 17 hours). and polypeptides extracted from the cytoplasmic fraction (track 1) and membrane fraction (track 2) showing an accumulation of the U90 in the cytoplasmic fraction. To visualise, anti-rabbit immunoglubulin conjugated to horse radish peroxidase was used with N'chloronapthol.

All western blots were reproduced by a combination of laser printing and photocopying which neither introduced nor removed any background material. Confirmation of this statement can be seen in Figure 33 where a blot has been re-produced using photography (Figure 33b) compared with laser printing (Figure 33a).

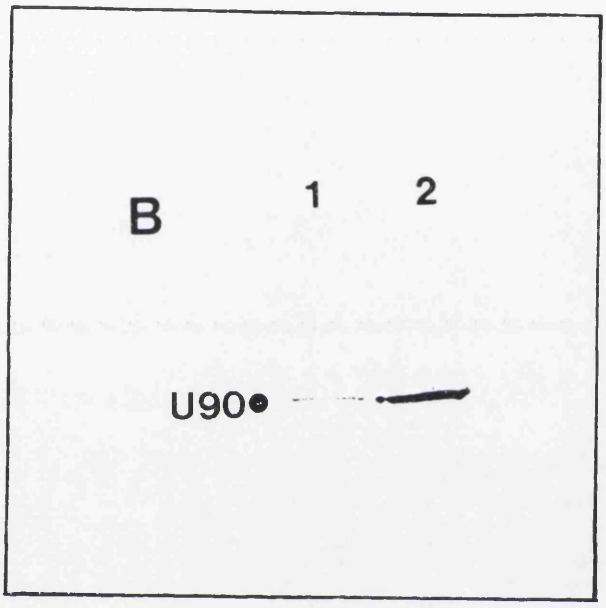


FIGURE 11b : Immunoprecipitation with TBS of polypeptides extracted from the cytoplasmic fractions of control (track 1) and HSV-2 infected Bn5T cells (track 2) followed by western blotting with U90 monospecific antiserum confirms that HSV infection results in the accumulation of the U90 in the cytoplasm and that the U90 antibody does in fact recognise the U90. Figure 11b was kindly completed by Dr. JCM Macnab.

U90. It should also be noted, that the alteration of the location of the U90 as a consequence of HSV infection, may have a significant effect on the cell.

DISCUSSION OF SECTION 1: CHARACTERIZATION OF U90

It was previously reported that the accumulation of the U90 polypeptide was a common feature of transformed and immortalized cells, but not of primary or secondary cells (Macnab et al, 1985a & Figure 1). It was therefore proposed that the accumulation of such a polypeptide is likely to be of importance at the level of immortalization of the cell (Macnab et al, 1985a). The U90 polypeptide was also seen to be recognised by antisera raised against HSV-2 infected cells, indicating that cellular polypeptides accumulating as a consequence of HSV infection may be implicated in the initiation of transformation by HSV infection (Macnab et al, 1985a).

The characterization studies described in Section 1 were carried out to provide a better understanding of the U90. In addition, these findings were utilized to determine the conditions under which maximal U90 was expressed in order to obtain larger amounts of U90 as starting material for the purification of the polypeptide.

These studies showed that the U90 polypeptide can be radiolabelled and immunoprecipitated using TBS not only from rat tumour cells but also from mouse and human cells. Cells of different lineages also displayed the U90 (as seen by IP reactions) which included adrenal, epithelial, fibroblastic and lymphoid cells. The presence of the U90 in such a diverse range of cells indicated that the U90 is highly conserved and as such, is likely to play an important role in the cell.

In these studies the U90 could by detected not only in transformed and immortalized cells but also in cells which are neither immortalized nor transformed but are only capable of passage in cell culture for a finite period of time. U90 was not immunoprecipitated from primary and secondary cell cultures under similar radiolabelling conditions despite exhaustive attempts. This indicated that the ability of TBS to immunoprecipitate the U90 from radiolabelled cells using TBS does not require immortalization of the cell line, but is correlated with the ability to passage cells in culture over a considerable number of doublings. From these results it was concluded that the change the U90 undergoes to enable it to be visualized by radio-immunoprecipitation in transformed, immortalized and cells established in culture, is an early stage in one of the many steps towards the progression of a "normal" cell to that of a cancerous phenotype.

Experiments with HSV infection showed that external factors can alter the cell's control of U90 expression in Bn5T cells. HSV infection with either type 1

or type 2 virus was found to increase the U90 detected in the cell. In order to investigate if the increase in the U90 was an increase in the incorporation of radiolabel in the U90 or a real increase in the total amount of U90 synthesized, comparative studies using radiolabelling ([^{35}S] L-methionine) and staining for total cell protein (Coomassie blue staining) were carried out.

Autoradiography of control and infected Bn5T polypeptides immunoprecipitated by TBS showed the presence of a greater total amount of radiolabelled U90 in the infected cell compared to the control cell. At least an equivalent increase was observed when the gel was stained with Coomassie blue (Figure 7b) and analyzed by scanning densitometery. This confirmed that HSV infection increased the total amount of U90 polypeptide in the cell as opposed to increasing the metabolic turnover of the U90 and hence the amount of radiolabel it incorporated.

The increase in U90 following HSV infection could have been a consequence of transcriptional activation or post-translational modification resulting in a stabilization of the polypeptide. As no sequence data for U90 was available, the levels of the U90 mRNA before and after HSV infection could not be investigated. As an alternative, the half life of the U90 polypeptide was investigated to establish if HSV infection resulted in a post-translational modification which stabilized the polypeptide and thus increased the total amount of U90 seen in the cell. The half life of the U90 in the uninfected Bn5T cell was found to be in the order 10 hours with the U90 in the HSV infected cell having a half life of around 13 hours. This difference in half life, which is most likely to be due to experimental error, would not explain the 8 fold increase of U90 found in the infected cell immediately after infection. It is therefore unlikely that the U90 is increased by a post-translational event but rather a transcriptional or post-transcriptional event. Both of these events would result in an increased amount of U90 message being present, either by transcriptional activation or by increasing the stability of the message.

The investigations with HSV indicated that the U90 was increased as a consequence of an early event in viral infection, with the greatest increase in U90 synthesis being prior to or at the onset of DNA replication (which is detected at 3 hours post-infection). Neither viral adsorption nor viral entry were sufficient for this induction of the U90, but expression of immediate early genes appeared to be implicated in the U90 induction as over-expression of IE polypeptides by temperature sensitive mutants resulted in increased U90 expression. Vmw175 which was functional for transactivation of early gene expression however, was not found to be essential for U90 induction. Although a mutant in1411 expressing a non-functional greatly truncated Vmw175 could not

induce U90, tsK which over-expresses a non-functional Vmw175, is still capable of U90 induction. Whether Vmw175 acts in a direct or indirect manner could not be determined. Vmw175 has been previously suggested to act in an indirect rather than direct manner in viral replication (Wu et al, 1988). In this study Wu and co-workers used transient assays to determine the essential functions required for viral replication. Vmw175 has also previously been established as having the ability to transactivate gene expression (reviewed in Everett, 1987). This could explain the requirement for Vmw175 in U90 induction, however experiments with transfected Vmw175 and Bn5T cells completed by both Dr J.C.M. Macnab and Helen Ritchie were disappointingly inconclusive.

In subsequent work (Dr J.C.M. Macnab), HSV viral replication was inhibited by phosphonoacetic acid (PAA), resulting in an accumulation of U90 (Grassie et al, 1993). From this it has been postulated that the U90 which is increased by viral IE synthesis is depleted by viral replication. This corresponds to the observation in this section that the U90 is increased by up to 8 fold between 1-3 hours post adsorption, but at later stages in the infection the radiolabelled U90 detected falls to a 3 fold increase (compared to control cells).

A further consequence of HSV infection on U90 in the cell was that it altered the sub-cellular location of the polypeptide. Previous studies (Hewitt et al, 1991), have shown U90 to be primarily located in the membrane associated fraction of the cell. However, following HSV infection, these studies showed that the majority of U90 was found to be in the soluble cytoplasmic fraction of the cell. Analysis of the detergent-solubilized membrane fraction and the nuclear fraction also showed a small amount of U90 to be present in these fractions. It was impossible however to determine if the presence of the U90 in these fractions was due to contaminating U90 from the cytoplasm or whether small amounts of U90 were also present in both of these fractions. Previous work which generated monoclonal antibodies (MAB), raised against DNA binding proteins extracted from HSV-2 infected cells and affinity purified by a DNA binding matrix, facilitated the isolation of an antibody, TG7A which recognised U90 (Macnab et al, 1985a). This indicates that the U90 detected in the nuclear fraction maybe a consequence of U90 associating with a DNA binding protein. It is also possible, that in fact more U90 is present in the nucleus than the IP reaction detected. This could be the case if the U90 were to bind to the DNA or become associated with a DNA binding complex, so resulting in a conformational change or masking the antigenic site recognised by the antibody such that the U90 was not recognised in an IP reaction. The small proportion of the U90 immunoprecipitated in the nuclear fraction is probably not complexed with other proteins or DNA and hence can react with the antibody in an IP reaction.

The presence of the U90 in the nucleus could now be investigated by western blotting the three sub-cellular fractions, an experiment which could not be carried out at the time as an antibody which recognised U90 under western blotting conditions was not yet available.

In summary, the work described in this section to characterize the U90, showed the U90 was present in a number of cell lines of different types and species, including human cells. The total amount of U90 isolated from the cell could be increased by HSV-1 and HSV-2 infection in all cells tested, where the increase in the total amount of U90 occurred in a manner which did not alter the half-life of the polypeptide. An early event in HSV infection was responsible for this increase with Vmw175 playing a essential role. However, whether Vmw175 acts in a direct or indirect manner is uncertain.

Following HSV infection the increased U90 was altered in its location and found predominantly in a soluble form in the cytoplasmic fraction. This solubility compounded with the increase in the total amount of U90 offered the opportunity to purify the U90 with much greater ease from the cytoplasmic fraction of HSV infected Bn5T cells as compared to purifying it from the membrane fraction of uninfected Bn5T cells.

These results were used to define the conditions of the starting material to give high levels of expression of the U90 and hence optimize the purification of the U90. The purification strategy and how it was achieved will be discussed in section 3.

SECTION 2 : HSV-1 INFECTION ALTERS GRP94 EXPRESSION

1. HSV-1 Infection Alters GRP94 Expression

During the characterization of the U90 polypeptide expression in RE and Bn5T cells, it was consistently noted that a polypeptide of a slightly higher molecular weight than U90 (as seen by SDS page) was immunoprecipitated by TBS from HSV-1 infected Bn5T cells and HSV-1 infected RE cells. This section outlines the analysis of this polypeptide.

Infection of control RE cells with 5-10pfu/cell of HSV-1 for 17 hours at 37°C resulted in the induction of a polypeptide of approximately 94,000 molecular weight, as seen by immunoprecipitation with TBS of [^{35}S] L-methionine radiolabelled polypeptides and analysis by SDS page. On going studies at this time were investigating the possibility that the U90 was a stress protein, related to either the heat shock protein hsp90, or the glucose regulated polypeptide GRP94. This was possible as TBS had been previously shown to possess activity against GRP94 (Amy Lee, personal communication). To investigate this, GRP94 was induced in RE cells by treating with either 50mM tunicamycin, (Olden et al, 1979, Duskin & Mahoney, 1982), 6X10^{-7}M calcium ionophore A23187 (Welsh et al, 1983, Drummond et al, 1987) or growing the cells in medium lacking glucose (Pouyssegur et al, 1977). Tunicamycin induces GRP94 as a consequence of the inhibition of glycosylation of cell polypeptides (Olden et al, 1979). This has recently been reported to cause the malfolding of polypeptides which in turn induces GRP94 (Kozutsumi et al, 1988). Calcium ionophore A23187 is thought to induce both GRP94 and GRP78 which are coordinately regulated (Chang et al, 1989), by depleting intracellular calcium stores (Drummond et al, 1987). Hsp90 was induced by heat shocking the cells at 42°C for 2 hours then transferring them to 37°C where they were radiolabelled either immediately, or after 30 minutes for 6 hours (Hewitt et al, 1991). Comparison of the U90 to the induced stress proteins by SDS page showed the U90 to be quite distinct (Hewitt, 1988). Peptide mapping analysis and comparison of the U90 partial amino acid sequence (discussed later in Results Section 4.6) to the published GRP94 and hsp90 sequences also showed U90 to be distinct from either of these proteins. However comparison of RE and Bn5T cells infected with HSV-1 with cells treated with either tunicamycin (Bn5T cells) or the calcium ionophore A23187, showed that the polypeptide of molecular weight 94Kda induced by HSV-1 migrated at the same position as GRP94 on SDS page (Figures 12 & 13a &b respectively).

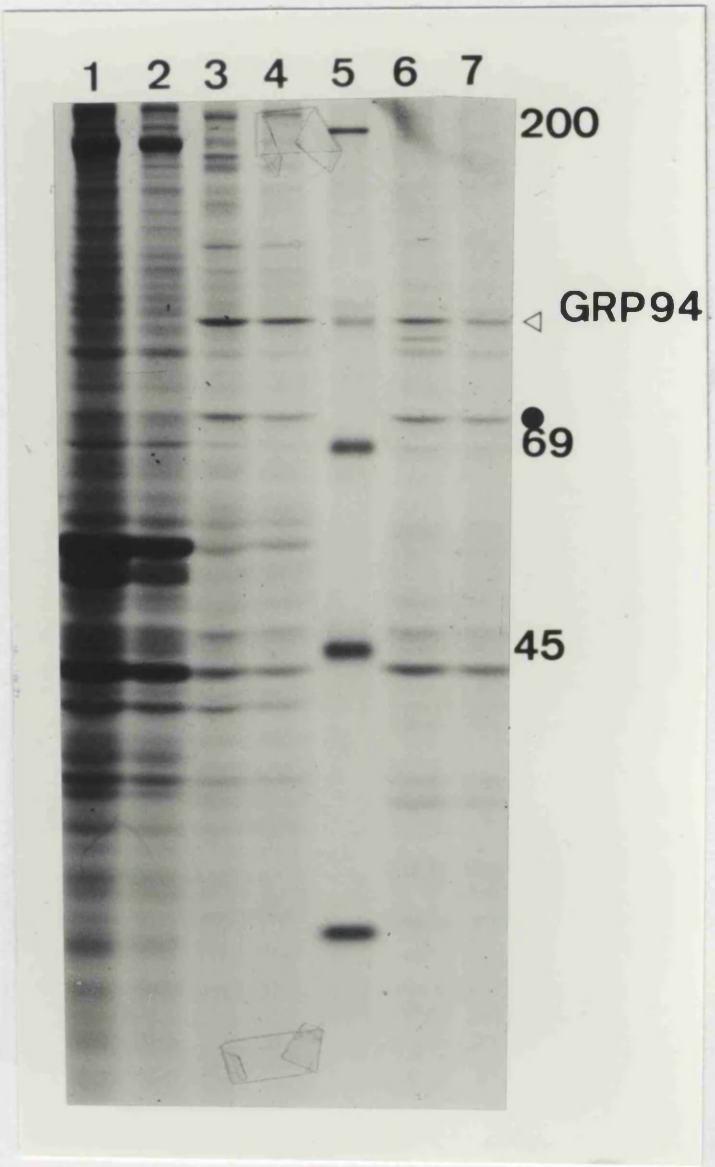


FIGURE 12 : Autoradiograph of IP reactions with TBS (tracks 1, 3 & 6) and Mab PR-6 specific for GRP94 (tracks 2, 4 & 7) using $4X10^6$ cpm each of [^{35}S] L-methionine radiolabelled polypeptides extracted using RIPA buffer from control Bn5T cells (tracks 1 & 2), tunicamycin treated Bn5T cells - final concentration 2ug/ml for 17 hours (tracks 3 & 4) and HSV-1 infected Bn5T cells - with 10pfu/cell for 17 hours at 37°C (tracks 6 & 7). GRP94 is indicated by a triangle and GRP78 by •. Molecular weight markers are shown in track 5 and values in kilodaltons indicated to the right of gel.

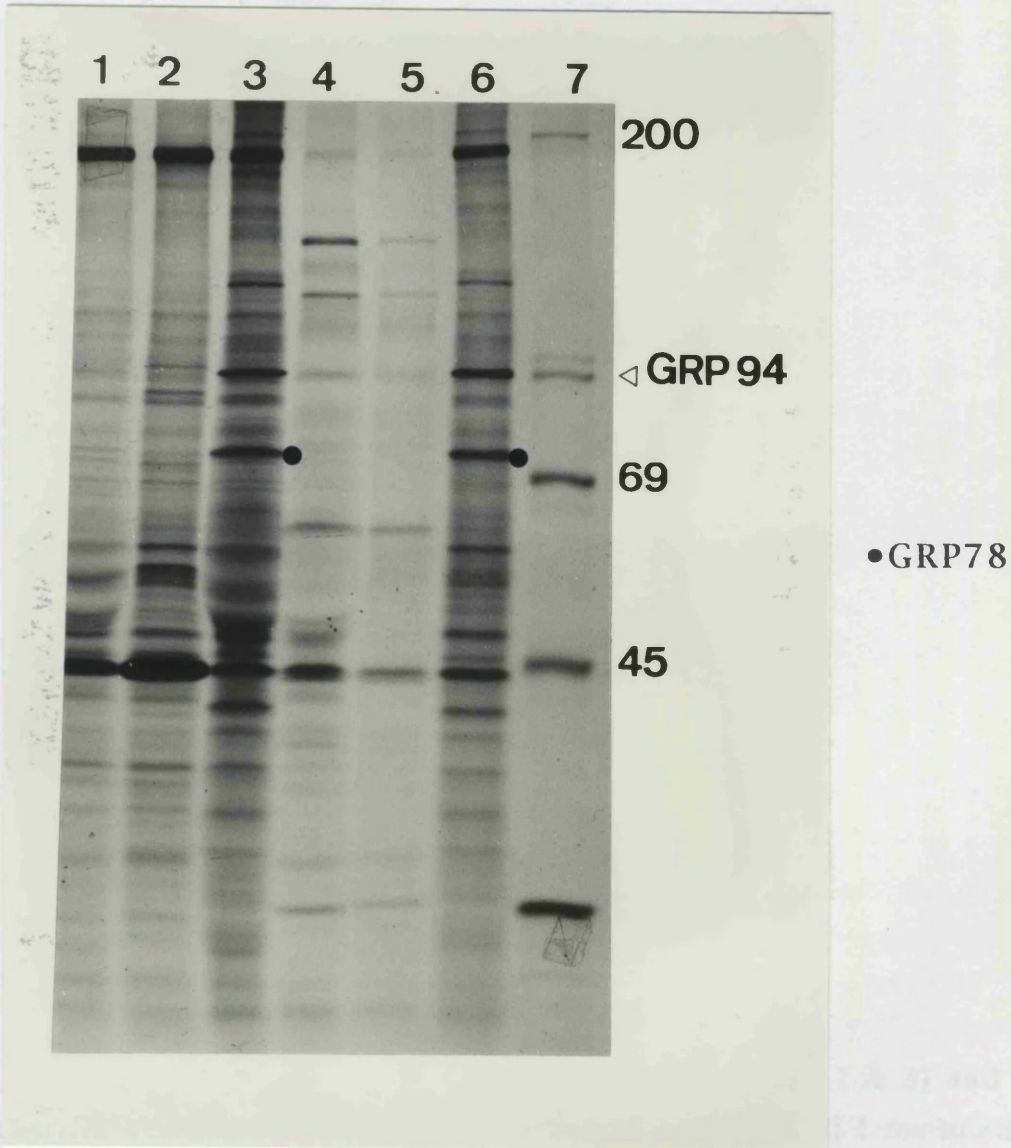


FIGURE 13a : Autoradiograph of IP reactions with TBS (tracks 1, 3 & 4) and the polyclonal monospecific antibody against GRP94 (tracks 2, 5 & 6) using $4X10^6$ cpm each of [^{35}S] L-methionine radiolabelled polypeptides extracted using RIPA buffer from control RE cells (tracks 1 & 2), tunicamycin treated RE cells - final concentration of 2ug/ml for 17 hours (tracks 3 & 6) and HSV-1 infected RE cells - with 10pfu/cell for 17 hours at 37°C (tracks 4 & 5). GRP94 is indicated by a triangle and GRP78 by •. Molecular weight markers are shown in track 7 and values in kilodaltons indicated to the right of gel.

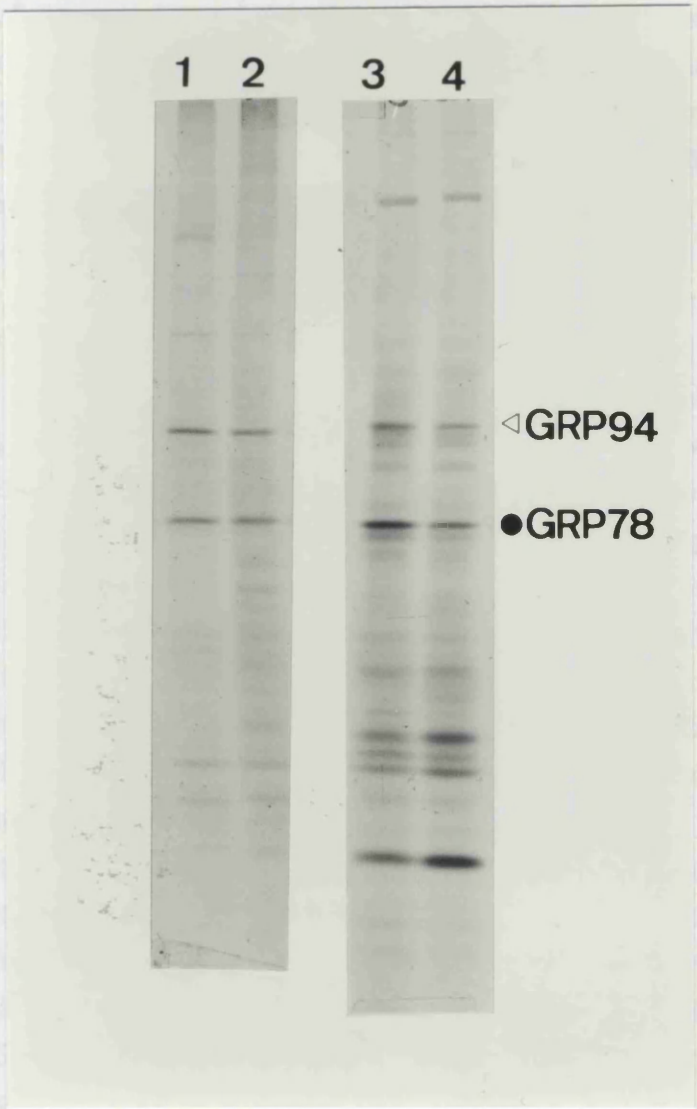


FIGURE 13b : Autoradiograph of IP reactions with TBS (tracks 1 & 3) and Mab PR-6 against GRP94 (tracks 2 & 4) using $4X10^6$ cpm each of [^{35}S] L-methionine radiolabelled polypeptides extracted with RIPA buffer from tunicamycin treated RE cells, 2μg/ml final concentration for 17 hours (tracks 1 & 2) and calcium ionophore treated RE cells - at a final concentration of $6x10^{-7}$M for 17 hours (tracks 3 & 4). Both GRP94 (triangle) and GRP78 (•) can be seen in all four tracks as they are coordinately regulated (Chang et al, 1989). In tracks 3 & 4 a number of forms of GRP94 can be seen due to the varying extent of glycosylation induced by the calcium ionophore. GRP94 is not expressed in control cells as shown in Figures 13a and 14.

As indicated above TBS had previously been shown to have antibody activity against GRP94 (Amy Lee, personal communication). This was confirmed by a series of immunoprecipitation reactions of tunicamycin treated radiolabelled cells using different antibodies with activity against GRP94 after a control experiment was completed to ensure that GRP94 did not non-specifically bind to Pansorbin protein A complexes. IP reactions using TBS, a monoclonal antibody MAb PR-6 against GRP94, a gift from Prof. D. Edwards and a monospecific polyclonal antibody raised against endoplasmin (ie GRP94, Smith & Koch, 1987), a gift from Dr Gordon Koch, indicated that TBS did in fact recognize GRP94 (Figure 14). These observations suggested that the HSV-1 induced 94,000 molecular weight polypeptide may in fact be GRP94.

To investigate this further the GRP94 specific antibodies were used to see if they recognised the HSV-1 induced polypeptide. This included MAb PR-6, the polyclonal antibody raised against endoplasmin and an antipeptide antibody raised against the first 20 amino acids of the amino terminal region of GRP94, a gift from Drs Ullrich and Appella (detailed in Materials: Section 7). IP reactions were carried out using these antisera with RE cells and Bn5T cells radiolabelled with [^{35}S] L-methionine and treated with tunicamycin, calcium ionophore or infected with HSV types 1 and 2. Analysis of these IP reactions by SDS page followed by autoradiography, resulted in the visualization of a 94,000 molecular weight polypeptide in the HSV-1 infected cells and the tunicamycin treated cells (Figures 12 & 13a). This 94,000 molecular weight polypeptide could not be immunoprecipitated from either the control cells or the HSV-2 infected cells (Figure 15). This difference in induction between HSV-1 and HSV-2 was seen in a minimum of 5 experiments. In track 2 of Figure 15 a reduced amount of viral polypeptides can be seen suggesting that infection in this sample was less efficient. This explains the lesser amount of GRP94 induced under these conditions. From these experiments it was concluded that HSV-1 infection induced a polypeptide immunologically indistinguishable from GRP94.

To investigate the mechanism of induction of this polypeptide a series of temperature sensitive HSV-1 mutants were used.

2 Investigation Of GRP94 Induction Using HSV-1 Mutants

Infection of RE cells with temperature sensitive (ts) mutants of the Vmw175 gene at the permissive (PT) and non-permissive temperatures (NPT) resulted in the induction of GRP94 to various extents. Infection with HSV-1 tsD, tsK and tsT, all of which are mutated in the immediate early gene Vmw175, resulted in the induction of GRP94 at their PT (31^{o}C) as expected however at their NPT (38.5^{o}C), tsD and tsT induced GRP94 to a small degree, whereas tsK did

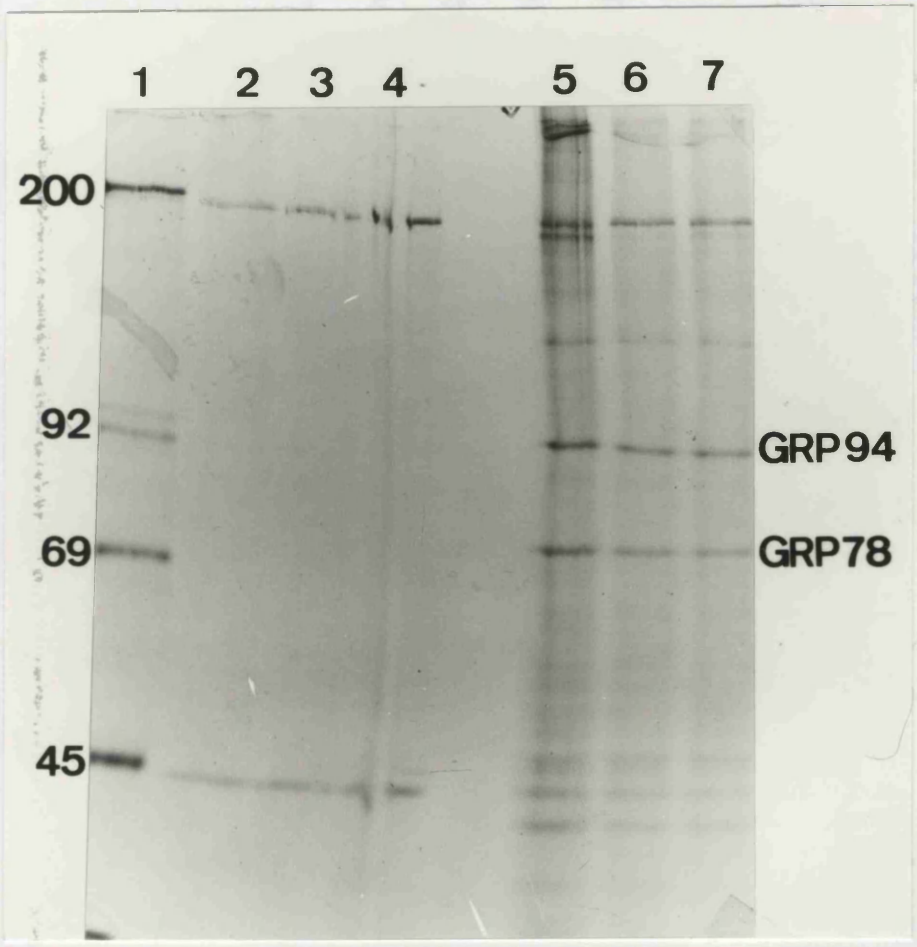


FIGURE 14 : Autoradiograph of IP reactions with TBS (tracks 2 & 5), polyclonal monospecific antibody against GRP94 (tracks 3 & 6) and Mab PR-6 specific for GRP94 (tracks 4 & 7) using $4X10^6$ cpm each of [^{35}S] L-methionine radiolabelled polypeptides extracted with RIPA buffer from control RE cells (tracks 2, 3 & 4) and tunicamycin treated RE cells - final concentration of 2ug/ml for 17 hours (tracks 5, 6 & 7). Molecular weight markers are shown in track 1 and values in kilodaltons indicated to the left of the gel.

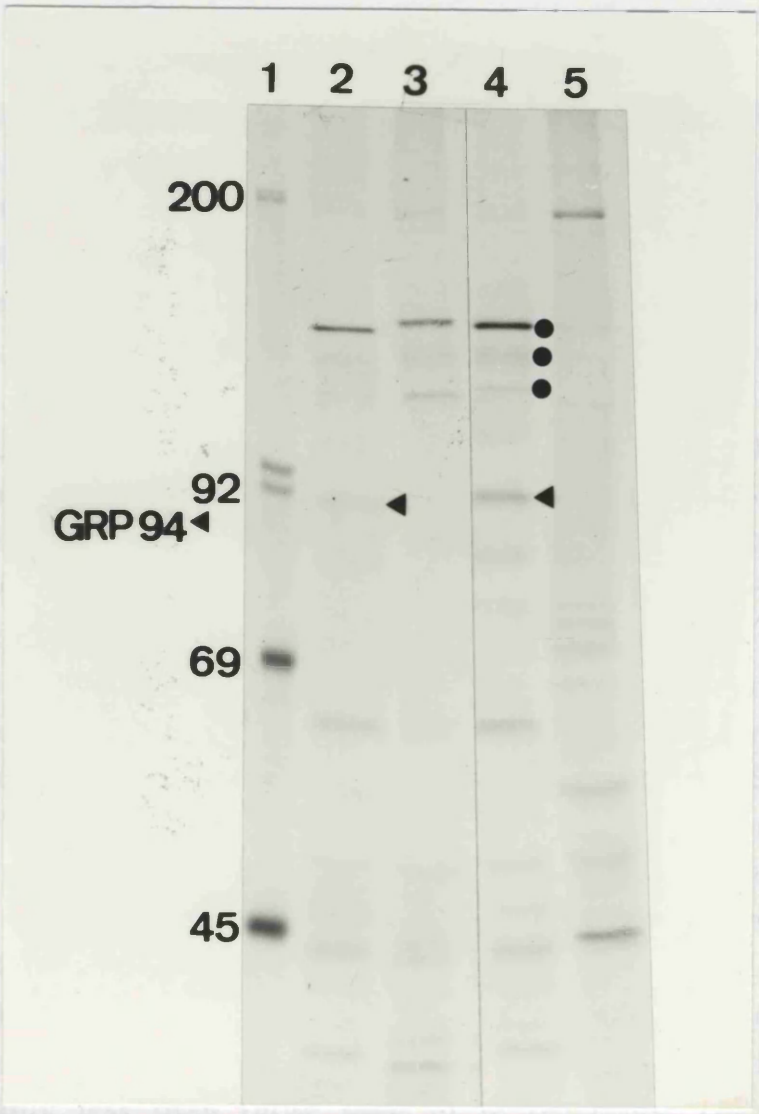


FIGURE 15 : Autoradiograph of IP reactions with TBS using $4X10^6$ cpm each of [^{35}S] L-methionine radiolabelled RE cell polypeptides extracted with RIPA buffer from control cells (tracks 5), cells infected with HSV-1 (tracks 2 & 4) and HSV-2 (track 3). Cells were infected at 10pfu/cells for 17 hours at 37^{o}C. GRP94 is less abudant in track 2 compared with track 4 because track 2 is less well infected as assessed by the presence of IE polypeptides. This is thought to be due to the reduced efficiency of HSV-1 infection in this sample as less viral polypeptides (175, 155 & 136 as denoted by •) are present. The corresponding molecular weights of HSV-2 IE polypeptides are 182K, 157K and 140K respectively. Molecular weight markers are shown in track 1 and values in kilodaltons indicated to the left of the gel.

not (Figure 16). Two possible explanations for this observation are proposed; (1) a functional wild type (WT) Vmw175 may be necessary for GRP94 induction. At the NPT no functional WT Vmw175 should be expressed by any of these three viruses, however tsD and tsT which are mutated at the carboxy terminus of Vmw175 are less efficient at preventing early and late viral protein synthesis (Preston, 1981) and induce some GRP94. This would suggest that the need for a functional Vmw175 is not an appropriate explanation for GRP94 induction. (2) An alternative explanation is that a later stage in viral infection than IE gene expression is essential for GRP94 induction. All three viruses over-expressed the IE genes, but tsD and tsT differ slightly from tsK in that they allow partial expression of early and late genes (Preston, 1981). This would suggest that the induction of GRP94 occurs after immediate early gene expression.

3 Does HSV-1 Infection Increase The Absolute Amount Of GRP94?

Quantitative analysis of GRP94 induction by HSV was carried out by western blotting. This investigated the total amount of GRP94 recognised by MAb PR-6 rather than the total amount of native radiolabelled GRP94 recognized in IP reactions (which is a function of half life) as was previously investigated. The same total number of cells were used when control RE cells or RE cells infected by HSV-1 were analyzed. Western blotting was carried out with these samples and the antibody MAb PR-6. Using 50μg and 25μg of each sample, the optimal protein-antibody concentration could be determined in order to compare the relative amounts of GRP94 in the control and infected samples (Figure 17). These blots confirmed that HSV-1 infection resulted in elevated levels of GRP94 being present in the RE cells when compared to control RE cells. Constitutively expressed GRP94 could also be detected in control RE cells by western blotting as the levels of background protein seen in such blots were considerably less than those seen in IP reactions. The western blotting experiment indicated that the total amount of GRP94 is increased as a consequence of HSV-1 infection. This shows that the increase in GRP94 as seen by radio-immunoprecipitation is unlikely to be due to an increased rate of [^{35}S] methionine incorporation, although alteration in the half life or stability of the protein cannot be excluded.

Northern and slot blot experiments were carried out by Carol Sievwright (a post-graduate student in the lab.), to investigate if HSV-1 induced GRP94 at the level of transcription. RNA was extracted from control RE cells, RE cells treated with calcium ionophore (which is known to induce GRP94) or infected with HSV-1 (for 6 hours at 37°C). The extracted RNA was run on a formaldehyde gel, blotted onto nitrocellulose then probed with a fragment of the GRP94 cDNA

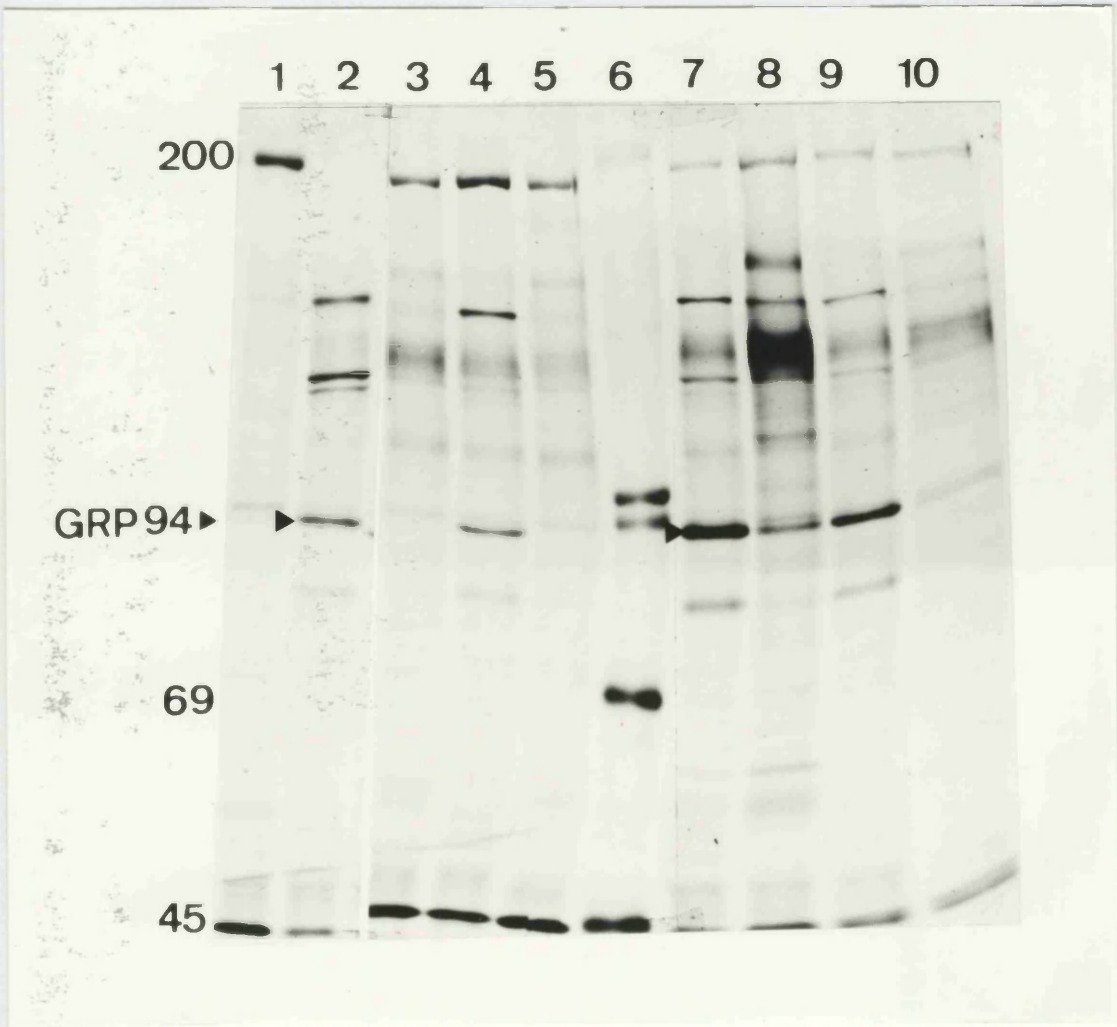


FIGURE 16 : Autoradiograph of IP reactions with TBS using $4X10^6$ cpm each of [^{35}S] L-methionine radiolabelled polypeptides extracted with RIPA buffer from control RE cells (track 1), RE cells infected with HSV-1 at 37°C for 17 hours (track 2), tsK at 38.5°C for 8 hours (tracks 3 & 5), tsK at 31°C for 17 hours (track 4), tsT at 31°C for 17 hours (track 7), tsT at 38.5°C for 8 hours (track 8), tsD at 31°C for 17 hours (track 9) and tsD at 38.5°C for 8 hours (track 10). Molecular weight markers are shown in track 6 and values in kilodaltons indicated to the left of gel. GRP94 is indicated by a triangle.

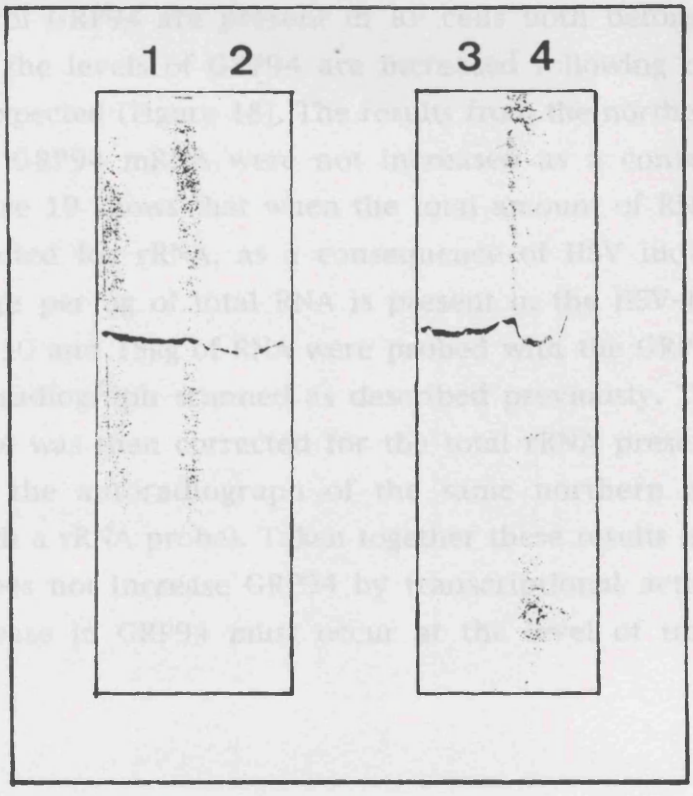


FIGURE 17 : Western blot with polyclonal monospecific GRP94 antibody and polypeptides extracted from a total cell extract of control RE cells (tracks 2 & 4) and HSV-1 infected RE cells using 10pfu/cell for 17 hours at 37°C (tracks 1 & 3). Tracks 1 & 2 represent 50µg and tracks 3 & 4 represent 25µg of cellular polypeptides as quantitated using the Bradford's method. To visualise, anti-rabbit immunoglubulin conjugated to horse radish peroxidase was used with N'chloronapthol. As stated previously western blots were reproduced by a combination of laser printing and photocopying which neither introduced nor removed any background material.

called p4A3. This 1.75Kb probe was generated following a Bam H1/Sal 1 digestion of a cDNA encoding GRP94 and was kindly supplied by Dr Amy Lee. The probe was then radiolabelled with $\alpha^{32}P$ by random priming (to a high specific activity of greater than $2X10^8$ cpm). To confirm that equivalent quantities of RNA were loaded on each track, a ribosomal RNA probe (Barbu & Dautry 1989) was used to reprobe the northern blot.

From the slot blot (using 20μg/track) it was seen on three occasions that similar levels of GRP94 are present in RE cells both before and after HSV-1 infection, but the levels of GRP94 are increased following calcium ionophore treatment as expected (Figure 18). The results from the northern blots also show that levels of GRP94 mRNA were not increased as a consequence of HSV-1 infection. Figure 19 shows that when the total amount of RNA loaded on each track is corrected for rRNA, as a consequence of HSV induced shutoff, less GRP94 message per ug of total RNA is present in the HSV-1 infected cells. In these blots 5, 10 and 15μg of RNA were probed with the GRP94 insert, and the resulting autoradiograph scanned as described previously. The GRP94 present in each sample was then corrected for the total rRNA present in each sample (by scanning the autoradiograph of the same northern after it had been hybridized with a rRNA probe). Taken together these results indicated that HSV-1 infection does not increase GRP94 by transcriptional activation but rather that the increase in GRP94 must occur at the level of translation or post-translation.

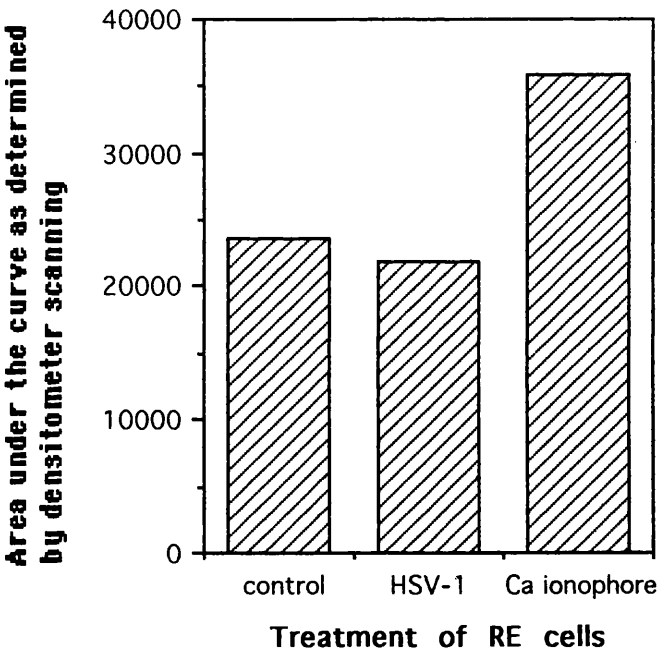


FIGURE 18 : Densitometer scan of autoradiograph of slot blot where 15μg of RNA extracted from control RE cells, HSV-1 infected RE cells (10pfu/cell for 6 hours at 37°C) and RE cells treated with calcium ionophore ($6X10^{-7}M$) were loaded on each track and the GRP94 cDNA (p4A3) used to probe the blot. Data corresponds to the area under the peak when quantitated by scanning densitometery (an average of 11 scans), as described previously. This slot blot was kindly probed by Carol Sievwright.

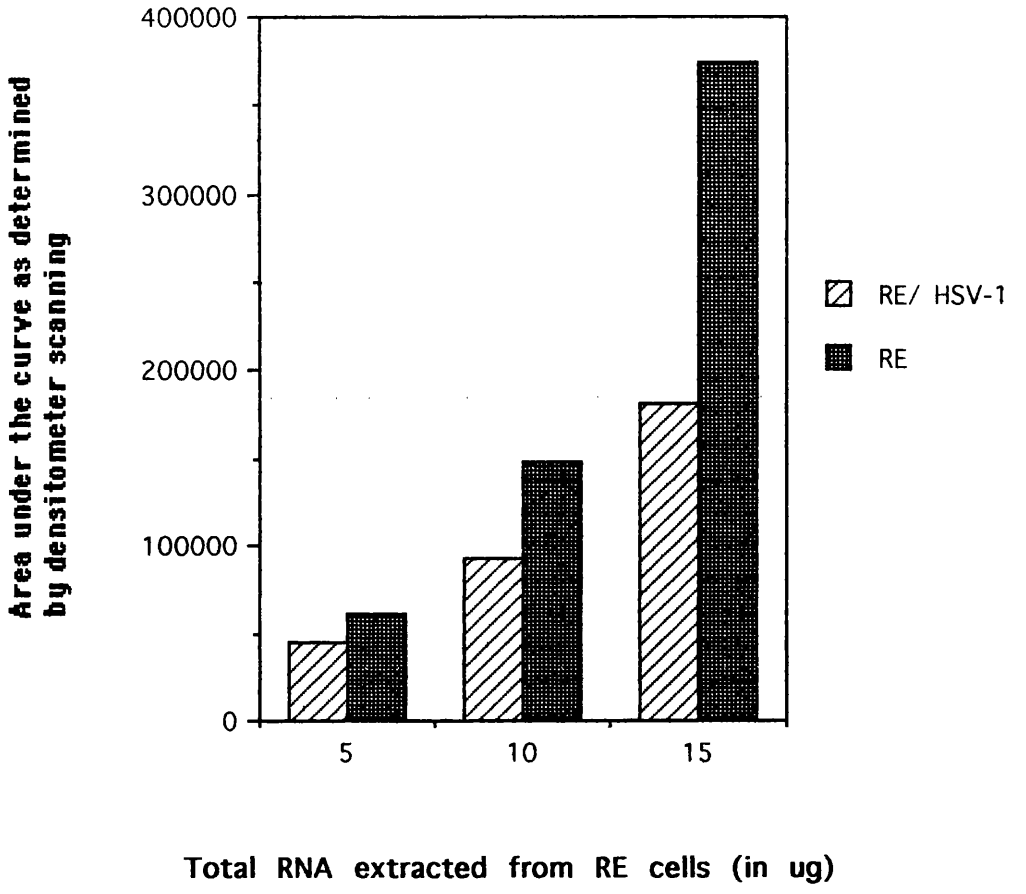


FIGURE 19 : Densitometer scan of autoradiograph of northern blot where 5, 10 and 15μg of RNA was extracted from control RE cells and HSV-1 infected RE cells (10pfu/cells for 6 hours at 37°C) and probed with the GRP94 cDNA (p4A3) probe. The blot was re-probed using a rRNA probe to ensure that similar quantities of RNA were loaded on the appropriate control and HSV-1 tracks. Data corresponds to the area under the peak when quantitated by scanning densitometery and corrected for rRNA. This northern blot was kindly probed by Carol Sievwright.

DISCUSSION OF SECTION 2 : HSV-1 INFECTION ALTERS GRP94 EXPRESSION

An interesting but complex observation is reported in this section. During the investigation of U90 expression in Bn5T and RE cells, a 94Kd polypeptide was consistently found to be induced by HSV-1, but not by HSV-2 infection. This polypeptide has been characterized as GRP94, which is a major structural component of the endoplasmic reticulum (ER) and the most abundant calcium binding protein in the ER (Koch et al, 1986). GRP94 is constitutively expressed at low levels in the cell and has enhanced expression following glucose deprivation, inhibition of glycosylation (Pouyssegur, et al, 1977; Chang et al, 1987), and pertubances of intracellular calcium stores (Welch et al, 1983; Drummond et al, 1987; reviewed in Lee, 1987). GRP94 is also thought to be induced by the presence of malfolded proteins in the ER (Kozutsumi et al, 1988). GRP94 together with a coordinately regulated glucose regulated polypeptide GRP78 (Chang et al, 1987) have been reported to regulate protein transport through the ER by their calcium binding capacities (Li & Lee, 1991).

GRP94 is a member of the stress protein family which is comprised of heat shock proteins and glucose regulated proteins. These polypeptides show a 50% homology with each other at the amino acid level (Mazzarella & Green, 1987; Sorger & Pelham 1987). This family of polypeptides has been previously implicated with HSV infection. Notarianni & Preston (1982) and Notarianni, (1986) reported the induction of stress proteins following HSV infection in chick embryo cells, La Thangue et al, (1984) reported the accumulation of heat shock proteins following HSV infection, Kennedy et al, (1985) reported the accumulation of heat shock proteins on acute HSV infection of cultured human neural cells and La Thangue & Latchman (1988) reported the induction of a hsp90-like polypeptide following HSV infection.

At the time of these investigations the mutant HSV-1 viruses tsD, tsK and tsT were being utilized to investigate the induction of U90. Experiments using these mutants were carried out to determine if IE gene expression was sufficient for GRP94 induction. Using immunoprecipitation techniques and TBS which has activity against GRP94 (Amy Lee, personal communication) immediate early expression seen with tsK at 38.5°C was found to be insufficient for GRP94 induction. As indicated in the previous Result Section 2.2, tsD and tsT do allow partial early and late gene expression at 38.5°C (Preston, 1981). This suggested that either early or late viral gene expression must occur for the alteration of GRP94 expression, or that some additional difference between tsK and tsD & tsT which is as yet undetermined is involved in GRP94 induction. This area could

not be expanded as I decided to concentrate on the induction of the U90 by HSV infection, which was transformation associated.

The northern blot results indicate that the increase in GRP94 is not a consequence of transcriptional activation or an increase in mRNA stability as measured during the first 6 hours after infection. The observed increase in GRP94 must therefore either be due to increased rate of translation of the mRNA or a post-translational modification which results in the stabilization of the protein, eg phosphorylation, glycosylation, sulphation or lipid modifications. In order to investigate if the total amount of GRP94 expressed in the cells was increased following HSV-1 infection or if the metabolic rate of [^{35}S] methionine incorporation was increased, western blots using a GRP94 specific antibody were carried out. Comparison of the results obtained in the western blot and those obtained following IP studies indicated that the increase seen in the radio-immunoprecipitated GRP94 corresponds to the increase in the total amount of GRP94 seen in the western blot. From these experiments it could be concluded that an increase in the total GRP94 translated occurred rather than an increased in radiolabel incorporation and turnover of the protein.

It can be clearly seen that HSV-1 but not HSV-2 infection increases GRP94. This further confirms that differences exist between the two viruses and their host cell interactions. It may be a distinction such as this ability or lack of ability to induce GRP94 that plays a role for example in the association of HSV-2 with certain tissue types, and HSV-1 with others. It can be concluded from this section that an interesting distinction between HSV types 1 and 2 host cell interaction has been uncovered, and further investigations may yield important results with regard to the differences which exist between HSV types 1 and 2 host cell interactions.

SECTION 3 : PARTIAL PURIFICATION OF U90

Partial purification of the U90 was initiated to enable the isolation of sufficient U90 as a discrete protein band, following western blotting, in order that N-terminal amino acid sequencing could be carried out. To purify the U90, 10 burlers of Bn5T cells infected with HSV-2 at 5-10pfu/cell were used to give maximal U90 expression. In addition to the increase in the total amount of U90 following HSV infection, the U90 was found in greater abundance as a soluble polypeptide in the cytoplasm of the cell. This greatly simplified the purification process. In developing the purification strategy, using HSV infected Bn5T cells to purify the U90 resulted in the elimination of a number of steps and so reduced the inevitable protein losses which occur at every stage in a purification scheme.

STEP 1 : Optimizing Protein Extraction

Ion exchange chromatography and selective protein precipitation (or salting out) methods were used to purify the U90 polypeptide. The buffers used to extract the U90 from the cells therefore had to be compatible with these purification techniques and were chosen, or developed with this in mind.

Previously general analysis of cell polypeptides was carried out using radio-immunoprecipitation (RIPA) buffer (Macnab et al, 1985a; Hewitt et al, 1991) as described in the Methods, Section 6a. Although RIPA buffer was very efficient at solubilizing cell polypeptides (including the U90), it contained the ionic detergent SDS which was incompatible with automated ion exchange chromatography using a mono Q column and therefore RIPA could not be used. This was as a consequence of irreversible binding of SDS to the mono Q column. The detergent SDS is almost impossible to remove from a protein sample once it has been added, although a method has been devised which claims to remove the vast majority of SDS (Fischer et al, 1989). After using this method however, it cannot be determined if all the SDS has been removed. Furthermore, this procedure also requires the sample to be passed over a "fast desalt" column (Pharmacia, England) to remove excess urea, an extra step which would also increase the loss of material.

An alternative buffer lacking SDS, had to be developed which was suitable for use on the different chromatography systems, but was still able to efficiently extract the small quantities of U90 present in the cell. Several non-ionic detergent containing buffers were analyzed for their ability to extract the U90. These included Michelson's buffer, (50mM tris pH7.4, 1mM beta mercaptoethanol, 2mM EDTA, 1% NP40, 1% sodium deoxycholate, 0.1mM PMSF,

1mM DTT), Toft's buffer (50mM tris pH7.4, 10mM EDTA, 50mM NaF, +/-10mM $NaMoO_4$) and PBS A plus 1% triton-X-100. This last buffer was found to give very good extraction of the U90 from whole cells (Figure 20), and would allow the U90 to be extracted from uninfected Bn5T membranes. In addition to triton-X-100 the efficiency of two other detergents (NP40 and octyl glucoside) at extracting U90 was investigated by Dr J.C.M. Macnab. To determine if either NP40 or octyl glucoside were better at solubilizing the U90, IP reactions were carried out and comparison of the total amount of U90 immunoprecipitated and the amount of background protein present in each reaction investigated. Of the three, triton-X-100 was found to give the best results with the least background protein being present at around 90K in IP reactions (Macnab, unpublished results). Triton-X-100 was therefore chosen to be used as a 1% solution in PBS A in the purification strategy.

In order to reduce the number of proteins in the starting material it was decided to fractionate the cells by using various combinations of hypotonic buffer (25mM tris pH7.5, 5mM KCl, 0.5mM $MgCl_2$, 0.5mM PMSF, 1mM DTT), dounce homogenization (using pestle B to break the cell membrane which was followed by pestle A to break the nuclear membrane), sonication and addition of detergent containing buffers such as Michelson's, Toft's or triton-X-100 buffer (1% triton in PBS A). These were used to generate the cytoplasmic, nuclear and membrane fractions of harvested cells (Method 6b). The U90 was detected by IP reactions of Bn5T cells extracted in all the buffers investigated, however when IP reactions were carried out considerable differences in the amount of background protein and the total U90 extracted from the cell was seen depending on which buffer was used.

The most efficient cell fractionation method for the isolation of U90 (described in Method 6b) used hypotonic buffer (as above) to swell the cells (on ice for 30 minutes) which were then lysed by dounce homogenization with pestle B to break open the cell membrane and release the soluble cytoplasmic fraction. The nuclear and membrane associated proteins were then removed from the cytoplasmic sample by centrifugation (3000rpm for 10 minutes at $4^{o}C$) generating a pellet which was then extracted using the nuclear extraction buffer (20% glycerol, 25mM tris pH 7.5, 0.6mM KCl plus 1mM PMSF + 1mM DTT) for 30 minutes at $4^{o}C$ and followed by dounce homogenization with pestle A. The centrifugation was repeated and finally the resulting pellet was treated with the membrane extraction buffer which contained the detergent triton-X-100 and sonicated to disrupt the cell membranes and release membrane associated polypeptides.

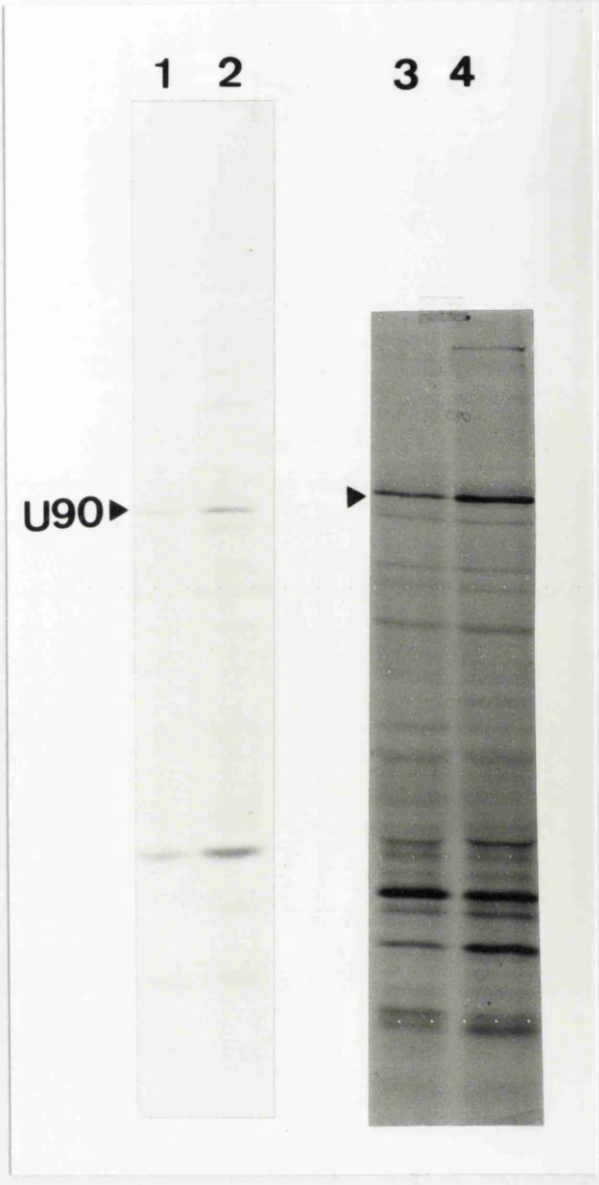


FIGURE 20 : Autoradiograph of IP reactions with TBS using $4X10^6$ cpm each of [^{35}S] L-methionine radiolabelled polypeptides extracted from Bn5T cells using either 1% triton X-100 in PBS (tracks 1 & 2) or RIPA buffer (tracks 3 & 4). Control Bn5T cells can be seen in track 1, HSV-2 infected cells in tracks 2 and 3 and HSV-1 infected cells in track 4. Virus was added at 10pfu/cell for 17 hours at 37°C.

In order to increase the speed of cell fractionation, and in doing so reduce the possibility of protein degradation, the above method was modified. This modified version which was used for the large scale extraction of the U90 from HSV infected and control cell membranes is described Methods 6c. This method differs from the cell fractionation described above as one step is removed. In the large scale extraction the nuclear fraction is not generated, but instead the membrane associated U90 is extracted from a sample which contains both the membrane and nuclear fraction. This had very little effect on the amount of background polypeptides in the membrane fraction, as seen in IP reactions, nor did it affect the total amount of U90 recovered, but most importantly the speed of the fractionation process was increased considerably by using this modification.

STEP 2 : Ammonium Sulphate Precipitation of U90

Following cell fractionation, the first step in the purification of the U90 was the "salting out" or selective precipitation of proteins by ammonium sulphate (AS) saturation of the cytoplasmic fraction of the HSV infected Bn5T cells (Method 8a). AS was gradually added at a consistent rate to the sample containing U90 over a period of 30 minutes to give a 30% saturated solution. This was then stored on ice with agitation for 1 hour, which resulted in the precipitation of the U90 together with a number of other cellular polypeptides. The addition of the AS was carefully monitored to ensure that it was added to the sample in an identical manner in each experiment. This was necessary as a change in the rate of addition of AS could result in an altered set of polypeptides being precipitated due to the different conditions.

The precipitated U90 was then isolated from the 30% AS saturated sample by centrifugation at 10,000rpm for 15 minutes ($4^{o}C$). The pellet containing the U90 was then resuspended in a detergent containing resupsension buffer composed of 1% triton-X-100 in PBS A.

At this stage the partially purified U90 was found to form highly insoluble aggregates if detergent was not included in the resuspension buffer. This may have been as a consequence of the AS precipitation disrupting protein-protein interactions which may have stabilized the U90 in its native form. For this reason, all buffers from this stage onwards contained a minimum of 0.1% triton-X-100 to prevent the U90 forming aggregates and precipitating out of the sample.

STEP 3 : Purification By Ion Exchange Chromatography

As indicated above the AS precipitated U90 was resuspended in 1% triton-X-100 in PBS A. This was stored on ice for a minimum of 30 minutes then sonicated until clear to disrupt any protein aggregates which had formed. The sample was then desalted (to remove the AS) by passing it over a PD10 sephadex G-25 column and the protein eluted into running buffer A (50mM tris pH8 +/- 1% or 0.1% triton-X-100) which was to be used on the FPLC, as described in Method 8b. If the samples were not injected onto the FPLC column immediately after desalting, they were sonicated again just before use. Prior to loading the sample onto the automated FPLC ion exchange system (Pharmacia, England) the sample was centrifuged for 5 minutes at 12,000rpm to remove any particulate material which might clog the filters within the FPLC system. The supernatant was then ready to be injected onto a 1ml anion exchange column, (mono Q HR5/5, Pharmacia, England) using a "superloop" which enabled a volume of up to 50ml to be loaded at one time onto the column. This proved extremely useful as when the precipitated U90 was resuspended in a small volume of buffer, the recovery of the polypeptide was found to be reduced. The superloop therefore allowed the protein to be resuspended in a larger volume and still be injected onto the column in one run.

After desalting the sample and prior to injection onto the column, the total protein content of the sample was determined. This was done using the Bradford method of protein quantitation (Method 7h) and was used to ensure that no more than approximately 10mg of protein was ever loaded on the column, as the column could only accommodate a maximum of 20mg of protein.

Three different sets of buffers were used in the FPLC system throughout the period in which the purification strategy was being developed, as detailed in Method 8(a), The first set, A.1 (50mM tris pH8) and B.1 (50mM tris pH8 + 1M NaCl) contained no detergent and was used in the initial attempts to purify the U90.

In initial experiments the U90 sample was injected onto the mono Q column in buffer A.1 and eluted using a 17ml linear gradient of 0-100% buffer B.1 with 1ml samples being collected. SDS page analysis of the samples, which contained a small proportion of [^{35}S] L-methionine radiolabelled polypeptides followed by autoradiography revealed a 90K protein to be eluted from the column between 76-94% buffer B, when the gradient was run over 17 minutes at a flow rate of 1ml buffer/per minute (Figure 21).

A very faint 90K polypeptide isolated from the mono Q column in fractions 20 and 21 (tracks 11 & 12 of Figure 21) reacted with TBS in IP reactions and migrated on SDS gels at a similar rate to the U90 (Figure 22). However, such a

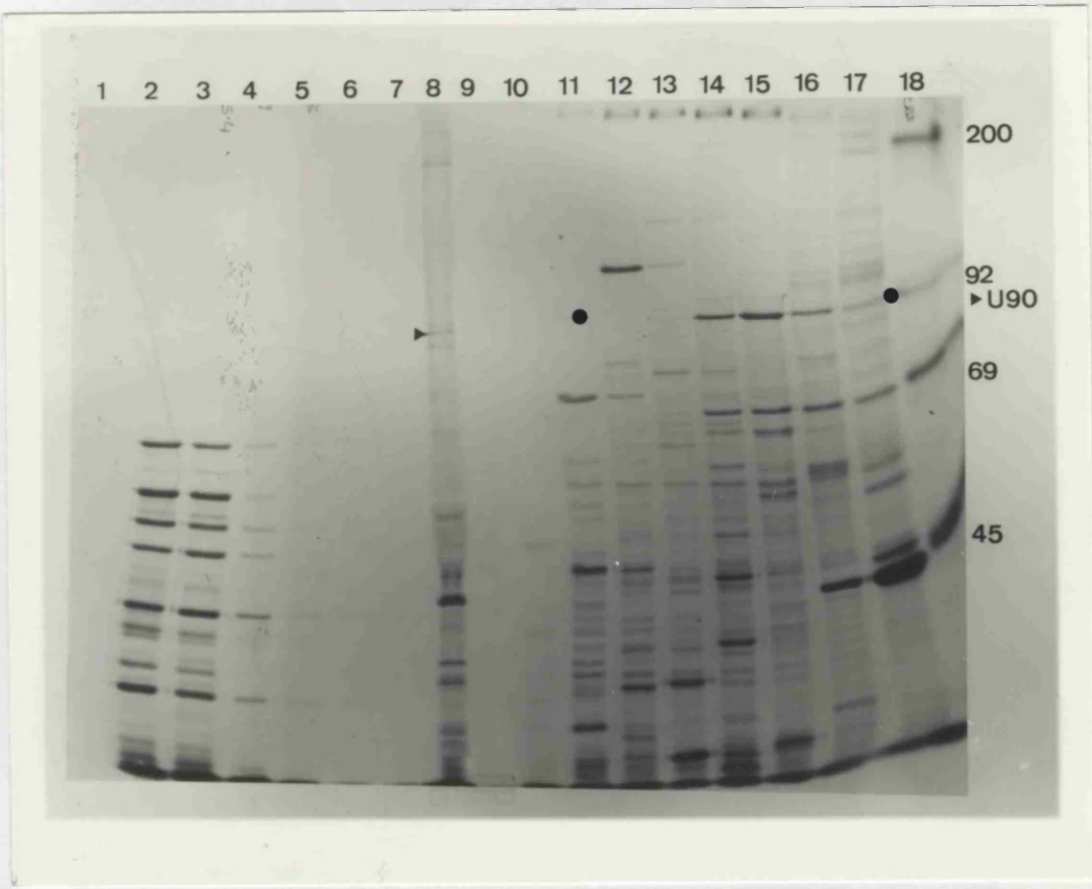


FIGURE 21 : Autoradiograph of 7.5% SDS page of Bn5T polypeptides extracted from 10 burlers of HSV-2 infected cells (1 burler of cells radiolabelled with 100μCi of [^{35}S] L-methionine) which were fractionated and treated as described in the text. A 30% AS precipitation reaction was completed on the cytoplasmic fraction, this sample resuspended in 1% triton/PBS, desalted, centrifuged (12K) to remove any particulate material, then injected onto the column in a volume of 7ml. 1ml samples were collected off the mono Q column. Fractions 1-7 represent the flow through with fraction 8-25 corresponding to a 0-100% gradient of buffer B (50mM tris, 1M NaCl). One tenth of each sample was analysed by 7.5% SDS-page. Track 1- fraction 1, track 2- fraction 2, track3- fractions 3-5, track 4- fractions 6-9, track 5- fractions 10-13 track 6- 14, track 7- 15, track 8- IP of Bn5T with TBS showing U90 (indicated by a filled triangle), track 9- fractions 16-17, tracks 10-17 corresponds to fractions 18-25 respectively. Track 18 shows molecular weight markers, with values in kilodaltons indicated to the side of the gel. • denotes the area in which 90K polypeptides migrate.

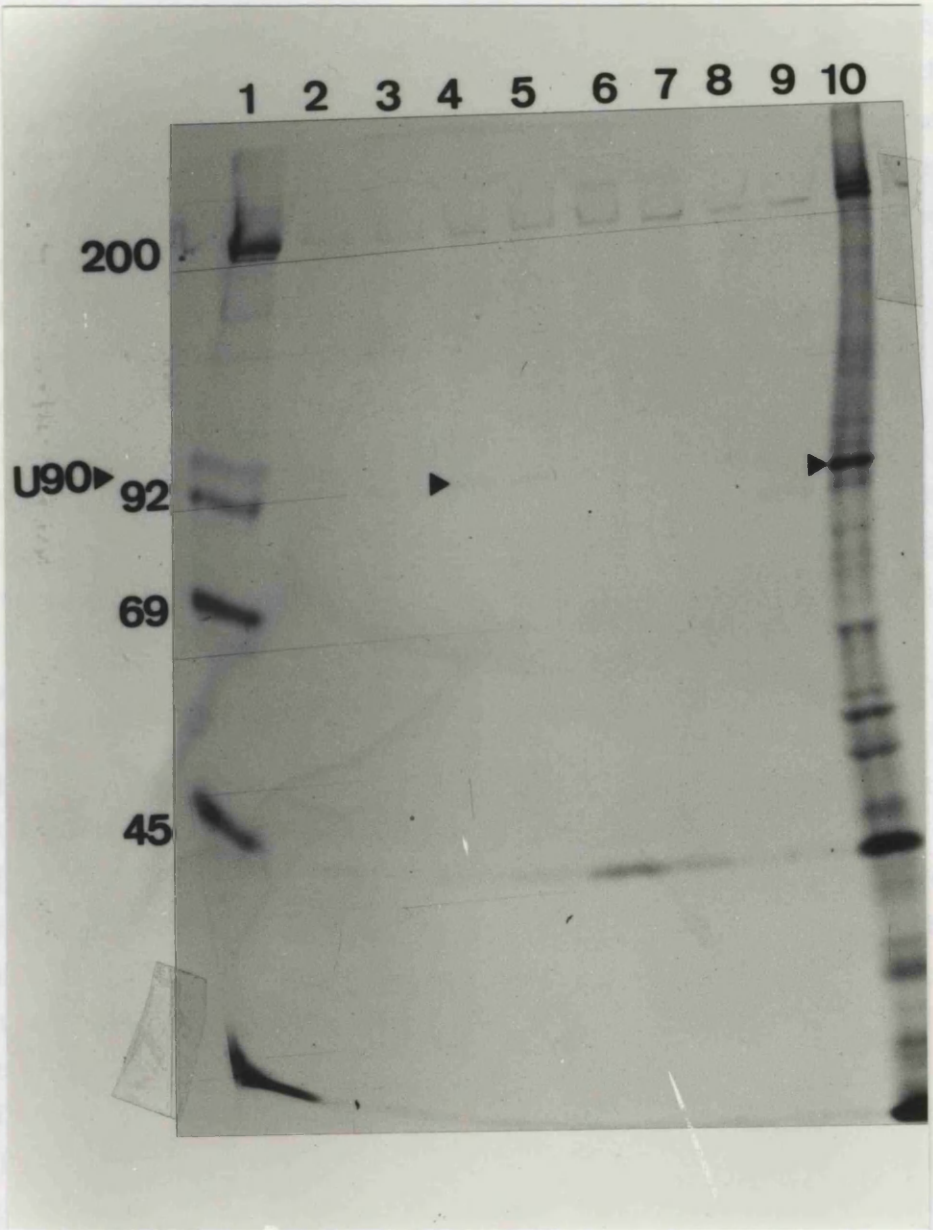


FIGURE 22 : Autoradiograph of IP reactions with TBS using $4X10^6$ cpm of [^{35}S] L-methionine radiolabelled samples isolated from the FPLC mono Q (Figure 21) in which a 90K polypeptide corresponding to the U90 may have been present. Tracks 2-9 represent IP reactions of samples run in tracks 10-17 of Figure 21. A total cell extract of radiolabelled Bn5T cells IP with TBS is seen in track 10. Molecular weight markers can be seen in track 1 and their values indicated in kilodaltons to the left of the gel. A filled triangle denotes the area where a faint band at 90K can be seen in track 4, and where the U90 is in track 10.

small amount of protein was recovered from these samples that insufficient ^{35}S was incorporated into the proteins to allow visualization following peptide mapping analysis. The stronger 90K band seen in fractions 22-24 did not react with TBS. Compared to the total amount of protein added to the column, only a very small proportion of the U90 was recovered. This indicated that only a small proportion of the U90 loaded onto the column was in this 76-94% buffer B sample. A second problem was encountered with these experiments in that on the addition of the sample, the column became blocked very quickly resulting in the build up of very high back-pressure. This back-pressure eventually prevented the elution of the sample off the column from being completed and also caused the column to be very difficult to clean. The reason for this may have been that once removed from detergent containing buffer, the U90 formed aggregates which then precipitated out of solution and eventually blocked the column and its filters. Enzymatic digestion with pepsin (1mg/ml pepsin in 0.1M acetic acid, 0.5M NaCl) at 37^{o}C for a minimum of 1 hour, was required to unblock the column after every experiment. This was then followed by careful washing of the column with a combination of high salt and acid followed by equilibration with buffer A (Method 8b). Using the buffer conditions described above, the column usually had to be cleaned after every experiment.

To resolve this problem, detergent was introduced into the running buffers A.2 and B.2, which were identical to A.1 and B.1 except that they also contained 1% triton-X-100. The addition of detergent to the running buffers had a dramatic effect on the U90 polypeptide, its interaction with the mono Q column and its subsequent purification.

The addition of detergent to the running buffers altered the U90 such that it no longer bound to the column but in fact was isolated in the column's void volume referred to hereafter as the flow through. The flow through contained polypeptides which did not bind to the column, and so were washed through the column by buffer A prior to the commencement of the salt gradient. In the flow through, only a small number of other cellular proteins were present in addition to the U90, which allowed the U90 to be isolated as a discrete band, when the samples were analyzed by SDS page and visualized by both Coomassie blue staining and autoradiography (Figures 23 & 24 respectively). Visualization of the polypeptides in the flow through by silver staining resulted in no further proteins being seen. This technique of protein staining was not used routinely, as unless the U90 could be easily visualized by Coomassie blue staining insufficient material was present to justify continuing towards amino acid sequencing experiments.

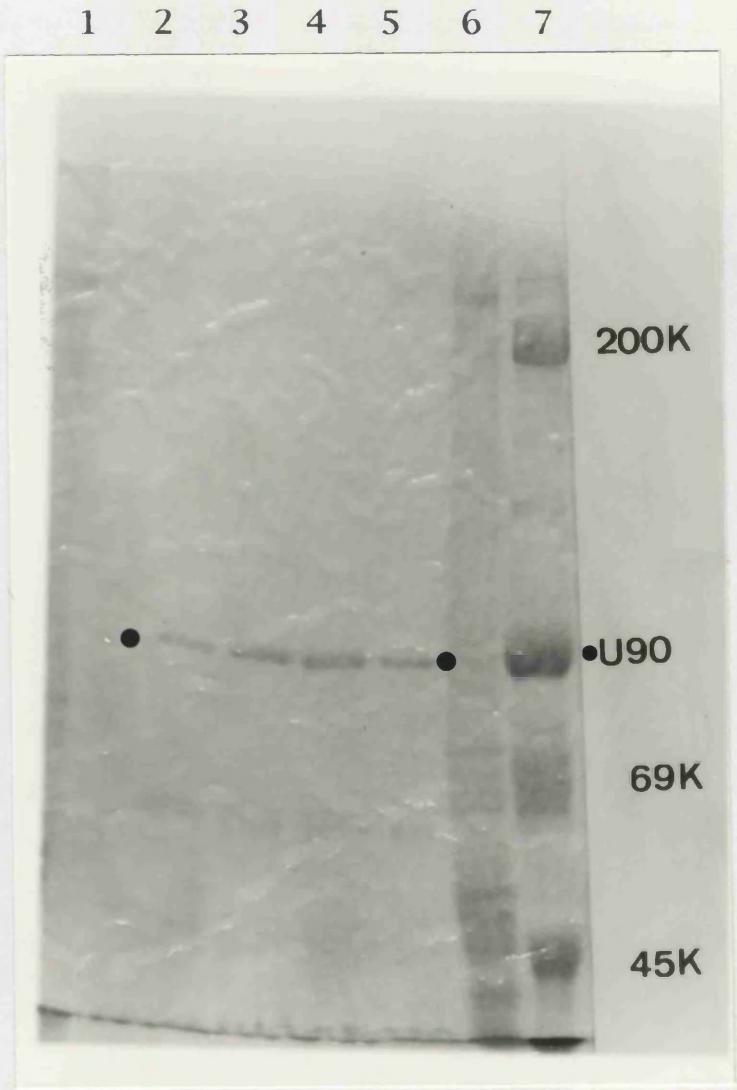


FIGURE 23 : Coomassie blue stained 7.5% SDS page showing Bn5T polypeptides extracted from 10 burlers of HSV-2 infected cells which were fractionated and treated as described in the text. A 30% AS precipitation reaction was completed on the cytoplasmic fraction, this sample resuspended in 1% triton/PBS, desalted, centrifuged (12K) to remove any particulate material, then injected onto the mono Q column in a volume of 5ml. 1ml samples were collected off the column, with 100ul of each of the flow through samples being analysed on a Coomassie blue stained 7.5% polyacrylamide gel. Flow through samples 1-5 can be seen in tracks 1-5 when 1% triton detergent was added to both buffers A and B. Track 6 contains Bn5T cells immunoprecipitated with TBS however, insufficient U90 is present to be visualized. Molecular weight markers are shown in track 7 and values in kilodaltons indicated to the right of the gel. U90 is indicated by •.

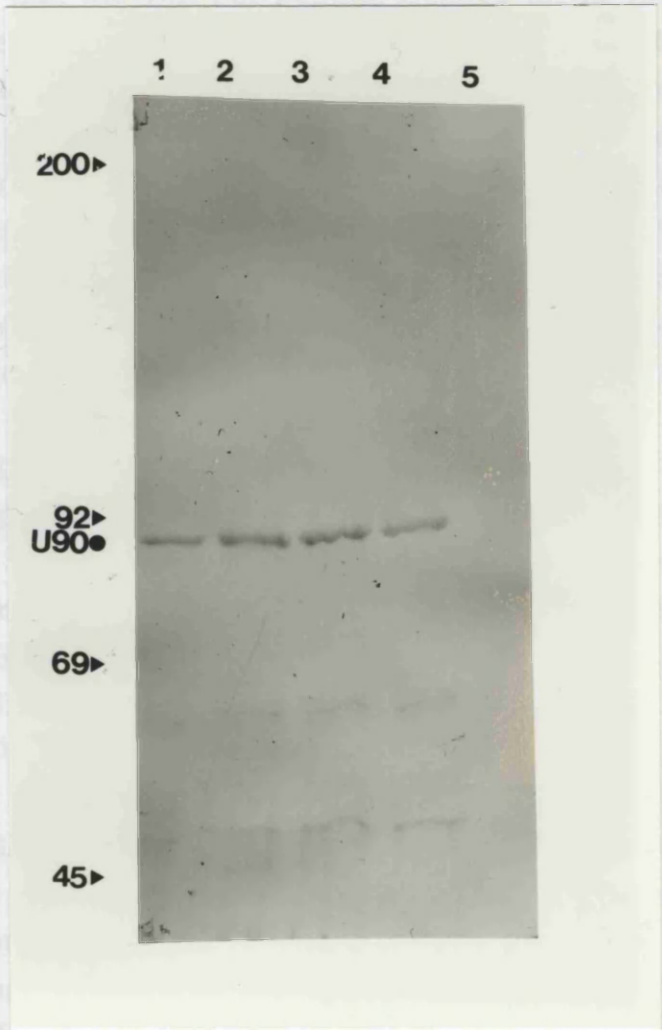


FIGURE 24 : Autoradiograph of gel shown in Figure 23, ie 100ul of each of the mono Q flow through samples of cytoplasmic fraction of HSV-2 infected Bn5T cells when 1% triton detergent was added into the FPLC buffers A and B. Flow through samples 1-5 are seen in tracks 5-1 respectively. Values of molecular weight markers are shown to the left of the gel. U90 is indicated by •.

The addition of detergent to a column chromatography system is believed to alter the capacity and selectivity of the column. This is a consequence of the proteins in the samples being surrounded by detergent and weakening the interactions which would normally occur between the protein and the column material (Yanagita & Kagawa, 1986). It was this phenomenon which greatly aided the purification of the U90.

To confirm that the 90K polypeptide isolated in the flow through was in fact the U90, the samples were first reacted with TBS in an IP reaction (Figure 25) and the resulting 90K bands in each flow through sample were peptide mapped with V8 protease to ensure that they had an identical digestion pattern to the U90 (Figure 26). These experiments confirmed that the 90K polypeptide isolated in the flow through was indistinguishable from the U90.

A representative trace of before the sample was injected into the mono Q and a trace of the proteins eluted from the mono Q column and the conditions in which this was carried out are illustrated in Figures 27 & 28 respectively.

Step 4 Purification Based On Hydrophobicity

To further purify and concentrate the U90 isolated from the flow through samples, reverse phase chromatography was attempted. This was done using an Applied Biosystems microbore HPLC system using a 2.1mmX30mm aquapore RP-(reverse phase) 300 column. This system separates proteins on the basis of the distribution of the solute between a polar mobile phase and an organic phase fixed to the matrix. The buffers used were A, (0.1% TFA in H_2O) and B, (0.1% TFA, 80% acetonitrile in H_2O) with a gradient of 0-100% buffer B being run with a flow rate of 50ml/min over a defined period time. The length of time over which the gradient was run was varied between 15 and 60 minutes in order to obtain maximum separation of eluted polypeptides.

The starting material for this purification was 1ml of sample of the partially purified U90 isolated in the flow through of the FPLC mono Q run. The flow through samples were reduced in volume by approximately 50% by partial lyophilization in a "speedi-vac", then loaded onto the reverse phase column by multiple manual loading This was essential as only a 50µl injection loop was available. Initial studies using this system were unsuccessful as the U90 was not retained on the column, but in a similar manner to the mono Q the U90 was found in the flow through. Analysis of these samples eluted from the HPLC was carried out by either electroblotting onto PVDF (polyvinyl dinitrofluoro) membranes followed by visualization with either Coomassie blue or by western blotting onto nitrocellulose. This was necessary as the small amount of material present would be difficult to detect by SDS page analysis. In addition as a

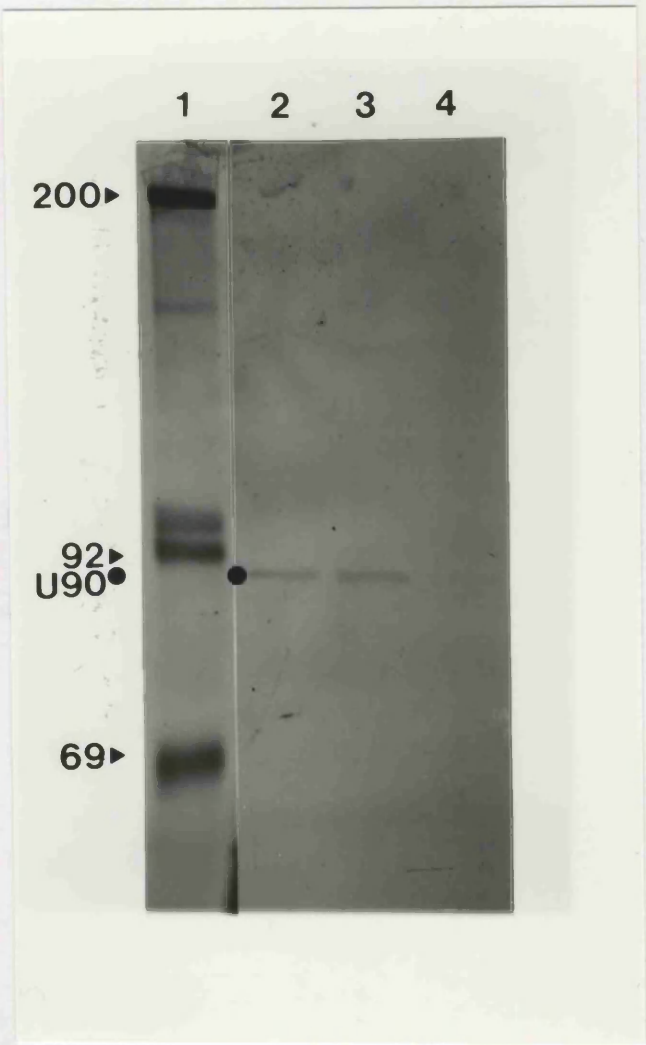


FIGURE 25 : Autoradiograph of IP reactions with TBS using 80ul of a 1ml sample isolated as the mono Q flow through samples (as seen in Figures 22-24). Fraction 2 is seen in track 2, fraction 4 - track 3 and fraction 7 - track 4. Molecular weight markers are seen in track 1 and their values in kilodaltons indicated to the left of the gel. U90 is marked •.

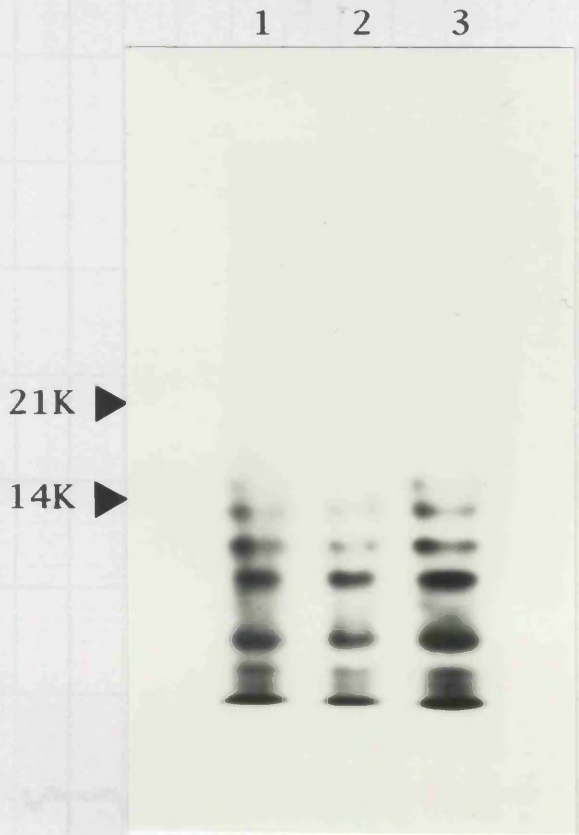


FIGURE 26 : Autoradiograph showing a comparison of the peptide digestion patterns of U90 polypeptide immunoprecipitated by TBS from the flow through fractions of the mono Q run, after the cytoplasmic fraction of HSV-2 infected Bn5T cells had been passed over it. This was carried out by peptide mapping analysis and 18% SDS-page. Digestion was carried out using 5μg of V8 protease/track. The U90 bands were cut from an IP reaction similar to those seen in Figure 25. Fraction 2 is seen in track 1, fraction 4 - track 2 and control U90 band from an IP reaction using total Bn5T cell extract in track 3. Molecular weight values in kilodaltons are indicated to the left of the photograph.

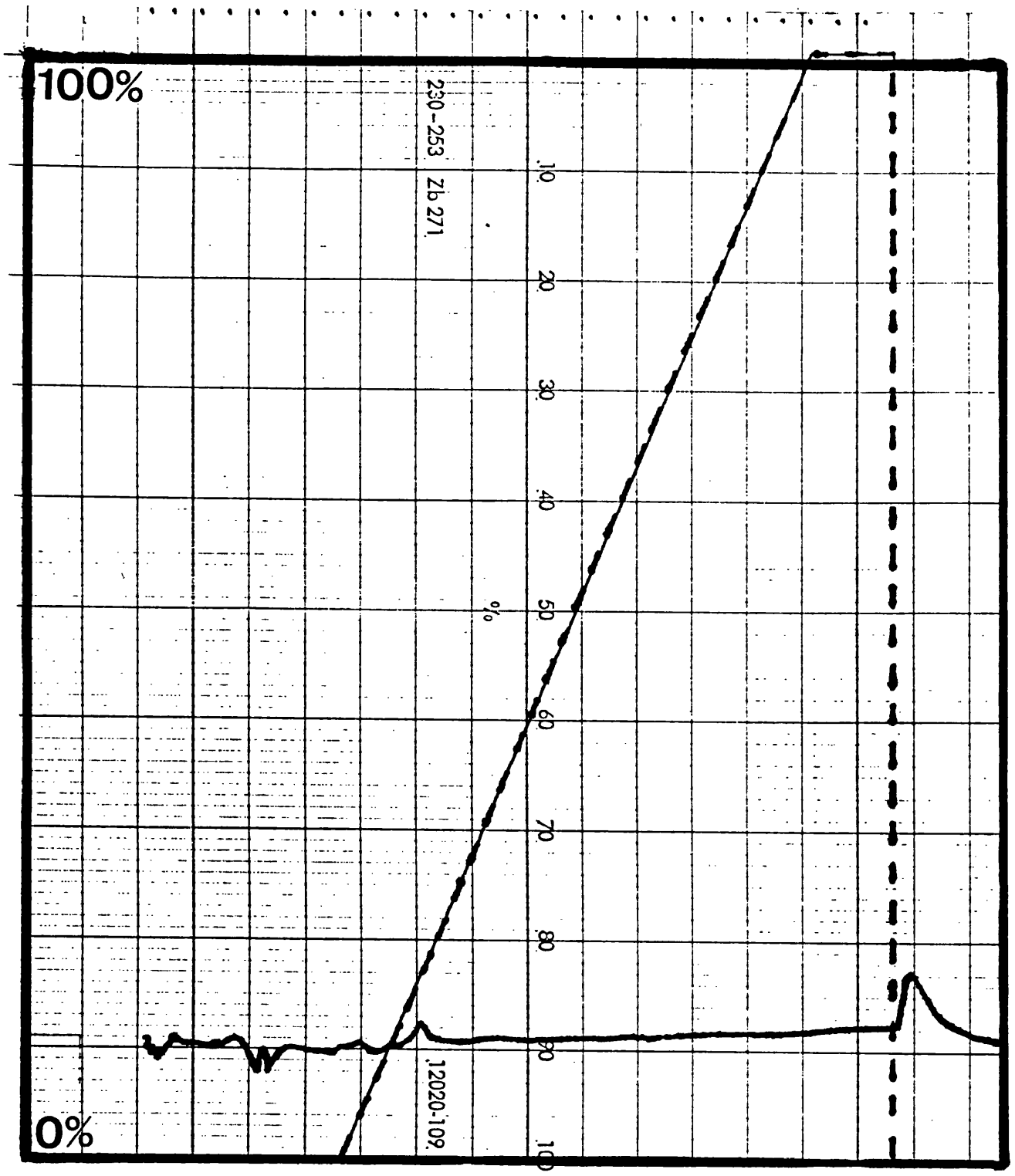


FIGURE 27 : FPLC trace generated when buffer A alone was injected onto the mono Q. - - - - represents the buffer B concentration - 0% buffer B for 7 minutes initially (flow through), 0-100% linear gradient over a period of 17 minutes, 100% for 3 minutes, returning to 0% buffer B for final 5 minutes. Flow rate =1ml/minute. Full scale deflection sensitivity = 0.5 Aufs

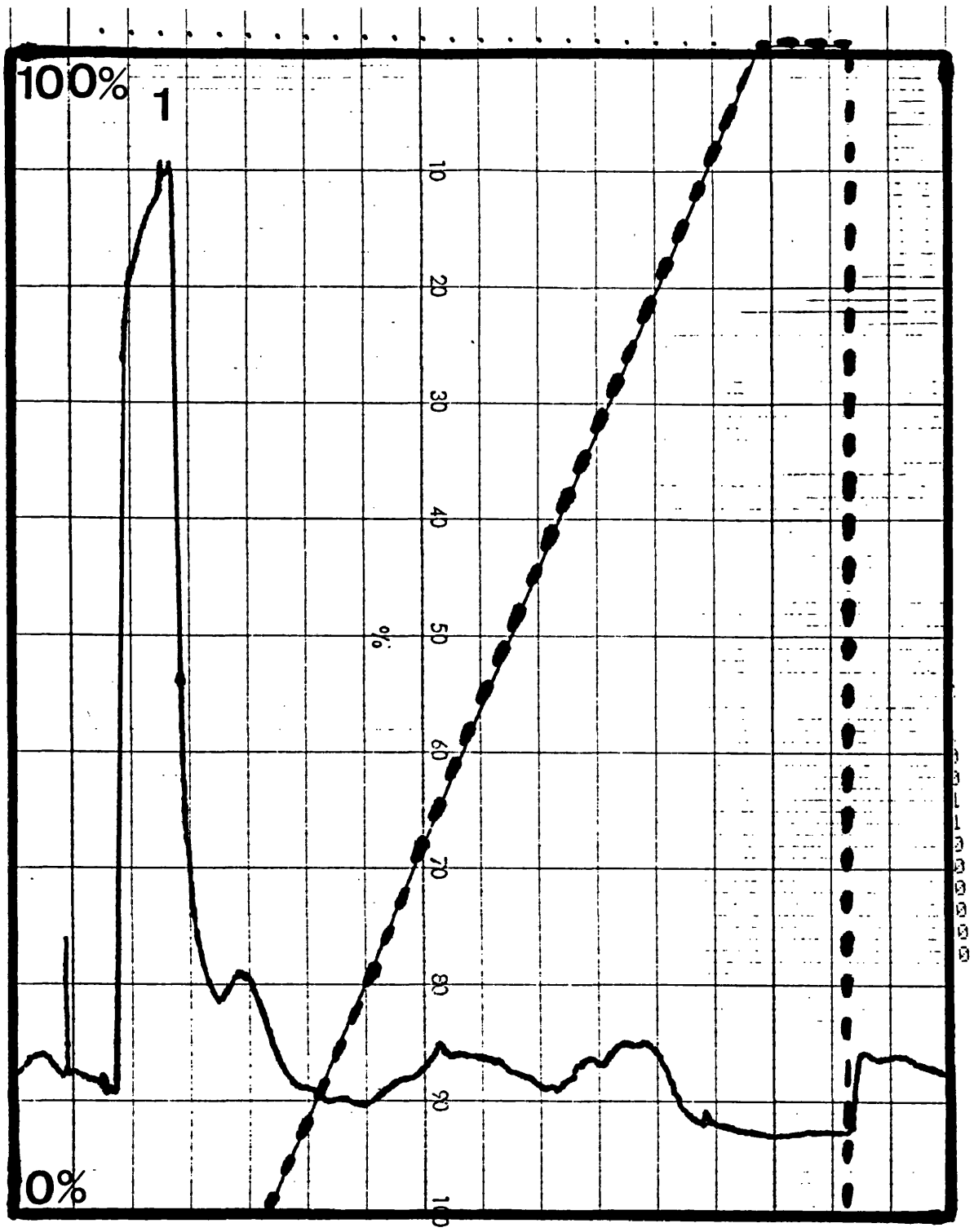


FIGURE 28 : FPLC trace generated when 7ml of sample generated from the desalted 30% AS precipitated cytoplasmic fraction of HSV-2 infected Bn5T cells (10 burlers) was injected onto the mono Q column in buffer A (1% triton-X-100, 50mM tris pH 8). Using a flow rate of 1ml/minute, the sample was injected and 0% buffer B run for 7 minutes followed by a linear gradient of 0-100% buffer B over a period of 17 minutes, 100% buffer B for 3 minutes and returning to 0% buffer B for 5 minutes. Peak 1 corresponds to U90 as determined by Coomassie blue staining (Figure 23), IP reaction (Figure 25) and peptide mapping analysis (Figure 26). - - - - represents buffer B concentration, solid line represents eluted protein. Full scale deflection sensitivity = 2.0 Aufs

considerable amount of material was required in order to progress to this point (taking approximately 3 months to generate the material - including the propagation and titration of virus), blotting the eluted samples onto PVDF meant that the sample could be sent for protein sequencing if sufficient was present. However, in contrast to the situation found with the FPLC mono Q column, it appeared that all the protein in the sample was in the flow through of the HPLC system, i.e. none of the U90, nor the contaminating proteins had bound to the column (Figure 29). A small proportion of this unbound material may have been a consequence of the 50µl injection loop, however the vast majority of unbound sample was attributed to the large amount of triton in the sample, as the FPLC buffer A.2 in which the U90 was isolated in the flow through contained 1% triton-X-100. This meant that following concentration, the U90 sample would contain a minimum of 2% triton. By changing the FPLC buffers to A.3 and B.3 which only contained 0.1% triton-X-100 and reducing the samples using centricon-30 micro-concentrator tubes (Amicon, UK) as opposed to lyophilization, the final concentration of the triton in the samples loaded onto the HPLC was maintained at 0.1%. Centricon tubes desalt and concentrate small volumes by ultrafiltration through a hydrophilic membrane. Centrifugal forces (5,000g) drive the solvent and low molecular weight solutes (of less than 30K) through the membrane. This results in a decreased sample volume and increases the solute concentration. A second buffer can also be introduced to the sample using this system, allowing the sample to be recovered in a new solvent.

Even after reducing the U90 sample by this method, the U90 would still not bind to the column and further attempts to remove even more of the triton were made as the detergent was still present at greater levels than its critical miscelle concentration of 0.01%. These are described below.

To remove as much as possible of the triton-X-100 from the sample, the U90 sample was concentrated in a centricon tube, then washed with 3 X 2ml of the HPLC buffer A. Despite the fact that the manufacturers of the centricon tubes claim that the membranes in these tubes have low adsorption properties, this was not found to be the case when small quantities of the U90 were used. It was noted that a large amount of the U90 was lost in the centricon tube and it was consequently accepted that in using the centricon tubes between 20-50% of the U90 could be lost through adsorption onto the membrane. Despite this the centricon tube did have the advantage of all manipulations being carried out in the one tube and so the inevitable loss of some protein due to adsorption onto the side of any vessel used was minimized. To compensate for the loss of protein

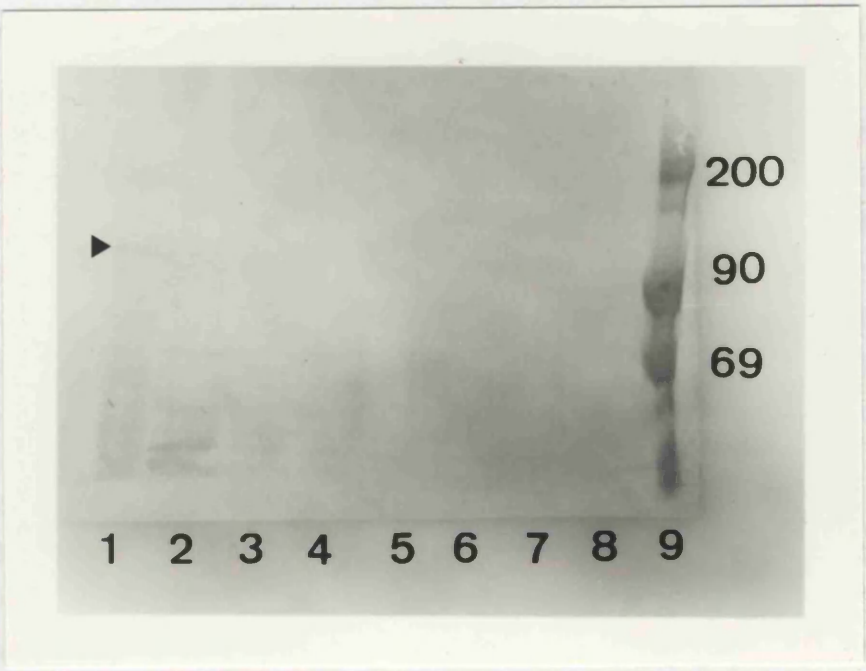


FIGURE 29 : Samples recovered from the reverse phase column on the HPLC after the injection of one flow through fraction of partially purified U90 (volume reduced by 50%). Samples recovered from the HPLC were run on a 7.5% mini-gel, electroblotted onto PVDF and stained with Coomassie blue. Tracks 1 & 2 represent the flow through, where almost 100% of the eluted protein can be seen, tracks 3-8 are samples recovered corresponding to a linear 0-100% buffer B gradient. Over-exposure was required in order to visualize the very small quantities of protein stained by Coomassie blue. Molecular weight markers are seen in track 9 and values in kilodaltons indicated to the right of the gel.

due to membrane adsorption, a larger quantity of U90 was therefore required as starting material.

Washing the sample three times with buffer A in a centricon tube removed sufficient triton-X-100 to enable the U90 to bind to the column. By using multiple manual loading of the TFA washed U90 sample, up to 100pmoles of the U90 was estimated to have been loaded onto the column. Multiple manual loading was required as only a 50μl loading loop was available for this machine.

Several different non-linear gradients of buffer B were used to find the optimum method of eluting the U90 as a discrete peak representing the purified polypeptide. As the U90 was the major component of the sample added to the reverse phase column, its point of elution should have been obvious with the largest peak corresponding to elution of the U90. The largest peak thought to correspond to U90 appeared between 80-90% buffer B (Figure 30), and so the gradient was extended over this range. Samples were collected manually either at regular intervals or to correspond to the point at which the U90 had previously been thought to elute. Following collection, the samples were analyzed by SDS page, electroblotted onto Problot PVDF membrane and protein visualized by Coomassie blue staining (this method, as opposed to silver staining, disclosed whether the eluted sample consisted of sufficient protein for protein sequencing).

When the gradient was extended between 80-90% buffer B, the U90 was thought to elute at 80-82.5% buffer B (Figure 31). However, the U90 was not isolated in one single sample, but trace amounts could be found in a number of samples isolated at higher buffer B concentrations later in the gradient - as visualized by Coomassie blue staining of a PVDF membrane (Figure 32). In addition, a peak corresponding to the U90 was also seen between 80-82.5% buffer B from the column while it was being cleaned. Even by extending the gradient for 30 minutes at the point where U90 started to be eluted from the column, some U90 still remained uneluted in the initial run and a peak could be seen at this point if the run was repeated without the addition of any further sample. Several wash runs were required to remove all the protein from the column. This inability to isolate the U90 from the reverse phase column as a single sharp peak resulted in further losses of the U90 in the purification process.

As seen in Figure 32, the gels containing the samples taken from the reverse phase column also had a number of additional proteins present in the same fraction as the U90. These were visualized by Coomassie blue staining of the PVDF membrane following western blotting of the polyacrylamide gels containing these samples. Extension of the gradient at this stage would not

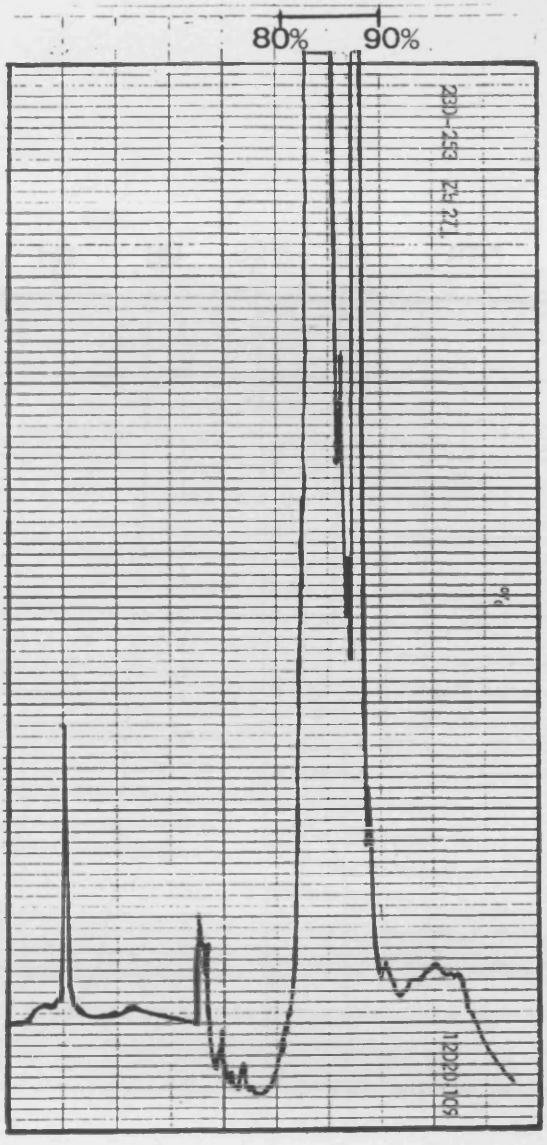


FIGURE 30 : HPLC trace of elution profile following injection of an estimated 100pmoles (equivalent of 9ug) of U90 isolated in the flow through of the FPLC. Large peaks between 80-90% buffer B are thought to correspond to the U90. Flow rate used was 50ul/minute with the following gradient: time 0-3 minutes 0% buffer B, 3-20 minutes linear gradient of 0-80% buffer B, 20-25 minutes 80-88%, gradient held for 1 minute , 26-30 minutes 88-92%, 30-43 minutes 92-100%, gradient held at 100% for 2 minutes then reduced from 100-0% from 45-60 minutes. Full scale deflection sensitivity = 0.1Aufs

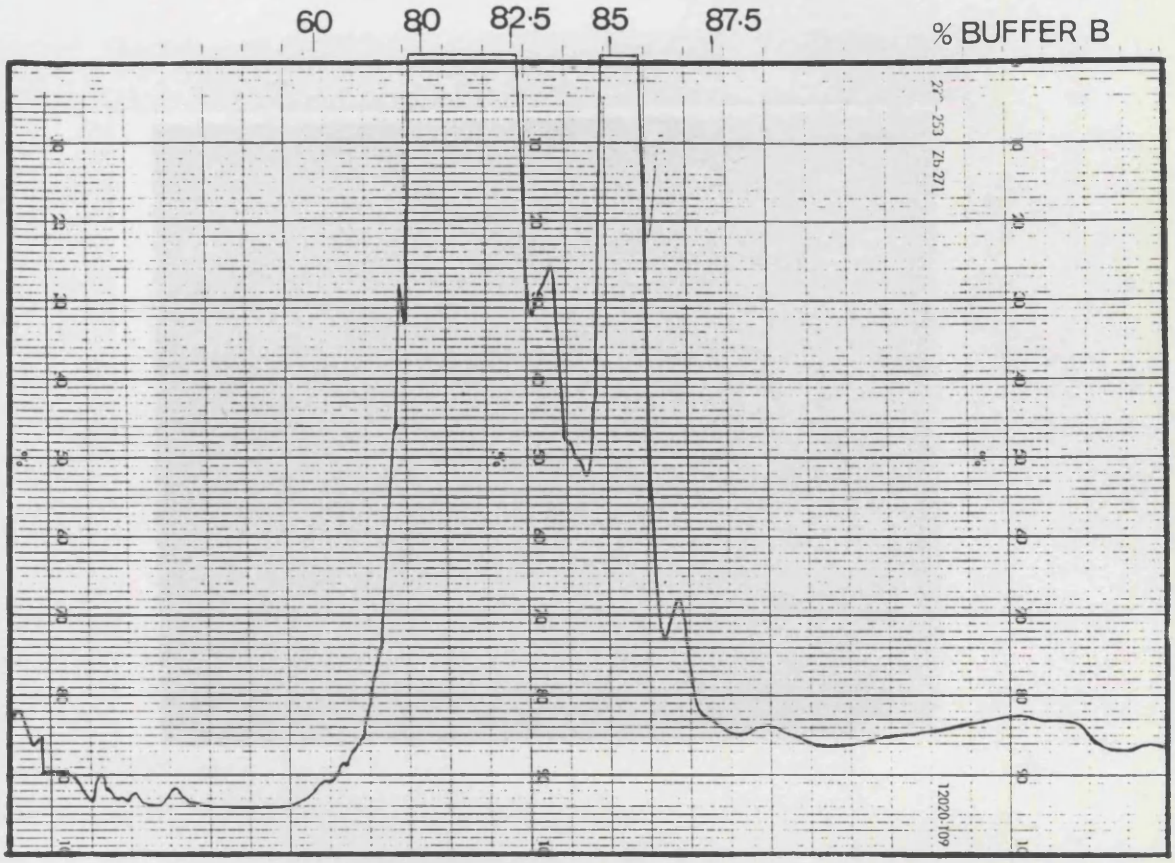


FIGURE 31 : HPLC trace of the elution profile following injection of an estimated 100pmoles (equivalent of 9ug) of U90 isolated in the flow through of the FPLC onto an HPLC reverse phase column. The gradient was extended between 80-90% buffer B. Flow rate used was 50ul/minute with the following gradient: time 0-3 minutes 0% buffer B, 3-20 minutes linear gradient of 0-80% buffer B, 20-30 minutes 80-85%, 30-40 minutes 85-90%, 40-50 minutes 90-100% then reduced from 100-0% from 50-55 minutes. Full scale deflection sensitivity = 0.1Aufs

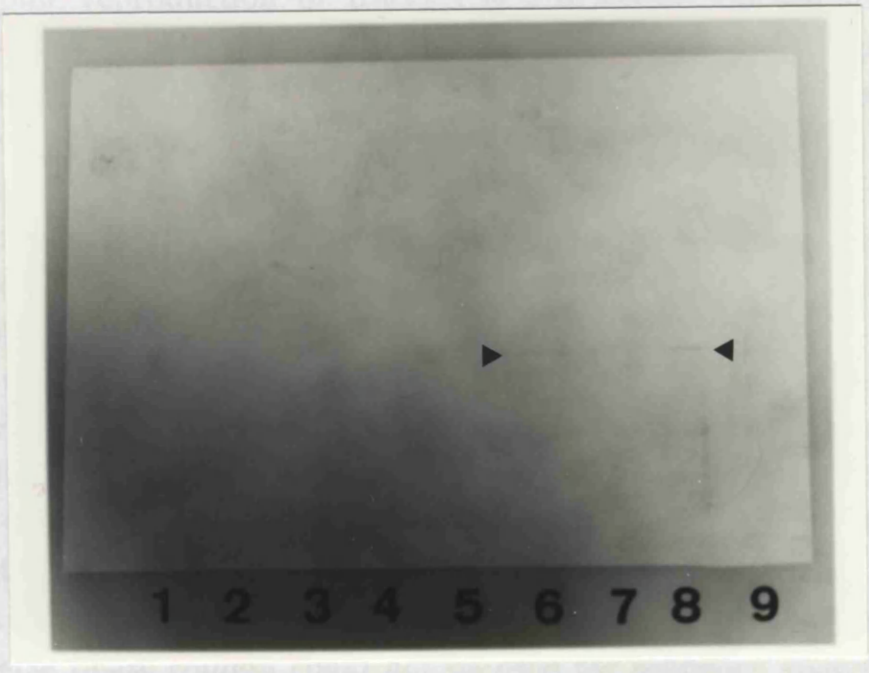


FIGURE 32 : Samples recovered from the reverse phase column on the HPLC after the injection of an estimated 100pmoles of partially purified U90. 25% of each sample recovered from the HPLC was run on a mini-gel electroblotted onto PVDF and stained with Coomassie blue. Track 1 =flow through, 2=0-20%, 3=20-40%, 4=40-60%, 5=60-80%, 6=80-82.5%, 7=82.5-85, 8=85-87.5%, 9=87.5-90%, 10=90-95% buffer B. Protein can be detected in tracks 6-8, with the band which is believed to be U90 indicated by a closed triangle. Over-exposure was required due to the very small quantities of protein eluted from the reverse phase column.

separate the U90 from these polypeptides, nor could it be explained from where they originated. When the samples were analyzed by SDS page then western blotted using the U90 monospecific antibody in addition to TBS, not only did the monospecific antibody confirm that the protein of molecular weight 90,000 was the U90, but it also recognised the proteins of lower molecular weight present in the sample (Figure 33). This western blot and all others in this thesis were reproduced by a combination of laser printing and photocopying which neither introduced nor removed any significant background material. A photographic reproduction of tracks 1 & 2 of Figure 33a are also shown in Figure 33b to confirm this. By comparing the sample before washing with TFA (to remove triton), and the eluted sample, the most likely explanation of the peptides origin was that they were breakdown products of the U90. These were thought to be generated by proteolytic digestion by TFA which was present in the buffer A used to wash the U90 sample when removing the triton-X-100. Comparison of the sample before injection onto the reverse phase column and the samples collected from the column after being eluted in TFA by western blotting using the monospecific U90 antibody showed that all the lower molecular weight proteins were present both before and after elution from the column. However, greater protein degradation can be seen in the eluted sample (Figure 34). This suggested that the U90 was not stable in 0.1% TFA and that prolonged contact with TFA resulted in U90 degradation.

As a consequence of these breakdown products the U90 isolated from the HPLC reverse phase column could not be used for sequence analysis since the U90 was not isolated in sufficient amounts nor as a discrete protein. Had time allowed, this might have been overcome by altering the buffer used to wash the sample to remove the triton-X-100, for example, to 10mM ammonium bicarbonate (instead of a buffer containing TFA). These changes still might not have allowed the U90 to be sequenced directly from the HPLC, as at least 50% of the material added to the column previously was not recovered in the final sample, yielding insufficient material for the U90 to be sequenced. The lost 50% may be accounted for in the breakdown products visualized following western blotting.

As the FPLC flow through samples contained no contaminating proteins at 90K, these samples were used to attempt to sequence the N-terminal of the U90 without the use of the HPLC.

STEP 5 : U90 Concentration

The proposed method for determining the N-terminal amino acid sequence of the U90 was based on that proposed by Matsudaira, (1987). This technique

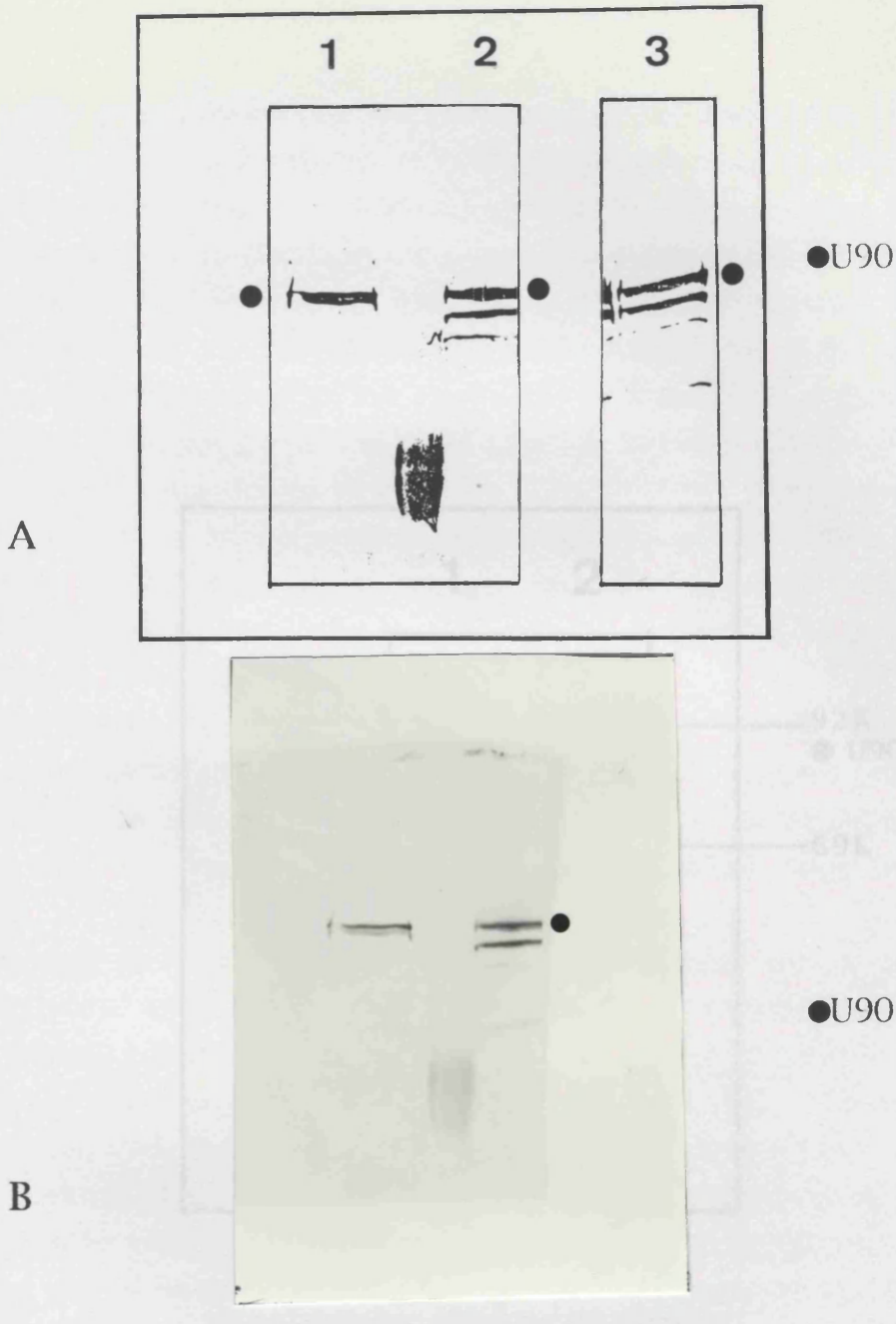


FIGURE 33a : A western blot of U90 sample eluted from HPLC reverse phase column (tracks 2 & 3) visualised with anti-rabbit immunoglubulin conjugated to horse radish peroxidase and developed with N'chloronapthol, compared to U90 in the FPLC flow through sample prior to washing with TFA (track 1). TBS was used in the blot containing tracks 1 & 2, and U90 mono-specific antibody used in blot containing track 3. The western blots were reproduced by laser printing and photocopying. This did not alter the background as compared to a photograph of the blot (Figure 33b). This technique was subsequently used for all western blots. U90 is indicated by ● with lower molecular weight bands believed to be U90 breakdown products caused by the presence of TFA.

FIGURE 33b : Photographic reproduction of tracks 1 & 2 seen in Figure 33a.

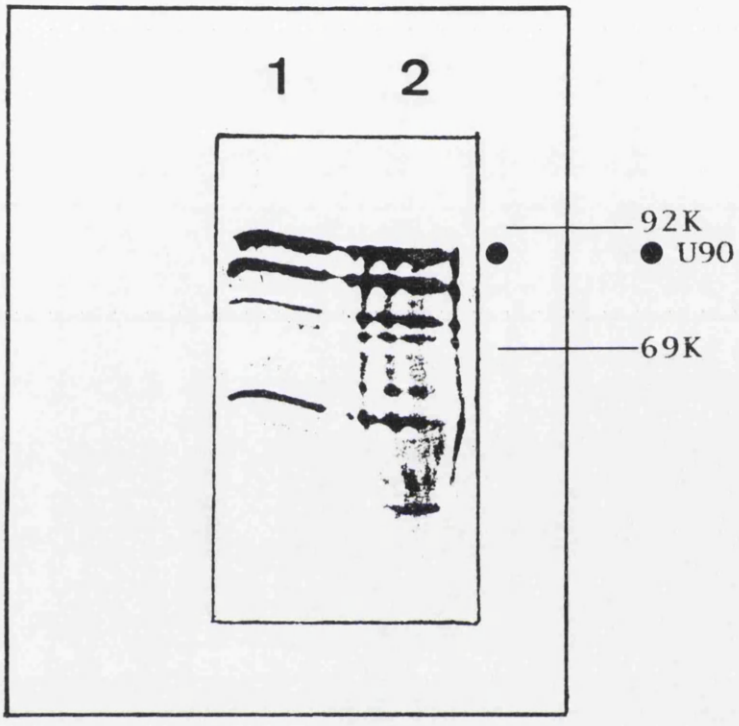


FIGURE 34 : Western blot of U90 sample prior to injection onto the HPLC reverse phase column (track 1) and U90 eluted from HPLC reverse phase column (track 2) using the monospecific U90 antibody, visualised with anti-rabbit immunoglubulin conjugated to horse radish peroxidase and developed with N'chloronapthol. The increased number of immunoreactive bands in track 2 is due to TFA degrading the U90 polypeptide. U90 is indicated by ● and molecular weight markers are indicated in kilodaltons to the right of the blot.

involves SDS page followed by electroblotting of the electrophoresed proteins onto a polyvinyl dinitrofluoro (PVDF) membrane. The proteins are then visualized by Coomassie blue staining and the required band(s) excised. The PVDF sections are then placed in the reaction chamber of the protein sequencer and the protein of interest eluted from the membrane and sequenced.

The size of the reaction chamber is the limiting factor in the process, as it can only accommodate up to the equivalent of 8 tracks in a Biorad mini gel system. The sequencing reaction itself requires between 10-100 picomoles of protein, and it is also found to be more efficient if the protein in question is blotted onto as small an area of membrane as possible. The protein for sequencing, which was run on the gel and electroblotted onto the PVDF membrane therefore had to be as concentrated as possible to enable it to be loaded onto a small number of lanes on the gel.

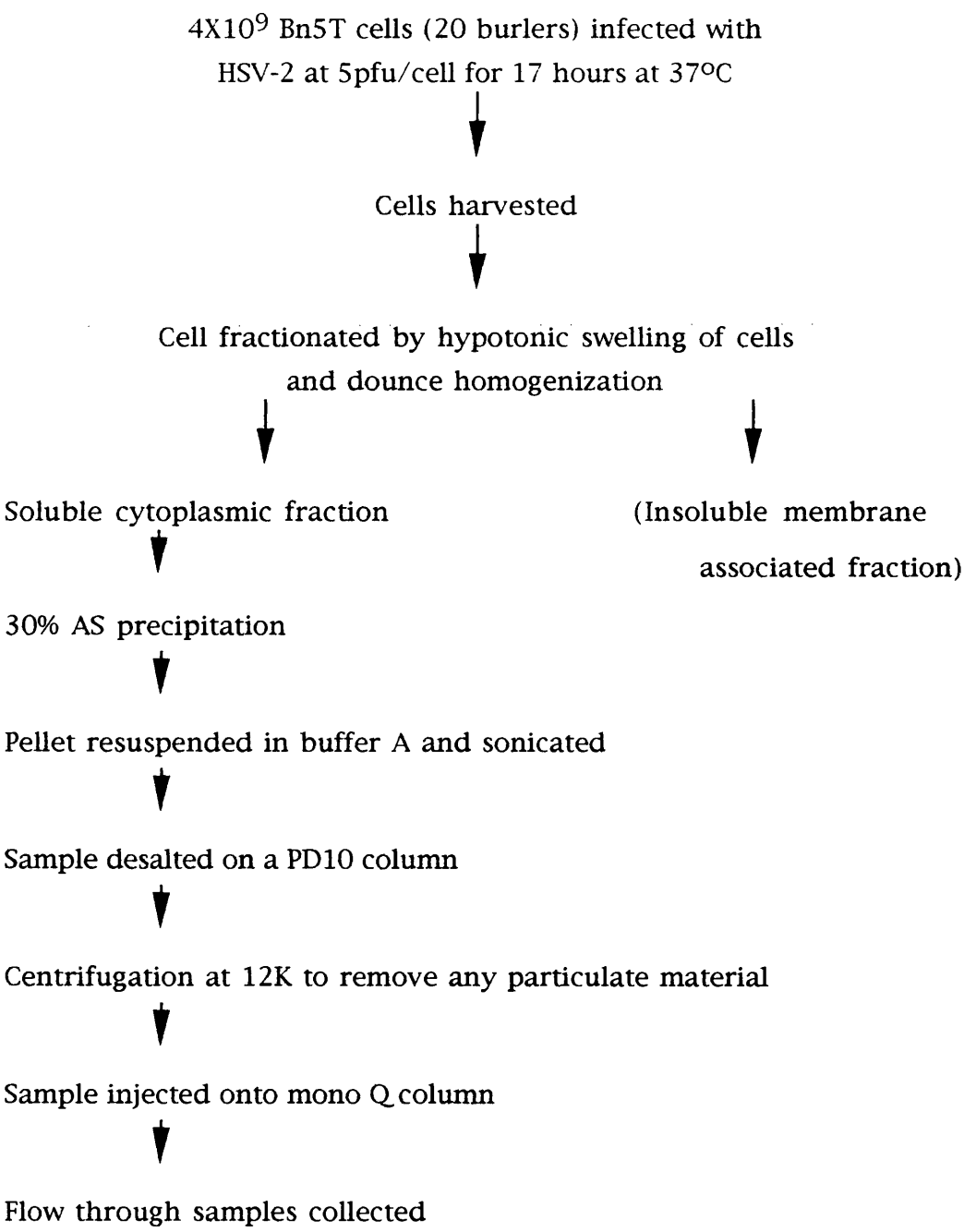
Unfortunately insufficient U90 was isolated in the flow through of a single FPLC run to obtain 10-100 picomoles. This meant that samples containing the U90 had to be reduced in volume to enable more U90 to be loaded on the gel at the one time. This concentration of U90 was approached using two different methods.

The first method involved reduction of the sample volume from 1ml to 0.1ml by partial lyophilization. This however, also concentrated the triton-X-100 in the sample resulting in up to 10% (v/v) of triton in sample which then dramatically affected the mobility of the U90 on SDS page. This effect was so great that the U90 could no longer be isolated as a discrete band by SDS page. To try to overcome this, the samples were reduced in volume using an Amicon centricon tube which concentrated the protein sample by allowing the buffer including the triton-X-100 to pass through the membrane within the tube, but not the protein (Method 7g). Unfortunately, the U90 was found to bind to the membrane, resulting in a large percentage of the U90 being lost during this procedure.

The solution found to this problem of excess detergent, was to once again change the FPLC running buffers to a third version of the original buffers, A.3 and B.3. These buffers still contained 50mM tris pH8 +/- 1M NaCl, but contained only 0.1% triton-X-100 (instead of 1%). As the critical miscelle concentration of triton-X-100 is between 0.01 and 0.001%, the detergent was still present in excess in the samples, and therefore in theory should give the same results. Using uninfected Bn5T cells and the purification method described in this thesis, Mr David McNab confirmed that these buffers containing 0.1% triton-X-100 did in fact give similar yields of U90 in the flow through.

The buffers A.3 and B.3 were then incorporated in the purification strategy of the U90, which enabled the flow through samples to be reduced ten fold in volume by lyophilization and still maintain the triton-X-100 concentration at levels of 1% (v/v) or less. This method was found to be the most suitable for the purification of U90 and so was incorporated into the purification strategy which is summarized in Figure 35.

FIGURE 35: SUMMARY OF PURIFICATION OF U90



DISCUSSION OF SECTION 3: PARTIAL PURIFICATION OF U90

The main objective in purifying the U90 was to enable partial amino acid sequencing to be obtained and hence allow molecular studies of the U90 to commence. In order to proceed with the N-terminal amino acid sequencing an order of 10-100pmoles of protein, void of all contaminating factors was required. Despite this, the purification of the U90 did not in fact require the U90 to be purified to 100% homogeneity but to a degree of partial homogeneity that allowed the polypeptide to be resolved as a discrete isolated band following SDS page and subsequent electroblotting.

The polypeptide characterization studies described in Section 1 determined the optimal starting conditions for the purification of U90. An important disclosure from these studies was that HSV infection of Bn5T cells resulted in increased U90 being isolated in a soluble form in the cytoplasm. This was of particular relevance as previous work to purify the U90 from the membrane of Bn5T cells had failed. This was because purification of the U90 had proved too difficult due to the small quantities present and the insoluble nature of the polypeptide in the membrane fraction. The cytoplasmic fraction of HSV-2 infected Bn5T cells was therefore used as the starting material to purify the U90.

To obtain sufficiently pure U90 with as little loss of material as possible it was aimed to keep manipulations of the sample to a minimum. By using the cytoplasmic fraction as starting material, ie with no membrane or nuclear proteins present, a large proportion of cellular protein was removed from the sample before the purification procedure had started.

The first step in the purification strategy was to selectively precipitate protein by increasing salt concentration. Although this technique is very crude, it allowed the removal of a considerable number of contaminating cell polypeptides. The use of ammonium sulphate precipitation in this system was established by J.-F. Lucasson, a post-graduate student within the laboratory who showed that the proteins precipitated in a 30% ammonium sulphate saturated sample of Bn5T cell polypeptides contained the U90.

This work was then applied to the HSV-2 infected Bn5T cells, and it was also found to be the case that when the cytoplasmic fraction of HSV-2 infected cells was saturated to 30% with ammonium sulphate, the U90 was precipitated from the sample. Further investigations showed that when 25%, 30% and 35% ammonium sulphate saturated samples were run on the FPLC mono Q and the subsequent flow through samples analyzed, the 25% saturated samples also contained a small amount of U90, as did the precipitated proteins in the 35% ammonium sulphate samples. The FPLC trace of protein eluted from the monoQ

after injection of the 35% AS cut showing a peak corresponding to the U90 in the flow through, can be seen in Figure 36. However analysis of these samples by SDS page showed that in comparison with the total amount of U90 isolated in the flow through of the 30% cut sample, the U90 found in the 25% and 35% cut was only a small proportion of the U90 found in the 30% cut. In addition, the number of background polypeptides seen in the flow through of the 35% cut was greater than the 30% cut, and therefore the 30% cut was used.

After the U90 had been selectively precipitated by saturating salt concentrations, it was found that the polypeptide was no longer in a soluble form and addition of detergent was required to solubilize the protein. Following the resuspension of the precipitated polypeptide a further step of sonication was included to disrupt protein aggregates which had formed. The insolubility of the U90 after selective precipitation may be a consequence of the U90 under normal circumstances being complexed with additional polypeptides which enabled it to remain in solution. The increased salt concentration and selective precipitation of the U90 may have disrupted such interactions resulting in the insoluble U90 forming aggregates and precipitating out of solution. Addition of detergent to the sample and sonication to disrupt any protein aggregates overcame this problem. It was in fact this hydrophobic nature of the U90 which helped in the partial purification of the U90 by FPLC.

Some time was devoted to trying to isolate the U90 as a single polypeptide eluted from a reverse phase column on a microbore HPLC, in order that the polypeptide could be sequenced directly without further manipulations. To achieve this, considerably more time would have had to be spent on this particular aspect to overcome a problem of protein degradation by TCA which was encountered. To sequence the U90 under the conditions which it was eluted from the HPLC, the sample would have still needed to be run on a gel, then blotted onto a PVDF membrane. No advantage was therefore obtained by using the HPLC compared to using U90 isolated from the flow through of the FPLC where conditions for isolating partially purified U90 were already well defined. As a consequence of this, it was decided to use the U90 isolated from the FPLC in attempts to obtain N-terminal amino acid sequencing.

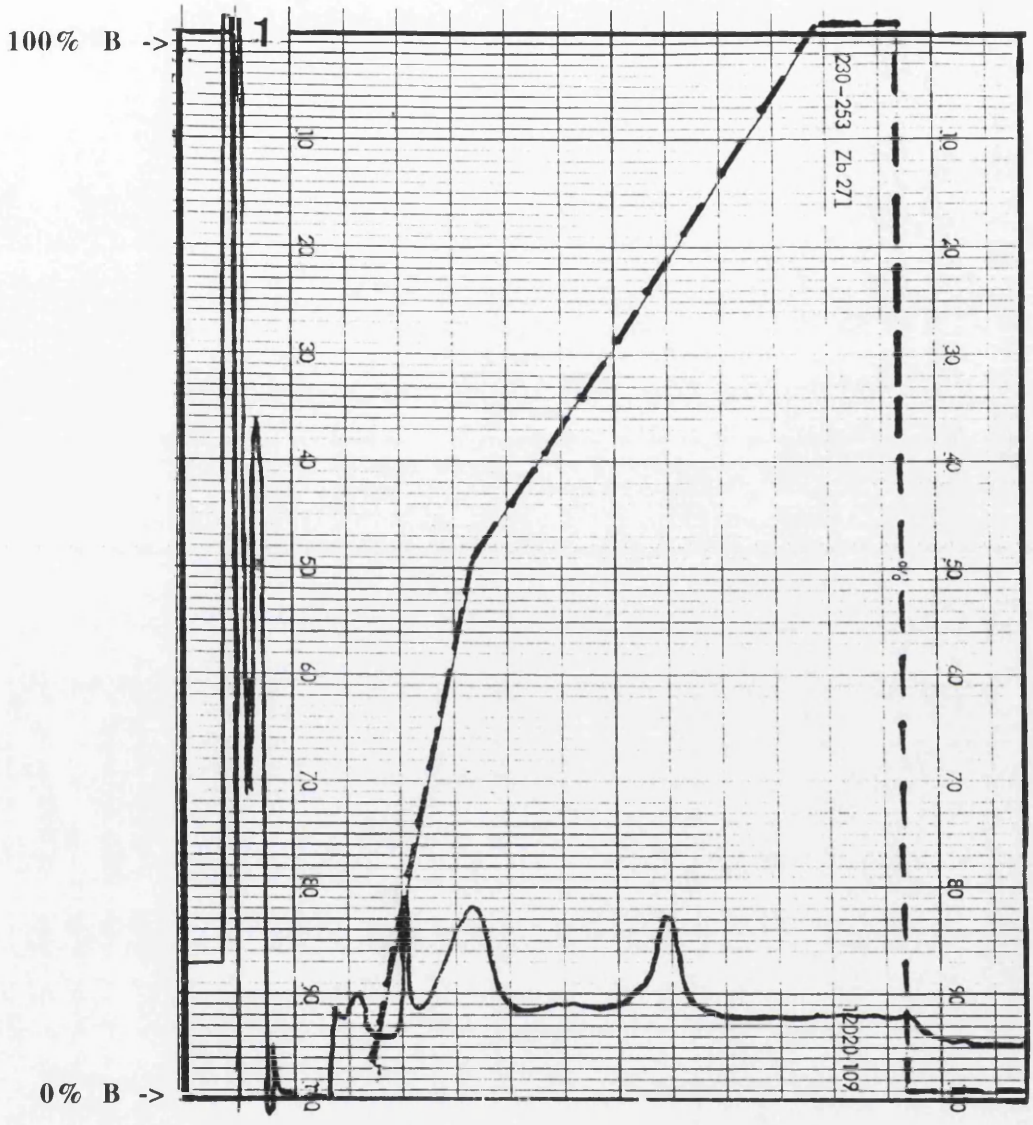


FIGURE 36 : FPLC trace generated when 7ml of sample generated from the desalted 35% AS precipitated cytoplasmic fraction of HSV-2 infected Bn5T cells (10 burlers) was injected on to the mono Q column in buffer A (1% triton-X-100, 50mM tris pH8). Using a flow rate of 1ml/minute, the sample was injected and 0% buffer B run for 7 minutes followed by a linear gradient of 0-50% buffer B over a period of 4 minutes, 50-100% over the following 13 minutes, 100% buffer B for 3 minutes and followed by 0% buffer B for 5 minutes. Peak 1 corresponds to U90 as determined by Coomassie blue staining. - - - - represents buffer B concentration, solid line represents eluted protein. Full scale deflection sensitivity = 1.0 Aufs

SECTION 4 : N-TERMINAL AMINO ACID SEQUENCING OF THE U90

Amino acid sequencing of purified U90 was carried out on an ABI 477A pulsed-liquid protein sequencer using the Edman degradation reaction. This reaction which is outlined in Figure 37 consists of a coupling reaction of phenylisothiocyanate (PITC) to the N-terminal amino acid of the protein to be sequenced followed by a wash step, to remove excess reagents, a cleavage reaction which generates a PTC derivative and a thiazolinone incorporating the original N-terminal amino acid. The thiazolinine is then converted to a more stable phenylthiohydantoin for identification by HPLC (Yarwood, 1989). Amino acid analysis was carried out on an ABI 420H amino acid analyzer. Amino acid sequencing and amino acid analysis were carried out by Drs Gordon Currie and Maggie Cusack in the Department of Geology, University of Glasgow.

1 U90 Isolated From the Cytoplasm of Infected Bn5T Cells

Using approximately $2X10^9$ HSV-2 infected Bn5T cells, U90 was purified from the cytoplasmic fraction which had been passed over the FPLC using buffers A.3 and B.3 containing 0.1% triton-X-100, and the flow through samples containing the U90 concentrated 10 fold by lyophilization. These samples were then loaded onto a 7.5% SDS mini-gel using a comb which produced one large deep well, almost the full length of the gel and a second smaller well in which marker proteins were run. This large track enabled more material to be run on the gel at the one time, so concentrating the U90 further in a single band. This gel was run as normal then transferred by electroblotting onto Problot (Applied Biosystems, England), a PVDF membrane (Method 7e). Following transfer, the U90 was visualized by Coomassie blue staining (0.1% w/v Coomassie blue, 10% methanol, 1% acetic acid) for 30-60 seconds, then quickly destained (10% methanol, 1% acetic acid), with several changes of buffer. If the U90 band was stained strongly enough with Coomassie blue such that it was easily visible within 3 minutes of destaining, it was predicted that a minimum of 10pmoles of U90 was present. (Coomassie blue staining will visualize a minimum of approximately 200ng of protein on a PVDF membrane (Matsudaira, 1990) where 900ng of U90 is equivalent to 10pmoles.)

To confirm the quantity of U90 present, a known proportion of the band was sent for amino acid analysis (in general 10% of the total U90 was analyzed). This accurately determined the total amount of protein which was present in a sample. If this analysis indicated that at least 10pmoles of U90 was present in

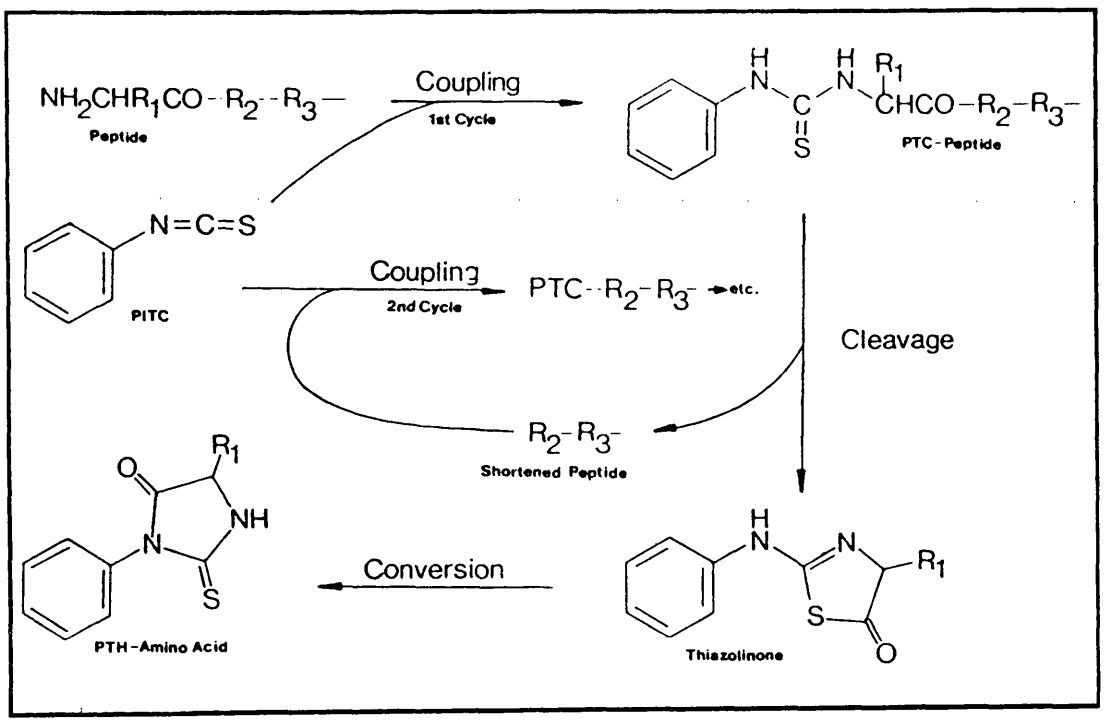


FIGURE 37 : Schematic representation of the Edman degradation reaction used in amino terminal protein sequencing (Yarwood, 1989). PTC= phenyl thiocyanate, PITC = phenylisothiocyanate.

one sample in total, (the lower limit for amino acid sequencing), amino acid sequencing was carried out. Ideally 25-100 pmoles of polypeptide was required.

This experiment was completed on 4 separate occasions with amino acid analysis being completed on three different occasions revealing that quantities between 60 and 88pmoles of U90 were present on the membrane sections sent for sequencing. This indicated that sufficient material was present to allow detection of the individual amino acids eluted from the HPLC after sequencing. However, when the samples were sequenced, on all 4 occasions, only 4-8pmoles were detected to be eluted from the HPLC following sequencing and hence no readable sequence was obtained. From this it was concluded that the amino terminus of the U90 isolated from the cytoplasmic fraction of HSV-2 infected Bn5T cells was "blocked".

Blockage occurs as a result of a modification of the amino terminus (eg acetylation), in such a manner that the Edman reaction used in N-terminal amino acid sequencing is inhibited preventing amino acid sequence from being obtained. This modification of the N-terminal can either occur naturally by post-translational modification, or it can occur as a consequence of chemical modification during the purification procedure. As a large number of proteins which have been isolated from eukaryotes to date are blocked (Driessen et al, 1985), it was possible that the U90 was also naturally blocked. Steps were taken to minimize the possibility of blockage occurring during the preparation and purification of the U90, and attempts to sequence the U90 were repeated.

2 Prevention of N-Terminal Blockage

Several factors can prevent a successful Edman reaction. Some of these factors were eliminated and if this was not possible, the length of time during which the protein to be sequenced was in contact with such a blocking agent was minimized. Potential blocking factors are discussed below and the method of minimizing inhibition of the sequencing reaction by them presented:

(1) acrylic acid present in polyacrylamide gels may react with the side chains of the protein to be sequenced. To minimize this only the highest purity acrylamide was used, which was treated with amberlite (an ion exchange resin) to remove oxidising factors which could produce acrylic acid. The smallest and thinnest possible gels were used to minimize the amount of acrylamide which the protein came in contact with. The gels were pre-run, ie the gels were run as normal before the stacker (and the samples) were added so eluting any unpolymerized acrylamide through the gel. Reduced glutathione was also added to the tank buffer while the gel was being pre-run, (0.7g per 250ml of buffer) to react with acrylic acid or any free reactive sites and finally gels were

"matured" by storing at 4°C for a minimum of overnight (Ullrich, personal communication).

(2) oxidising agents present in the polymerized gel could also give rise to blockage of the protein. A free radical scavenger (3ul of thioglycolic acid, free acid/ 250ml of buffer) was added to the upper buffer to mop up any oxidising or reducing agents while the gel was being run (Ullrich, personal communication).

(3) contaminants in the gel solutions were minimized by using only chemicals and water of highest purity (FPLC grade), and were stored where possible in sterile plastic containers and not glass containers which may have had contaminants adsorbed to the glass as a consequence of the detergent containing fluids used during cleaning.

(4) the tank buffer was changed from the standard glycine buffer to a tris-tricine buffer (Methods, Table 1). This was necessary as the glycine in the buffer previously contaminated the protein sample and the resulting sequence had an artificially high glycine content (Curry & Cusack, personal communication).

Even after incorporating these conditions, the U90 was still found to be blocked. It was therefore accepted that the U90 was most probably naturally blocked, and so an alternative method for obtaining amino acid sequence of the U90 had to be found.

3 U90 Isolated From The Membrane Of Infected Bn5T Cells

As the U90 could also be isolated from the membrane fraction as well as the cytoplasmic fraction of Bn5T cells, the amino terminus of the U90 was purified from the membrane fraction. This was investigated in the hope that the U90 polypeptide contained a membrane signalling peptide which was removed once the U90 became associated with the membrane. Such signal peptides are normally 10-20 amino acids in length and situated at the amino terminal (Einfeld & Hunter, 1991). It was therefore anticipated that if such a peptide was removed from the U90 on its insertion into the membrane, an unblocked N' terminus might be exposed and sequencing therefore become possible.

The membrane sample was treated as described for the cytoplasmic sample, with the exception that 10 times more starting material was used. The purified U90 was electroblotted onto Problot membrane with at least 10pmoles estimated to be present, as determined by the intensity of staining by Coomassie blue. Amino acid analysis revealed that sufficient material was present for successful sequencing to be carried out, but in a similar manner to the cytoplasmic isolate of U90 no amino acid sequence was obtained from the purified U90 isolated from

the membrane fraction of HSV-2 infected Bn5T cells. The experiment was also repeated using U90 purified from control Bn5T cells, but no readable sequence was obtained from these attempts.

4 Attempts To Unblock the U90 Amino terminus

The majority of eukaryotic polypeptides which cannot be sequenced are blocked as a result of acetylation of the terminal amino acid. It has been claimed that treatment of such polypeptides with TFA will remove the blocked terminal amino acid from the polypeptide so leaving second amino acid free to be sequenced (Hulmes et al 1989). U90 isolated on ProBlot membrane was treated with TFA using a modification of the procedures outlined by Hugli (1989) Method, Section 8c. A 20% solution of TFA in water was added to the bottom of a small glass bottle containing U90 immobilized on the Problot membrane. This was made air tight and the TFA left to evaporate to produce gaseous TFA. The U90 was left in the gaseous TFA for either 10 days at room temperature or 2 days at 42°C. Following this treatment the U90 was sequenced.

No readable sequence was obtained from either of these two preparations as the TFA not only digested the terminal amino acid but also the U90 itself. This resulted in breakdown products creating background sequences and so prevented a single readable sequence from being obtained.

5 Internal amino acid sequencing

As no amino acid sequence of the U90 could be obtained using the conditions defined above, experiments were progressed to try to obtain internal amino acid sequence by enzymatically digesting the polypeptide then sequencing the N-terminal of each fragment. To obtain internal amino acid sequence U90 purified as described previously was digested using *Staph. aureus* V8 protease within a gel system in a similar manner to that described for peptide mapping analysis (Method, Section 7c). The major difference between the procedure to obtain internal sequence as opposed to amino terminal sequence was that an absolute minimum of 10 times more material was required for internal amino acid sequencing. This was to ensure that at least 10pmoles was present in each digested fragment to enable a readable amino acid sequence to be obtained. The U90 was therefore required to be further concentrated after the flow through samples had been reduced in volume by partial lyophilization. This was done by running the concentrated flow through samples on a 7.5% gel, using a large comb consisting of one well which ran almost the entire length of the comb and a second small well for protein markers. The U90 was then

visualized with Coomassie blue stain, the gel dried and the U90 band carefully excised from the dried gel.

One large U90 band the length of the gel was produced by this step, which was then cut into smaller pieces to enable it to be placed into the wells of a second 7.5% gel. The bands were rehydrated in the wells with tricine tank buffer (methods section, Table 1) then run into the gel as normal. Once the dye front had run the length of the gel, the gel was stained with Coomassie blue, dried, then the highly concentrated bands of U90 were cut out and digested within a third gel using the peptide mapping technique.

The gel used for the digestion varied from that of a normal peptide map in that it had a much larger stacker and so required larger gel plates (188x250mm). The peptide mapping gel used wells which were twice as deep as normal wells, and because of the depth of the wells the stacker gel was made larger than normal (approximately 150mm in length). The larger stacker gel was also necessary to enable the material being loaded onto the gel to form a tightly stacked band. If the protein did not form a tight band before it reached the interface of the stacker and resolving gel, it was found that only a proportion of the U90 was completely digested as the remainder of the protein ran behind the enzyme on the gel. If this happened the remainder of the sample which was only partially digested, produced protein smears the length of the gel, preventing discrete bands of digested peptides from being isolated. By using a larger stacker this was avoided.

Once the U90 had stacked together to form a tight band and had reached just above the interface of the two gels, the power was switched off for one hour to allow the digestion to take place. The power was then returned and the samples run as far as possible down the gel to give the maximum separation of peptides. These peptides were then transferred to Problot membrane as before by electroblotting, visualized by Coomassie blue staining, then carefully excised (using a scalpel blade) ensuring no smears of contaminating protein were included in the band. Figure 38 shows the electroblotted V8 protease digested U90 peptides as visualized by Coomassie blue staining prior to being excised and sent for sequencing.

The N-termini of 7 independent peptides in total were successfully sequenced over a period of three experiments the sequences of which are presented in Table 1. The sequences are represented by the single letter code for amino acids (which is outlined in Table 2). Four of the seven peptide sequences were confirmed at least twice. To ensure that the *Staph. aureus* V8 protease had not been mistakenly sequenced, all peptides were checked for

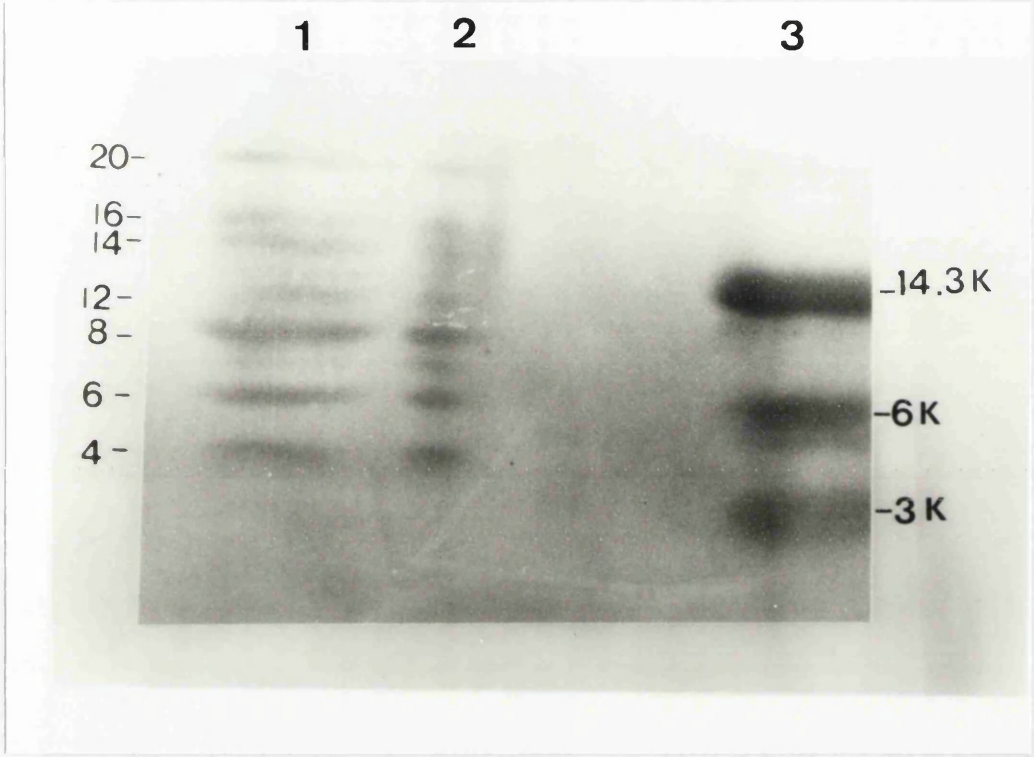


FIGURE 38 : U90 purified from the cytoplasmic fraction of an estimated $2X10^{10}$ HSV-2 infected Bn5T cells using the purification strategy as described previously (Figure 35). The U90 was digested with V8 protease using the modified version of the peptide mapping technique as described in the text. Half of the U90 was loaded into each of tracks 1 & 2 together with 50ug of V8 protease/track and digestion carried out as described previously. The 18% SDS gel was then electroblotted onto a PVDF membrane which was then stained with Coomassie blue. The approximate molecular weights of the resulting peptides are indicated to the left of the membrane, with molecular weight markers present in track 3 and their values in kilodaltons indicated to the right of the membrane.

TABLE 1 : Amino acid sequences of U90 peptide fragments

Amino acid sequences of the U90 peptides are listed below using the single letter code (see table 2), **bold** letters correspond to the sequence finally determined after careful comparison the different sequence data obtained and analysis of the amino acid elution profiles generated (where appropriate).

Size (Kd)	Sequence	Quantity (pmoles)
4	VGYLWYGEWP	43
4	VGYLWYGEKP STFLRAGP YSHW	33
	VGYLWYGEWP STFLRAGP YSHW	
6	SWWVTSDKNM RSDAQ	16
8	FQWYKEGNRV	25
8	FEEYKAGDRV TINWKTLDKA	47
8	FEEYRAGDRV TINWN	16
	FEEYKAGDRV TINWNTLDKA	
12	KIYPTLADEN GQEKV	47
14	KGYIKAADEN VQPHV	40
16	VGYWFYGTDP AWFFS	21
16	VGYWWYWWR	8
	VGYWFYGTDP AWFFS	
20	VIKINAQFYE	12
20	WIKISANFYE	23
20	?ILINANFYE	8
	VIKINANFYE	

TABLE 2 : Single and three letter code for amino acids

AMINO ACID	THREE LETTER CODE	SINGLE LETTER CODE
alanine	ALA	A
arginine	ARG	R
asparagine	ASN	N
aspartic acid	ASP	D
cysteine	CYS	C
glutamine	GLN	Q
glutamic acid	GLU	E
glycine	GLY	G
histidine	HIS	H
isoleucine	ILE	I
leucine	LEU	L
lysine	LYS	K
methionine	MET	M
phenylalanine	PHE	F
proline	PRO	P
serine	SER	S
threonine	THR	T
tryptophan	TRP	W
tyrosine	TYR	Y
valine	VAL	V

homology with the known amino acid sequence of the enzyme using the computer software described below.

6 Comparison of the U90 to documented sequences

The seven internal amino acid sequences of U90 were compared to the sequences entered in the NBRF databases using the Genetics Computer Group (GCG) Sequence Analysis software package version 7. The programmes Wordsearch and FastA were used, which both search for sequence similarity by either a Wilbur & Lipman (1983) or Pearson & Lipman (1988) style search respectively. No significant homology to any protein sequence entered in the database was found. This will be considered further in the following discussion section

The programme Backtranslate was also used to produce the nucleic acid sequences of the peptides, which were then compared to the nucleic acid sequences entered in the Gen EMBL databases. No significant homology was found with any of the nucleic acid sequences (including hsp90 or GRP94), suggesting that the U90 is a novel tumour associated polypeptide.

DISCUSSION OF SECTION 4 : Amino Acid Sequencing of the U90 N-terminus

The development of PVDF membranes and automated protein sequencing has enabled the sequencing of the N-terminal amino acids of many proteins. By electroblotting a partially purified polypeptide onto such a membrane, limited amino acid sequencing of an unknown polypeptide can be obtained, without the need for a 100% homogeneous sample of protein. The acquisition of such sequence data, even though it be potentially very small, facilitates further investigations of the protein in question using such techniques as PCR, cDNA library screening, northern and Southern blotting. The aim of the protein sequencing studies described in this section was therefore to obtain sufficient sequence of the U90 to allow some of these techniques to be applied to the expression of U90 in tumour cells and compare this with HSV infected tumour cells, infected RE cells and control RE cells.

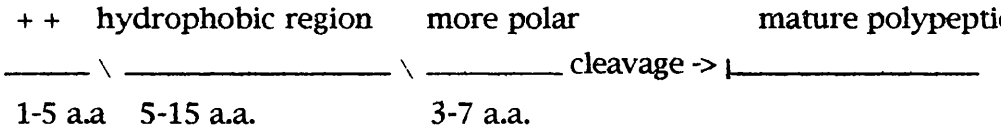
Initial attempts to sequence the U90 used the most abundant source of material available, U90 isolated from the cytoplasm of HSV-2 infected Bn5T cells. Following the protein's partial purification and electroblotting onto a PVDF membrane the amount of protein on the membrane was estimated and then sent for amino acid sequencing. Despite deliberately under estimating the total amount of U90 on the PVDF membrane (by Coomassie blue staining) to be in the very least 20 pmoles of protein, the results generated by amino acid sequencing indicated that less than 4 pmoles of protein were present. This suggested that either the estimation of protein present by Coomassie blue staining was somewhat inaccurate or that the protein was in fact blocked. The former which was subsequently disproved by amino acid analysis of the sample, will be discussed below.

Blockage can occur for example by the presence of a formyl group, a methyl group, a pyroglutamyl group or an acetyl group on the terminal amino acid, where the addition of a pyroglutamyl or acetyl group occurs more frequently. Considerable work has been done investigating acetylation and has been reviewed by Driessen et al (1985). It is thought that acetylation can occur shortly after the initiation of translation while the protein is still being translated. It has been proposed that a general function of N-acetylation may be to protect proteins from proteolytic degradation by amino peptidases (Jornvall, 1975) although contradicting evidence has been presented (Mauk et al, 1976).

In the first protein preparation to be sequenced, it was not known if the blockage in the N-terminal was a naturally occurring modification of the terminal amino acid or if the modification occurred as a result of protein

manipulation. To ensure blockage did not occur during the handling of U90 every possible precaution was taken to remove any "blocking factors" and minimize contact with those blocking factors which could not be removed. Before further amino acid sequencing was attempted an amino acid analysis of the sample was used to quantitate the total amount of U90 present on a small portion of the PVDF membrane. Approximately 10% of the total piece of PVDF membrane to be sequenced was analyzed and unless the amino acid analysis predicted that at least 25 pmoles of U90 was contained on the remaining 90% of the membrane, sequencing was not attempted. On the three occasions that amino acid analysis was completed the amounts of amino acids seen were between 60-88pmoles, but following sequencing the amino acid levels generated by the Edman degradation reaction and detected by HPLC were never found to be significantly above background levels. From this data it was concluded that the N-terminal of the U90 was blocked.

By varying the source of U90 starting material it was hoped that an unblocked form of the U90 could be isolated and amino terminal sequence derived. Alternative sources of U90 included membranes of infected Bn5T cells (a small amount of U90 was detected in this fraction) or membranes from control Bn5T cells. It was hoped that the membrane-associated form of the protein would contain a membrane targeting signal which would be cleaved from the N-terminus of the U90, once it had become associated with the membrane. Such signal peptides are thought to have a general pattern of amino acids (which is outlined below) as opposed to a specific consensus sequence.



If this were the case, the possibility existed that U90 isolated from the membrane associated fraction of the cell may have a number of its N-terminal amino acids cleaved and therefore expose an unblocked terminus. Unfortunately the membrane associated U90 isolated from HSV-2 infected Bn5T cells was also found to be blocked.

The final attempt to obtain N-terminal amino acid sequence was to apply the U90 purification strategy to control Bn5T cell membranes to overcome any modification which might occur within the U90 following HSV infection. Although characterization studies suggested that it was unlikely that HSV-2 infection resulted in the stabilization of the U90 by post-translational modification (Results Section 1.6) the introduction of the virus to the cell may

still have resulted in some modification of the polypeptide which could have prevented the Edman degradation reaction from being completed. U90 was purified from uninfected Bn5T cells (by using considerably more starting material than used previously) - but still the U90 was found to be blocked.

The alternatives available at this point in the project were to either try to unblock the N-terminal or to digest the protein in order to generate "new" N-termini of internal peptide fragment.

Attempts to unblock the amino terminal with TFA using a modification of methods described by Hugli (1989), not only removed the N-terminal amino acid but also cleaved the polypeptide in a number of places resulting in the generation of additional peptide fragments. The most viable option left was to try to generate internal peptide sequence by proteolytic digestion of the protein followed by electroblotting of the peptide fragments onto PVDF membrane which were then sequenced.

In order to obtain internal amino acid sequence of the U90, peptide fragments were generated by proteolytic digestion using V8 protease. This was completed by using a modification of the protocol as described for peptide mapping analysis. The modifications introduced aimed to increase the total quantity and purity of each peptide finally recovered and sequenced after electroblotting from the SDS gel to the PVDF membrane. In order to sequence the individual peptides, the Coomassie stained bands were carefully cut from the PVDF membrane.

The dimensions of the gel itself were modified for two different reasons. In order that maximum efficiency of transfer was achieved a "thin" gel of 0.75mm thick as opposed to the normal standard 2mm gels was used. This was necessary as it was discovered that electroblotting of a 2mm thick gel in the BioRad mini gel system at 120mA (constant voltage) for 1 hour resulted in at least 20% of the protein remaining in the gel, as seen by Coomassie blue staining, compared to almost no visible protein remaining in the gel when a 0.75mm gel was used. Due to the small quantities of peptides that were being dealt with, achieving 100% transfer could mean the difference between obtaining just sufficient material for amino acid sequencing or providing a little more than the minimum required and hence generating much more reliable sequence data.

The second change to the dimensions of the gel was required in order that a larger stacker region of the gel could be generated. This was necessary as the purified U90 polypeptide was loaded into the wells of the gel in the form of strips of dried acrylamide cut from 7.5% gels containing partially purified U90. As a result of the large amount of material required to be loaded in each well, the protein was rehydrated in the wells of the gel (as opposed to prior to

addition to the gel) and then electrophoresed through the 7.5% strips into the stacker of the 18% peptide mapping gel. In order that more gel slices could be loaded onto each track, the wells were made twice the depth as normal and therefore the remainder of the stacker had to be increased in length so that it was at least twice the length of the wells. This was necessary to allow all the protein which was eluted from the gel slices to "stack" correctly before entering the resolving gel. If this "stacking" process did not occur properly before the protein entered the resolving gel, a smear of protein was seen by Coomassie blue staining which ran the length of the track. This was a consequence of the different rates of migration of the polypeptides due to the very slightly earlier time at which the protein at the leading edge of the protein band entered the resolving gel compared to protein behind the leading edge.

Another modification introduced to the system was the addition of considerably more enzyme to each well containing U90 gel slices. 50μg of V8 protease was used for each track of the gel (as compared with 5μg which was used previously). It was estimated that up to at least 10 times more material was being digested under these conditions and as 5μg per track was in vast excess previously, it was anticipated that digestion to completion would be obtained using 50μg of enzyme per track.

The pH of the enzyme digestion buffer was altered from pH 6.8 (which allows digestion of peptide bonds on the C-terminal side of glutamate residues, cleavage of aspartyl bonds and some non-specific cleavage), to pH 8.1. This change restricted peptide bond cleavage primarily to glutamate residues (Aitken et al, 1989). By reducing the number of peptides generated during proteolysis, it was anticipated that some larger fragments would be obtained which would be able to be separated more easily than a number of small peptides of similar molecular weight.

During SDS page, the dye front was run as far as possible down the gel (without the loss of any low molecular weight peptides) to obtain maximum separation, the gel was electroblotted, quickly stained and destained using staining and destaining solutions containing only 1% acetic acid (Method Section 7e), to allow the peptide fragment to be visualized. The individual peptides were carefully excised using a scalpel blade, then sent for amino acid sequencing.

The resulting fragments seen in Figure 38, visualized by Coomassie blue staining appear different to digestion pattern of U90 when visualized by autoradiography of [^{35}S] labelled peptides. This is due to two factors, (1) the different digestion pH conditions and (2) the different method used for

visualizing the peptides. [^{35}S] L-methionine may be incorporated in some peptides, but others may contain no methionine residues resulting in some peptides not being visualized by autoradiography even though they were present. The different digestion conditions should also generate fewer cuts and therefore fewer peptides.

In total seven different peptides were isolated after V8 protease digestion, with the sequences for four of these peptides being generated on at least two occasions. The sequences from the other four peptides were only generated on one occasion due to the difficulties in obtaining sharp discrete bands on the PVDF membrane. This problem occurred because some of the peptides migrated very close to each other on the gel, ie the 12K, 14K and 16K peptides. A similar problem was obtained with the 6K peptide since the 4K and 8K peptides migrated below and above it on the gel, potentially "contaminating" the area around the 6K peptide. Due to the cost of sequencing each peptide, only bands isolated on the PVDF membrane which were free from contaminating smears of protein from above or below them were sent for sequencing.

As can be seen from Table 1 some variations in the sequence were obtained on different occasions. Despite more than adequate material being registered by the system as being present, the quality of the amino acid sequence obtained was not ideal. The sequence was determined by analysis of the HPLC amino acid elution profiles following Edman degradation by the inbuilt programme of the Applied Biosystems 477A protein sequencer. This compared the total amounts of individual amino acids as they were eluted from the HPLC system. Each amino acid is characterized by the length of time which they are retained on the column, eg serine is retained for 6.37 minutes with lysine being retained for 23.58 minutes. However, it was discovered that the system did not always accurately interpret this analysis (Curry & Cusack, personal communication) and therefore every elution profile had to be carefully analysed manually by comparing the picomoles of each amino acid eluted after every cycle in the Edman degradation reaction.

Disparity between the manually and computer predicted amino acid sequence occurred when the levels of more than one amino acid were elevated. Under these circumstances the best fitting sequence for these elution profiles were predicted manually. In some cases these were later modified when more sequence data became available and comparison with the second (or third) set of sequence data suggested that one amino acid was more likely to be correct than another for that particular position. This was particularly relevant when two amino acids were eluted from the HPLC at similarly increased levels, with no indication which was the most probably correct. These manual predictions also

took into account the lag period seen when proline was eluted from the column, which results in increased levels of proline in the system for several subsequent degradation reactions (Curry & Cusack, personal communication).

Analysis of the seven proposed amino acid sequences showed that some similarity existed between the 4K and the 16K peptides and also between the 12K and 14K peptides, as illustrated below (identical amino acid residues shown in bold). As the 12K and 14K peptides were only sequenced on one occasion, the possibility existed that the 14K peptide was derived from the same region of the U90 polypeptide as the 12K, but the 14K had not been fully digested. This was very unlikely as the proteolytic enzyme was added in vast excess and therefore digestion should have gone to completion and additionally, although the peptides were of similar molecular weights, it was certain that the peptides were not mistakenly identified as the alternative peptide since the sequences were generated from two very distinct bands isolated from one track on a single piece of PVDF membrane with the peptides being electroblotted after being separated on a 18% polyacrylamide gel.

4K **V G Y L W Y G E W P** S T F L R A G P Y S H W

16K **V G Y W F Y G T D P** A W F F S

12K **K I Y P T L A D E N** G Q E K V

14K **K G Y I K A A D E N** V Q P H V

It is therefore proposed that the similarities that arise between the 4K and 16K peptides and the 12K and 14K peptides are a consequence of repeat elements within the U90 polypeptides itself. These repeat elements may represent some functional domains within the protein. However, as the sequence data is limited, what these particular regions may be is impossible to predict.

The amino acid sequence of the seven peptides were compared to the 27,711 protein sequences entered in the NBRF data bases using two different programmes - Wordsearch and FastA which align regions of homology within two sequences. These use slightly different methods of alignment, but both indicate not only homology but will indicate conservative changes of amino acids and introduce spaces in one sequence in order to produce a better alignment of the sequences.

Each individual sequence was compared to the sequences entered in the database NBRF. Using the Wordsearch and FastA programmes discussed above, different levels of stringency were applied in order to identify homologous sequences to the U90 within the database. The stringency was varied by altering the number of "mismatches" which could be tolerated during the search. When only one or no mismatches were allowed, no sequences were identified as being homologous to the U90 peptides sequences. This suggested that the U90 had not been previously sequenced and therefore the searches were repeated allowing up to four mismatches in order to search for a sequenced protein which displayed homology with the known sequences of the U90.

When such low stringency searches were completed, a large number of entries in the database were found which met the search criteria. However the homologies found were very limited and only occurred over a small number of amino acids. the statistical significance of such homologies is very low, as the shorter the stretch of amino acids is, the greater the probability that such sequence will occur elsewhere. In order to assess if any of these homologies were significant, proteins which showed any degree of homology, no matter how small, to more than two of the U90 peptide sequences were analysed in greater detail

One sequence was found to have a small degree of homology with the peptides 4K and 16K. This sequence was derived from NADH dehydrogenase (ubiquinone) chain 1 which is 318 amino acids in length, isolated from the mitochondrial genome of the Norweign rat, accession numbers 504747 and 501954 (Gadaleta et al, 1989).

It should be noted that the 4K and 16K peptides themselves exhibit some degree of homology with 3 out of the first 5 amino acids being the same (see above) and it is possible that it was a consequence of this, that the alignment of NADH dehydrogenase was found to have homology with two of the peptide sequences. Further comparison of the U90 (taking all seven peptide sequences into account) with NADH dehydrogenase showed no other homologies to suggest that the U90 was in fact related to NADH dehydrogenase. By carefully considering conserved amino acid changes (eg lysine to valine or tryptophan to arginine) conserved motives were searched for - once again no homologies were found.

In further attempts to determine if the U90 was similar to any other proteins entered in the database not already searched the nucleotide data base GenEMBL was screened. In order to do this the seven peptide sequences had to be "Backtranslated" to predict their DNA sequences (using the GCG programme "Backtranslate"). This used the human foetal globin codon usage as determined

by Shen et al, (1981), analyzed by Smithies et al, (1982) and adopted for use in the Backtranslate programme by Dr J.C.M. Macnab. No entries were found that displayed any significant homology with two or more of the peptide sequences.

Comparison of the U90 sequences derived from the digested polypeptide to the documented sequence of V8 protease sequence confirmed that the enzyme had not been mistakenly sequenced. Further comparison of the sequences was also made to the published sequences of hamster GRP94 (Mazzarella & Green, 1987), with no homology being found. The predicted DNA sequences of the peptide fragments were also compared to the DNA sequence of hsp90 generated by Farrelly & Finkeslstein (1984), and later Jendoubi & Bonnefoy (1988). No homology was found confirming that the U90 was indeed distinct from both GRP94 and hsp90. This also agrees with subsequent work by Dr J.C.M. Macnab in collaboration with Goldberg (Harvard Medical School) who showed that the purified U90 did not cross react with antibodies raised against the La protease (hsp90 equivalent in *E.coli*), nor did the monospecific U90 antibody react with purified La protease. This is in contrast to the reported findings of La Thangue & Latchman (1988).

Throughout this section of the work, a considerable number of problems were encountered which could neither be avoided nor overcome by me. A number of technical problems were reported to have arisen with the protein sequencer itself, including one particular problem with the injector. This resulted in the complete loss of two of my peptide samples (which took up to 2 months to prepare). Despite the fact the the sequencing facility were aware that problems existed, 4 other peptides samples were sequenced under these circumstances without my knowledge and out with my control, resulting in very poor quality sequence data. further more, technical problems which arose with the sequencer resulted in considerable delays between a sample being sent to the Geology Department and the sample being analysed. An additional problem encountered was that of spurious peaks on the HPLC trace, generated during the sequencing. These peaks could have been caused by a "dirty" column, which should have been avoided by extended washing of the column - an point which I could not anticipate nor have any control over.

To ensure that the sequence data given by the Geology Department was as accurate as possible the amino acid profiles of every piece of sequence data were discussed at great length with Drs Cusack and Currie. This was particularly important when determining the relevance of dual amino acid peaks eluted from the column. These could be explained by an amino acid lag period seen after the initial elution of particular amino acids (eg proline). As a consequence, every piece of sequence data generated was discussed with the

protein sequencers in the Geology Department. A considerable time and effort that was therefore invested in every sample sent for sequencing, and the sequence data that was received, no matter how little, was very carefully analyzed not only by myself but also by Dr Maggie Cusack, Dr Joan Macnab and Mr David McNab. Amino acid analysis of the U90 provided no relevant information about the composition of the U90 because of the size of the polypeptide. This information could have been useful if it were obtained for the small peptide fragments generated by V8 digestion, however such small quantities of peptides were available that all the material generated was required to obtain amino acid sequence.

Based on the assumption that the sequence data eventually generated by Drs Curry and Cusack was correct it can be concluded that the U90 is a novel tumour associated polypeptide with no significant sequence homology to any sequence entered in the data bases to date.

SECTION 5 : UTILIZING THE U90 AMINO ACID SEQUENCE

1.Utilizing amino acid sequence for cDNA library screening

Dr J.C.M. Macnab had previously generated cDNA libraries of the Bn5T cell line in the expression vector λgT11. With the information obtained from the amino acid sequencing data, this library could now be screened. Although this expression vector was originally designed for screening cDNA libraries with antibodies, oligonucleotide probes have also been successfully used (Huynh et al, 1985). The experiments described in this section were not completed due to the short period of time remaining for this project.

Before embarking on any molecular biology experiments certain assumptions had to be made: firstly that the amino acid sequence data was correct in order to generate probes for the U90, and secondly that the cDNA library (generated by Dr JCM Macnab) contained the cDNA for the U90. Neither of these assumptions could be substantiated and due to the short period of time left to identify the U90 gene the sequence data had to be accepted as a close representation of the U90 and the cDNA library accepted as expressing the U90.

To generate synthetic oligonucleotides corresponding to the U90, the internal amino acid sequences were backtranslated to nucleic acid sequences using the eukaryotic codon usage of human foetal globins G and A gamma genes (Shen et al, 1981 , Smithies et al, 1982) and the GCG Sequence Analysis Backtranslate programme as detailed in Table 3. This resulted in either a "bestfit" sequence, which predicted the most likely codon usage for each amino acid, or a degenerate sequence which included all possible nucleotide sequences for that particular peptide's 5' amino acid sequence. Unfortunately none of the amino acid sequence generated had low redundancy which resulted in the necessity to synthesize degenerate probes.

The most reliable peptide sequences (ie where there was least ambiguity), were analyzed to determine which part of the U90 peptides contained the least degeneracy and these regions were chosen to generate oligonucleotides. By reducing the number of degenerate sites, all degeneracies could be incorporated into the nucleic acid sequences of the 4K and 8K peptides and used to produce synthetic oligonucleotides. In all circumstances oligonucleotide sequences were checked by at least one other person and the suitability of the particular sequence for PCR confirmed others who were experienced in PCR primer design (Dr Bill Carman, Institute of Virology). The degenerate synthetic oligonucleotides which were kindly made by Dr John Maclauchlan and their derivation is illustrated in Figure 39. At the time of synthesis of the initial oligonucleotides only one set of amino acid sequence data had been obtained and

TABLE 3: Eukaryotic codon usage

Eukaryotic codon usage as derived from the human foetal beta globins G and A (Shen et al, 1982; Smithies et al, 1982).

Amino acid	codon	% distribution	amino acid	codon	% distribution
Gly	GGA	46	Asn	AAT	40
	GGC	46		AAC	60
	GGT	1			
			Met	ATG	100
Glu	GAG	50			
	GAA	50	Ile	ATA	25
				ATC	75
Asp	GAT	63			
	GAC	38	Thr	ACC	50
				ACT	30
Val	GTG	69		ACA	20
	GTT	15			
	GTC	15	Trp	TGG	100
Ala	GCC	55	Cys	TGT	100
	GCT	27			
	GCA	18	Tyr	TAC	100
Arg	AGG	67	Leu	CTG	71
	AGA	33		CTC	18
				TTG	12
Ser	TCC	45			
	TCT	18	Phe	TTC	63
	AGT	18		TTT	38
	AGC	18			
			Gln	CAG	100
Lys	AAG	83			
	AAA	17	His	CAT	57
				CAC	43
			Pro	CCT	50
END	TGA			CCA	25
				CCC	25

FIGURE 39 : Derivation of synthetic oligonucleotides 4a & 4b and 8a & 8b.

The amino acid sequences and corresponding DNA sequences of the 4K and 8K peptides are shown below. The arrows indicated the DNA sequences from which the synthetic oligonucleotides originated. This data was generated from initial amino acid sequence data and used for cDNA library screening and PCR. As at this time only one set of sequence data had been obtained, oligonucleotides were directed against the most "reliable" sequences which were at the amino terminus of the peptides. Positions of degeneracy are indicated by showing the alternative base(s) in brackets.

4K PEPTIDE

	Val	Gly	Tyr	Leu	Trp	Tyr	Gly	Glu	Trp	Pro
4a	5'→→→→→→→→→→→→→→→→→→→→3'									
	GTG	GGA	TAC	CTG	TGG	TAC	GGA	GAG	TGG	CCT
	GTT	GGC		CTC			GGC	GAA		CCA
	GTC			TTG						CCC
4b	3'←←←←←←←←←←←←←←←←←←←←5'									

4a
5' GTG(T,C) GGA(C) TAC C(T)TG(C) TGG TAC GG 3'

4b (complementary to RNA in region indicated by arrows)
5' CC GTA CCA C(G)AG(A) GTA T(G)CC C(A,G)AC 3'.

8K PEPTIDE

	Phe	Gln	Trp	Tyr	Lys	Glu	Gly	Asn	Arg	Val
8a	5'→→→→→→→→→→→→→→→→→→→→→ 3'									
	TTC	CAG	TGG	TAC	AAG	GAG	GGA	AAC	AGG	GTG
	TTT				AAA	GAA	GGC	AAT	AGA	GTT
							GGT			GTC
8b		3'←←←←←←←←←←←←←←←←←←←←←– 5'								

8a
5' TTC(T) CAG TGG TAC AAG(A) GAG(A) GG 3'

8b (complementary to RNA in region indicated by arrows)
5' G(A)TT T(GA)CC C(T)TC C(T)TT GTA CCA CTG

therefore the amino acid sequence used to generate the probes does not exactly correspond to the final sequence present in Table 1. The 4a and 8a oligonucleotides were then used to try to identify any cDNA in the λgt11 libraries containing the U90 message.

As indicated cDNA libraries were constructed in an expression vector allowing both oligonucleotide and antibody probes to be used to screen for the U90 cDNA. For this reason, in conjunction with the degenerate oligonucleotide screening, Mr David McNab also used an antibody probe (the monospecific U90 antibody and TBS) to screen the library. This meant that any positive clone isolated could be confirmed by both an antibody and an oligonucleotide probe.

Up to 80,000 plaques were screened in total using end labelled probes (Method 9c) derived from both the 4K and 8K peptide fragment sequences and duplicate filters. A number of clones gave positive reactions. However, when these were isolated and grown up they were found to be non-specific positives. Repeating the experiments using duplicate filters and the two probes separately still resulted in no positive clone being consistently identified. Screening with the antibody probe also resulted in the isolation of several positive clones (McNab, unpublished) but disappointingly in a similar manner to the clones isolated by the oligo probes, none of these were found to be positive following plaque purification and further antibody screening.

A number of technical difficulties arose during these experiments including false positives. This particular problem was removed by using duplicate filters. Other problems included high non-specific background and difficulties in determining the optimal hybridization temperature of the probes (due to their degeneracy). These problems could only be overcome by synthesizing new probes using nucleotide sequences which would hybridize 100% with the cDNA of the U90. This could only be done by obtaining DNA sequence directly from the gene or the cDNA, ie sequence which was not inferred by the amino acid sequence which may have contained inaccuracies.

The other possible reason for not being able to identify the U90 cDNA could have been that either the cDNA construct expressing the U90 had been lost, that it was present in such low amounts that insufficient clones had been screened to allow its detection or that expression of the U90 was toxic to the bacteria such that any positive clone when it was attempted to be amplified, died. It is also possible that the cDNA for the U90 was not included in the library at the time of synthesis. This may have occurred if the message for the U90 had a long polyA tail. As a result of this the resulting large cDNA may not have been isolated intact. If time had not been a limiting factor, I would have liked to generate

further cDNA libraries using not only Bn5T cells, but also Bn5T cells infected with HSV and control RE cells.

In order to obtain some highly homologous DNA sequence and overcome these problems the oligonucleotides were used in polymerase chain reactions (PCR) to amplify either mRNA which was reverse transcribed into cDNA or genomic DNA, both of which were isolated from different sources as described below.

2 Utilizing Amino Acid Sequence For Polymerase Chain Reaction

Studies using the polymerase chain reaction (PCR) can be divided into two sections - either using DNA as a template or RNA as a template (from which cDNA was synthesized). These two conditions were then further divided depending on the primers used - either a set of primers derived from two different internal peptide sequences (Figure 39) or a set of primers derived from either end of a single peptide sequence (Table 4).

2(a) Bn5T DNA As A Template

DNA was isolated from $6x10^6$ Bn5T cells as described in Method 11, quantitated by optical density (OD_{260}), then used as a template in PCR reactions using degenerate synthetic oligonucleotide probes. Prior to PCR, the primers were purified on a 15% urea/acrylamide gel (Method 9a) then the molarity of the primer samples determined using their OD_{260} in the equation below (Steinthorsdottir, personal communication), where A=the number of adenine nucleotides, C=cytidine, G=guanine, T=thymidine.

$$Em = A(16,000) + G(12,000) + C(7,000) + T(9,600)$$

$$\text{where Molarity } M = \frac{OD_{260}}{E_m}$$

PCR was carried out using 15-30ug of Bn5T DNA, 0.2μM of each of the two primers, 0.125mM of each of the four nucleotides and 2 units of Ampli Taq polymerase/ reaction in PCR reaction buffer as described in Method 13(b) containing 4mM $MgCl_2$.

As the primers were derived from the amino acid sequence of two different internal peptides of U90, the orientation of the sequences with respect to one another was not known. To determine this orientation two complementary sets of primers were used to accommodate both possibilities (Figure 40a) using the synthetic oligonucleotides described in Figure 39. The amplification using both sets of primers was carried out in a total volume of 50ul, for 35 cycles, using the following temperatures - denaturation at 95°C for 6 minutes in the first cycle

TABLE 4 : Derivation of synthetic oligonucleotides 4.1 & 4.2 and 8.1 & 8.2.

Synthetic oligonucleotides generated from revised amino acid sequence data to enable PCR products the length of the known peptide sequence to be generated. Oligonucleotides were directed at the extremes of the known amino acid sequences in order to generate as large a PCR product as possible. In these oligonucleotides inosine (I) is used at positions where degeneracy is greater than two, and the arrows correspond to the regions against which the oligonucleotides were directed.

4.1 5'→→→→→→→→→→→→→→→→→→→→→3'

Val Gly Tyr Leu Trp Tyr Gly Glu Trp Pro Ser

Thr Phe Leu Arg Ala Gly Pro Tyr Ser His Trp

4.2 3'←←←←←←←←←←←←←←←←←←←←←5'

4.1

5' GTI GGI TAC C(T)TG(C) TGG TAC 3'

4.2 (complementary to RNA at position indicated by arrows)

5' CCA A(G)TG G(A)G(C)A(T) GTA IGG ICC 3'

8.1 5'→→→→→→→→→→→→→→→→→→→→→3'

Phe Glu Glu Tyr Lys Ala Gly Asp Arg Val Thr

Ile Asn Trp Asn Thr Leu Asp Lys Ala

8.2 3'←←←←←←←←←←←←←←←←←←←←←5'

8.1

5' TTC(T) GAG(A) GAG(A) TAC AAG(A) GCI GG 3'

8.2 (complementary to RNA at position indicated by arrows)

5' GC C(T)TT A(G)TC C(G)AG(A) IGT G(A)TT CC 3'

FIGURE 40: Determining the relative orientations of the 4K and 8K peptides.

Figure 40a and 40b differ with regards to the oligonucleotides used. In Figure 40a the oligonucleotides were derived from only 10 amino acids of sequence and therefore represent the vast majority of the peptide sequence and are partially complementary. In Figure 40b, the oligonucleotides were derived from peptides of at least 20 amino acids in length and therefore are derived from the amino and carboxy ends of each peptide sequence.

Figure 40a

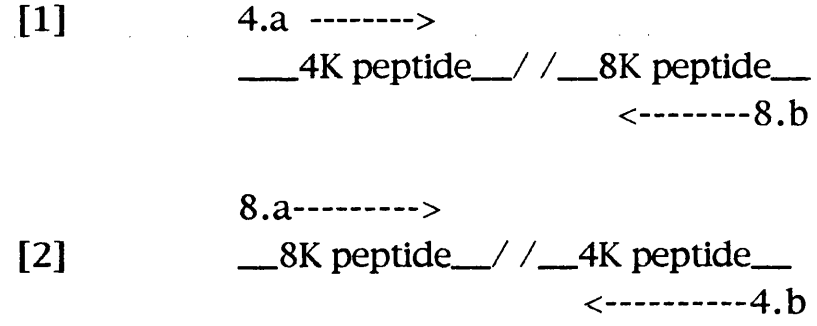
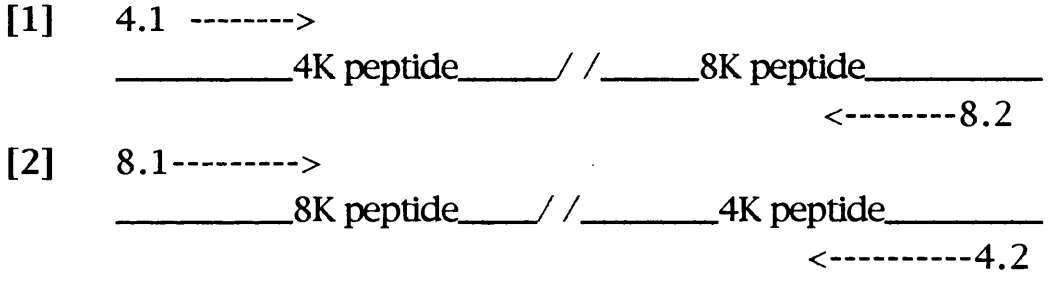


Figure 40b



and 1 minute thereafter, hybridization at 45°C for 1.5 minutes, and extended polymerization at 72°C for 4 minutes. The low hybridization temperature of 45°C was used to enable hybridization of low specificity to occur. This was essential as the oligonucleotides used were not 100% homologous to the U90 DNA. One tenth of the resulting samples were analyzed on agarose gels and DNA products visualized by UV illumination.

Using DNA as a template and primers from separate internal peptide sequences no DNA could be visualized by UV illumination. This may have been because insufficient DNA was present to be visualized or alternatively the reaction may not have worked for a number of reasons which are discussed below.

There was no way of determining how far apart the primers were on the U90 gene as it was impossible to predict the size of any potential PCR product when DNA was used as a template. This also made the PCR reaction itself more difficult as it was not known how much time should be assigned to the elongation period of each cycle. If the elongation period was too short, a full length product would not be made, but if it was too long the activity of the Ampli Taq polymerase would be unnecessarily reduced. Another problem with using DNA as a template was the possibility that introns existed between the two primers on the U90 gene. If this were so, the result would be the amplification of a DNA fragment which included an intron and hence would be too large to expect the PCR reaction to be successful. It was therefore concluded that the two probes were most probably too far apart on the DNA to expect a successful PCR reaction. However, the inability to isolate a product may have been due to insufficient cycles being completed or the oligonucleotides being too degenerate to permit efficient hybridization.

An alternative approach was taken using primers both of which were derived from the sequence of a single peptide fragment (Figure 40b). This was only possible because more amino acid sequence of the 4K and 8K peptides had been obtained. As a result of the additional sequence which had been generated alterations were made to the predicted DNA sequence (inferred from the corrected amino acid sequence) and new oligonucleotides made (Table 4). These were also modified to include inosine (Patil & Dekker, 1990), in positions where three or more different nucleotides were required due to degeneracy of the sequence. This meant that the orientation of the primers with respect to each other was known and the size of product could be predicted assuming an intron did not fall in the middle of the known amino acid sequence. Using primers 4:1 and 4:2 for one reaction and 8:1 and 8:2 (Table 4) for a second reaction, the PCR was repeated as before using 1 and 10ug of Bn5T DNA, 500uM dNTPs, 1mM of each

primer, 2.5 units of Tap polymerase and either 1.5 or 2.5mM $MgCl_2$. 45 cycles were completed using a hybridization temperature of 48°C for 1 minute and an extended polymerization temperature of 72°C for 1 minute. Following the first 45 cycles no DNA product could be visualized therefore 10% of each of the amplified samples were re-amplified for a further 20 cycles. Analysis of these samples revealed DNA products in the samples using primers 8:1 and 8:2 at a $MgCl_2$ concentration of 2.5mM. A representative line diagram and photograph of the resulting DNA products can be seen in Figure 41. The resulting DNA product of less than 100bp in size, as estimated by DNA markers run on the gel, was seen on two occasions. This corresponds to the expected size of the DNA generated using the 8.1 and 8.2 primers (17 bases between the primers plus 34 bases for 8.1 plus 31 bases for 8.2 = 82 bases in total), assuming no intron falls in this region The DNA bands seen running at approximately 40bp in tracks 6 and 7 are thought to be due to primer-dimer formation which is frequently seen (Carman, personal communication).

The reamplified DNA in the sample was purified on an low melting agarose gel and cloning into M13 was attempted by blunt end ligation (Method 13c) to allow the amplified DNA to be sequenced. The ligation reaction was attempted on 3 occasions and transformed into E.coli, however no positive clones were isolated by blue/white X-gal selection.

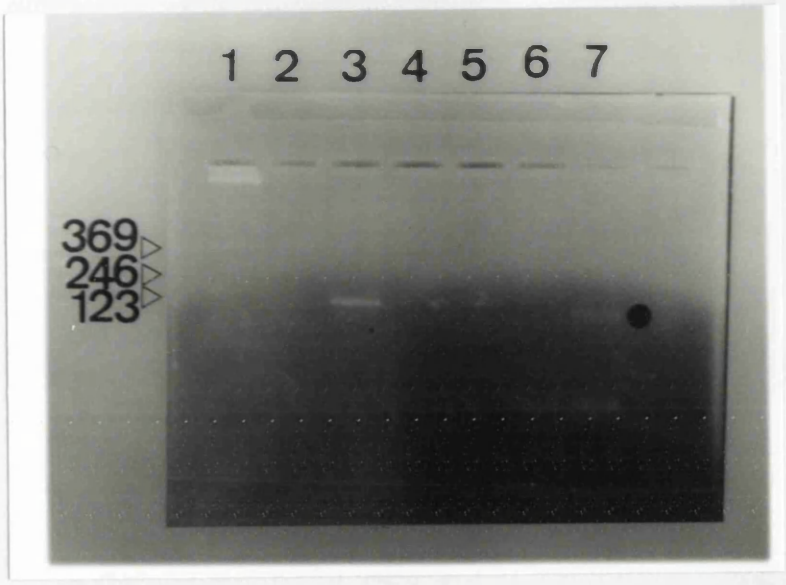
2(b) Bn5T RNA As A Template

To overcome the problems encountered when DNA was used as a template for PCR (ie the possibility of introns being present within the target DNA or the DNA product defined by the primers being too large to realistically expect the PCR to be successful), RNA was isolated from cells and used as a template. The RNA isolated was produced using two different methods and from cells either infected or uninfected by HSV-2. The two latter conditions were used to help determine if the U90 was transcriptionally activated by HSV infection.

(a) Cytoplasmic RNA was isolated from Bn5T cells as described in Method 12a.

(b) Poly A RNA was isolated using Dynabeads oligo $(dT)_{25}$ selective for the poly A tail of mRNA (Dynal, Ireland) as described in Method 12b. The Dynabeads which bind poly A tails of the mRNA were isolated from the sample by magnetism and the mRNA eluted from the beads using buffers supplied and conditions described by the manufacturer's protocol. This method of RNA isolation was used with both infected and uninfected Bn5T cells.

Once the RNA was isolated and quantified the RNA was reverse transcribed as described in Method 13a (Steinthordottir & Mautner 1991) using the primer



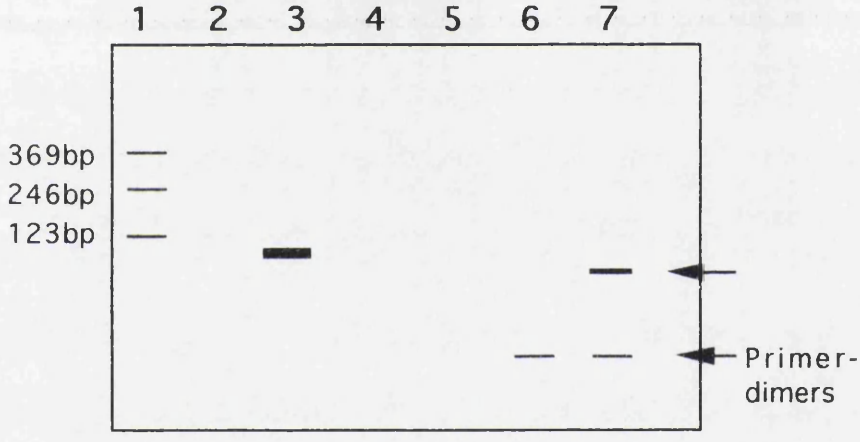


FIGURE 41 : Ethidium bromide stained agarose gel (3% nusieve, 1% agarose), containing PCR products generated using the primers 4.1 & 4.2 (tracks 4 & 5) and primers 8.1 & 8.2 (tracks 6 & 7). Track 1 shows DNA markers whose values are indicated to the left of the gel in bp, track 2, a negative sample and track 3, a positive sample of an expected size of 100bp. Tracks 4 & 6 used 1.5mM $MgCl_2$, and tracks 5 & 7 used 2.5mM $MgCl_2$. 10ug of Bn5T DNA was used as a template, 2.5 units of Taq polymerase, 1mM of each primer and 500uM dNTPs in each reaction. Initially 45 cycles were completed in a total volume of 50ul (94^oC denaturation for 1 minute, 48^oC hybridization for 1 minute, 72^oC extension for 1 minute), and 1/10 of the final sample was removed. A 20 further cycles were carried out on this material using the same conditions - after new primers, enzyme and dNTPs had been added. 5ul of each sample can be seen in the above gel. A DNA product (indicated by an arrow), of <100bp can be seen in track 7. A representative line diagram is shown.

which was complementary to the RNA to initiate cDNA synthesis. This first strand cDNA was then used as a template for the PCR reaction.

Various conditions were used over a number of experiments by changing the following parameters:

Starting material - 1 and 5ug of RNA was used to produce cDNA

The number of cycles used - 35 cycles were used initially. This was increased to 40 followed by a further 20 cycles to reamplify the product.

Denaturation temperature - this was maintained at 95°C for a period of 5-6 minutes in the first cycle and 1 minute in all other cycles

Hybridization temperature - this was kept low due to the degeneracy of the primers, between 45-54°C.

Extension temperature - maintained at 72°C

Time allowed for extension - this varied depending on the template and primers used, between 1 minute and 4 minutes. The larger the expected DNA product, the longer the extension period had to be, thus in reactions using primers derived from two different peptide fragments the extension was carried out over 4 minutes, and over 1 minute if the primers were derived from either end of the one peptide fragment.

Ampli Taq enzyme concentration - this was used successfully at 2.5 units per reaction.

$MgCl_2$ concentration - this was varied between 1 and 4mM with success most often at 4 mM.

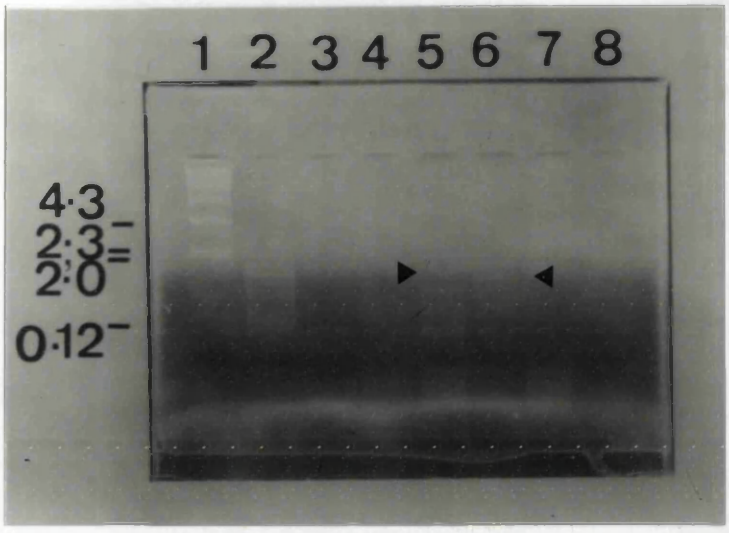
Using the two sets of primers (4.1 + 8.2 and 8.1 + 4.2) derived from peptides 4 and 8 (Table 4) the orientation of the peptides with respect to each other was investigated. To determine which was the correct location of the 4K and 8K peptides with respect to each other as illustrated in Figure 40b, two different 1st strand cDNA synthesis experiments followed by PCR were completed.

In order to investigate the situation where the 4K peptide is located to the amino end of the 8K peptide (ie to the left) primer 4.1 was used in the 1st strand cDNA synthesis reaction (seen in Figure 40b [1]), and 8.1 was used for 1st strand cDNA synthesis, to investigate the opposite situation (ie the 4K peptide lying to the carboxy or right side of the 8K peptide) seen in Figure 40b [2]. In this experiment 1 or 5ug of RNA was used for 1st strand cDNA synthesis, (isolated as described in Method 12a). Conditions used were 1.5mM $MgCl_2$, 1mM dNTPs, 10 units of RNasin, 10 units of AMV reverse transcriptase and 1uM of the relevant primer (in 10ul). The sample was incubated at room temperature for 10 minutes followed by 30 minutes at 42°C, 5 minutes at 100°C then placed immediately on ice (modified from Steinthordottir & Mautner 1991). To proceed with PCR more

of each primer was added to a final concentration of 0.2uM, 1 unit of Taq polymerase and the volume made up to 50ul. Using a hybridization temperature of 50°C and 36 cycles were completed and 10% of each sample analyzed on a 1.5% agarose gel as seen in Figure 42 (which also includes a diagramatic representation of this gel). It demonstrated on three separate occasions that orientation dictated by the primers 4.1 and 8.2 was correct, as only these primers gave any DNA products following amplification. The DNA amplified under these conditions included non-specific DNA products due to the low stringency of the primer hybridization temperature.

Attempts to reamplify the above PCR products to provide more material to allow shot gun cloning into M13 to be completed was unsuccessful. This was most likely due to the same primers being used in the reamplification as opposed to nested PCR being attempted with a second set of primers. This was not possible as insufficient sequence was available to synthesize further primers to carry out nested PCR.

A repeat of these experiments using mRNA isolated using Dynabeads oligo $(dT)_{25}$ was unsuccessful. The PCR reaction itself was successful as shown by the positive control, however it appears that the mRNA isolated using these conditions was not suitable for PCR. This may have been a consequence of the final buffer the RNA was isolated in, as it contained EDTA, or alternatively, the U90 mRNA may not have been isolated because the poly A tail was hidden, or, in a similar manner to histone DNA, the U90 may not have a poly A tail.



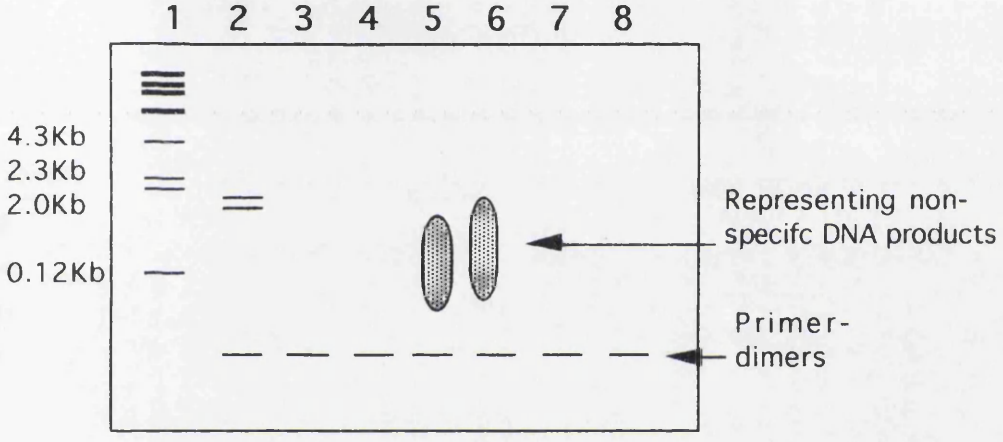


FIGURE 42 : Ethidium bromide stained agarose gel (1.5% agarose), containing PCR products generated using the primers 4a, 4b, 8a & 8b. Track 1 contains DNA markers, indicated to the left of the gel, track 2 a positive sample of an expected size of around 2Kb, track 3 - a negative sample. Tracks 4-6 used 5ug of HSV-2 infected Bn5T RNA as a template, and tracks 7 & 8 used 5 and 1ug respectively of uninfected Bn5T DNA. Track 4 used the primer 8a to generate cDNA, followed by 8a & 4a in PCR, with tracks 5 & 6 using primer 4b to generate cDNA, followed by primers 4b & 8b in PCR. Conditions for PCR were 1.5mM $MgCl_2$, 2.5 units of Taq polymerase, 1mM of each primer and 500uM dNTPs in each reaction. 40 cycles were completed in a total volume of 50ul (94^{o}C denaturation for 1 minute, 50^{o}C hybridization for 1 minute, 72^{o}C extension for 3 minutes), and 10% of the final sample analysed. The reverse transcription reaction where appropriate used 20 units of reverse transcriptase at room temp for 10 minutes, 42^{o}C for 30 minutes, 100^{o}C for 5 minutes and cooled quickly on ice. The PCR reaction was then carried out. A representative line diagram is shown.

DISCUSSION OF SECTION 5 : Utilizing the U90 amino acid sequence

This section outlined the molecular experiments initiated to utilize the information generated from the amino acid sequencing of the proteolytic peptide fragments of the U90. The long term aim of this work is to be able to obtain the complete amino acid and DNA sequence of the U90, either by isolating cDNA encoding the U90, by screening cDNA libraries, or by accumulating sequence data generated by PCR. On establishing the sequence of the U90, the data bases could be rescreened to search for other polypeptides which might contain functional domains which display a degree of homology with the U90. If this was achieved, progression towards elucidating the functional role of the U90 in the cell could be made. As stated previously these experiments depended on the accuracy of the amino acid sequence data generated by others and the presence of a cDNA representing the U90 in the cDNA library.

To screen the λgT11 cDNA library of Bn5T cells, which was previously generated by Dr J.C.M. Macnab, synthetic oligonucleotide probes were made. Using the sequence data of the 4K and 8K peptides, mixed oligonucleotides that encompassed all degenerate sequence possibilities were made, as described by Girgis, et al (1988). This increased the chance of the most homologous probe possible, which was complementary to the U90 cDNA being present. The amino acid sequence against which the oligonucleotides were made was chosen such that the number of degenerate sites in the probes were minimized (in order to reduce the total number of different probes required in the mixed oligos). Where possible this was done by choosing a region of amino acid sequence which incorporated the least amount of degeneracy, eg sequence containing tryptophan, tyrosine, methionine, cysteine and glutamine.

The mixed oligo probes were also to be used as primers in PCR experiments and so further considerations had to be taken into account when designing these probes. In PCR the most important region of the primer is the 3' region. When selecting the region of amino acid against which to make the probes, any large area of degeneracy was avoided in the 3' area and it was essential to select a region where there was no dubiety in the amino acid sequence. The length of primer should aim to be around 20 bases and where possible end in a guanine or cytosine. Latterly deoxyinosine was incorporated into the primers at points of degeneracy. Finally once these conditions have been met, the primer should be checked for obvious complementarity within itself to prevent hairpin bends forming. Most importantly, complementarity with the second primer should be minimized, as if this does occur primer dimers will occur and reduce the

number of primers in the sample free to align with the target DNA. Before selecting the probes to be synthesized these conditions were carefully applied, the sequences of the probes checked by at least one other person and the design of the probes confirmed to be suitable by Dr Bill Carman.

Two probes were used to screen the available cDNA library, one derived from the 4K peptide and the second derived from the 8K peptide. The use of two different probes proved to be a considerable aid, as screening of the nitrocellulose filters with degenerate oligos produced a large amount of background. By using two independent probes and duplicate filters it was possible to select regions on the duplicate filters which gave positive signals with both probes. As the cDNA of the U90 was thought to be of low abundance in the cells (Macnab, unpublished), it was possible that only a very small proportion of the library would correspond to the U90. To increase the chance of isolating U90 cDNA, as many colonies as possible were screened on any one occasion by plating the library out at a high number of colonies per plate. Any regions on the plates which were found to be positive with both probes were then picked, grown overnight and plated out at a lower density in order that single colonies could be isolated. However when these colonies were rescreened no positive colonies were found, suggesting that the original colonies were just background contamination. Despite attempts to remove the background by introducing more stringent washes, no positive colonies could be isolated.

The inability to isolate positive colonies could have been due to the low representation of the U90 in the library, the absence of U90 in the library or potentially inaccuracies in the original amino acid sequence. Low levels of U90 cDNA (or none at all) may have occurred if the U90 expression was found to be toxic to the bacteria. Efforts were then concentrated on trying to obtain more sequence data by generating a PCR product, cloning it into M13 and sequencing it.

Several different experiments were attempted using the synthetic oligonucleotide probes with both DNA or RNA as a template. Initial PCR experiments were unsuccessful when DNA was used as a template to try to amplify the DNA sequences lying between the 4K and 8K peptide fragments. However, as the relative location of the 4K and 8K peptide fragments to each other on the U90 gene were unknown, the size of any potential DNA product could not be predicted. It is probable that this product would have been too large to expect a successful amplification reaction, as the chance of introns being present between the two peptides was also possible.

Once further amino acid sequence had been obtained, primers of a known distance apart from each other were used by synthesizing oligos corresponding

to the beginning and the end of the sequence data of a single peptide. Some success was obtained using this approach as a small DNA product (Figure 41), of the expected size could be visualized when primers from either end of the 8K peptide were used.

To reduce the size of any potential DNA product when primers from the two different peptides were used, RNA was used as a template as opposed to DNA. These experiments allowed the relative positions of the 4K and 8K peptides on the U90 gene to be determined. This was done by using a primer complementary to the RNA to enable the reverse transcription of the mRNA, followed by PCR using the two primers from the 4K and 8K peptides to amplify up the synthesized cDNA.

A number of background bands were seen when the samples were analyzed by gel electrophoresis which were probably generated as a consequence of partial reverse transcription of the RNA (producing truncated products), and/or due to the low stringency of the annealing conditions as a consequence of the degenerate nature of the oligos.

Attempts to clone the DNA products generated by the different PCR experiments were unsuccessful. This may have been due to insufficient material in the ligation reactions or possibly incorrect conditions to increase the chance of the ligation reaction being successful. The ligation reactions were attempted on three different occasions using material isolated from low melting agarose gels. As the reaction depended on a blunt end ligation reaction being successful, perhaps if more material had been available repeating the experiment several more times would have resulted in the successful cloning of the amplified DNA.

SECTION 6 :CHARACTERIZATION & PURIFICATION OF U90 IN PRIMARY RE CELLS

1 U90 Visualized In Control RE Cells by Coomassie Blue Stain

When control RE cell polypeptides are radiolabelled with [^{35}S] L-methionine, immunoprecipitated with TBS, analysed by SDS page and visualized by autoradiography, the U90 polypeptide was never seen. Extensive investigations were initially carried out on RE cells in an attempt to isolate the U90 in control cells and are described below:

(1) Cells were radiolabelled at two different stages in the cell cycle, when they were either quiescent or in a logarithmic growth phase. This was to determine if the expression of the U90 was cell cycle regulated.

(2) Cells were radiolabelled in 1% or 10% serum to investigate the possible involvement of a serum responsive element in the regulation of the U90. This was prompted by the discovery of such an element 5' of the transcriptional initiation signal of the c-fos gene (Treisman, 1985, 1986; Gilman et al, 1986; Greenberg et al, 1987).

(3) RE cells were also treated with a glucocorticoid, dexamethasone, as glucocorticoid responsive elements have also been reported to act as regulatory elements in gene expression (Kasambalides & Lanks, 1983). This has been well characterized in the case of the LTR of mouse mammary tumour virus (reviewed by Offord, 1988).

None of these conditions resulted in the visualization of the U90 in RE cells by immunoprecipitation of radiolabelled polypeptides.

In contrast to the above, when a similar IP reaction was repeated with RE control cells using at least 10 times more material and antibody than normal (around $4X10^7$ cell per reaction) and the polypeptides visualized by Coomassie blue staining, a polypeptide of similar molecular weight to that of the U90 immunoprecipitated from Bn5T cells could be seen in the RE cells (Figure 43). This was believed to be the correct band as the U90 from Bn5T cells was radiolabelled and could be localized by aligning the autoradiograph with the stained gel. This observation was made before the U90 purification strategy had been developed and as no antibody was available to test on a western blot if this 90K polypeptide in RE cells was in fact the U90, this could not be tested further at this point. However, once the U90 purification strategy had been developed, Mr David McNab was able to raise a monospecific antibody against the U90 (Methods Section 3b), by using the U90 purified by the method described in Results Section 4, and injecting rabbits with polyacrylamide gel slices

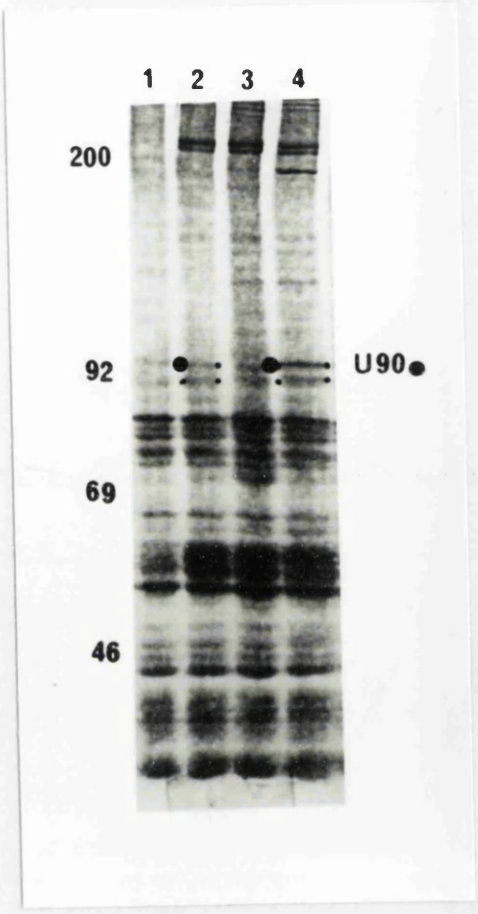


FIGURE 43 : IP reaction using TBS of RE cells (tracks 1 & 2) and Bn5T cells (tracks 3 & 4) using an estimated $4X10^7$ uninfected cells (tracks 1 & 3) or HSV-2 infected cells (tracks 2 & 4) in each reaction. The IP reactions were analysed as normal by 7.5% SDS-page and the gel stained with 0.2% Coomassie blue as described previously. The additional Coomassie stained bands are bacterial polypeptides generated by the use of Pansorbin, (which is a suspension of *S.aureus* Cowan 1 strain which express a coat of protein A), as a alternative for protein A.

containing the protein. This antibody was then used in conjunction with purification studies to investigate the presence of the U90 in control RE cells.

2 Purification of U90 From RE Cells

Using the purification strategy devised to isolate U90 from HSV infected Bn5T cells, attempts were made to try and purify the U90 from HSV-2 infected RE cells. The RE cells were infected by HSV-2 at 5-10pfu/cell for 17 hours at 37°C, the cytoplasmic fraction isolated, a 30% ammonium sulphate cut performed, the sample desalted into FPLC buffer A (50mM tris 0.1% triton), centrifuged, passed over the FPLC mono Q column and the flow through samples analyzed as described in Results Section 4 and summarized in Figure 35.

Coomassie blue staining of the flow through samples indicated that a polypeptide of a similar molecular weight to U90 was present in the cytoplasmic fraction of HSV-2 infected Bn5T cells (Figure 44). This 90K protein was then further analyzed by western blotting using the monospecific U90 antibody.

3 U90 Isolated From Control RE Cells Is Recognised By The Monospecific U90 Antibody

The U90 isolated from the flow through of HSV-2 infected RE cells was analyzed by western blotting using the monospecific U90 antibody raised in rabbits against purified U90. To confirm that the monospecific U90 antibody did in fact recognise the U90, western blots using this antibody were carried out following immunoprecipitation of the U90 from Bn5T cells and Bn5T cells infected with HSV-2. The resulting blot (Figure 45a) demonstrated that the U90 antibody recognised the immunoprecipitated U90 and shows a quantitative difference in the amount of U90 in the infected cell compared to the uninfected Bn5T cell. The 90K protein seen in the RE cells was also recognised by TBS in IP reactions where the RE cells were immunoprecipitated with TBS, run on an SDS gel and then western blotted using the monospecific U90 antibody (Figure 45b). The former experiment was kindly done by Dr Joan Macnab confirming that the monospecific U90 antibody recognised the 90,000MW RE cell polypeptide immunoprecipitated by TBS. This showed that control RE cells express a U90 homologue despite the fact that it had not been visualized by [^{35}S] L-methionine radiolabelling overnight.

4 U90 In RE Cells Is Also Increased By HSV Infection

U90 was purified from both HSV-2 infected and uninfected control RE cells, and the cytoplasmic and membrane associated fractions from each compared. 2X10^{9} cells were harvested as usual (approximately 10 burlers of primary RE

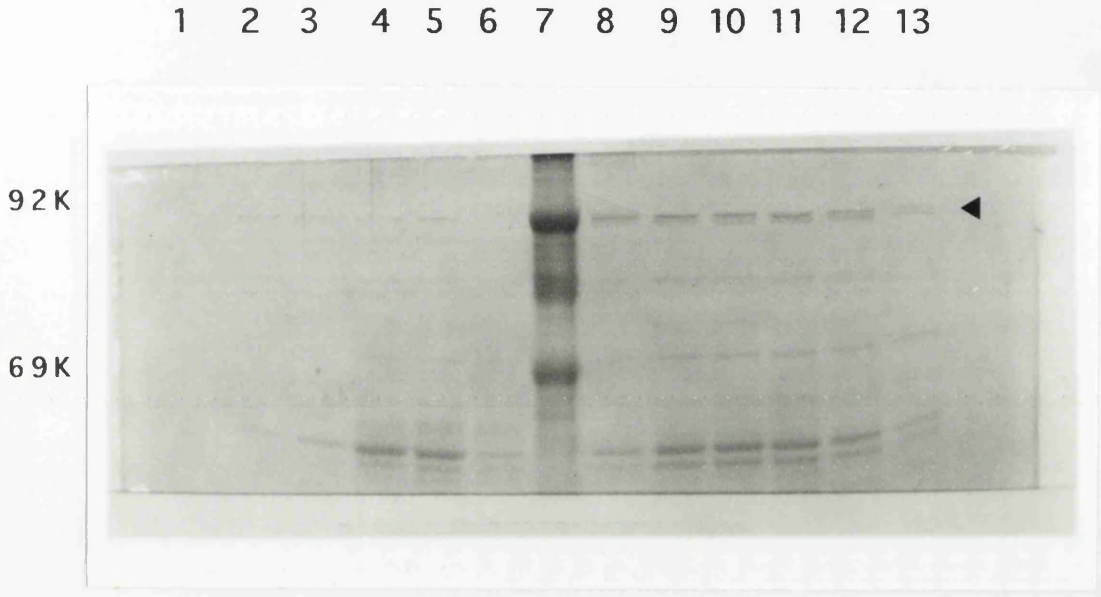


FIGURE 44 : RE cells were infected by HSV-2 at 5-10pfu/cell for 17 hours at 37^{O}C, the cytoplasmic and membrane fractions isolated, a 30% ammonium sulphate cut performed on each, the samples desalted into FPLC buffer A (50mM tris 0.1% triton), centrifuged and injected onto the FPLC mono Q column as two separate runs. This was carried out in the same manner as described for Bn5T cells in Results Section 4 and summarized in Figure 35. The 1ml flow through samples were collected and 100ul of the membrane fraction flow through samples 1-6 analysed by Coomassie blue staining of a 7.5% SDS-page (tracks 1-6 respectively) The similarly treated cytoplasmic flow through samples 1-6 can be seen in tracks 8-13 respectively. Molecular weight markers are present in track 7 and their values in kilodaltons indicated to the left of the gel.

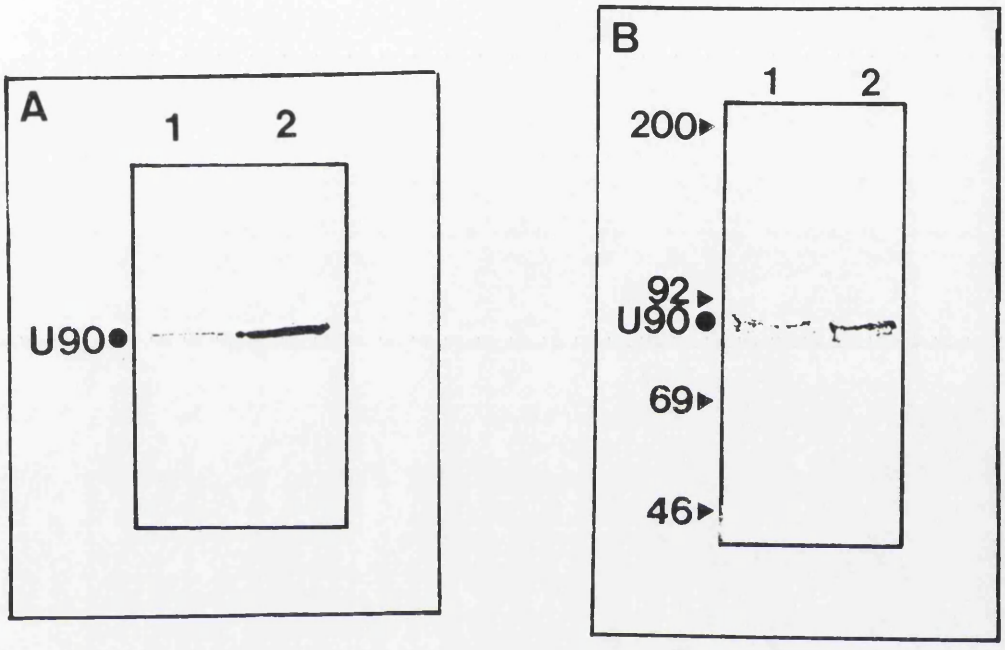


FIGURE 45A : IP reactions with TBS of polypeptides extracted from the cytoplasmic fractions from each of Bn5T cells (track 1) and HSV-2 infected Bn5T cells (track 2) followed by western blotting with U90 monospecific antibody confirming that the U90 antibody does recognise the U90.

FIGURE 45B : IP reaction with TBS of polypeptides extracted from the cytoplasmic fractions from each of control RE cells (track 1) and HSV-2 infected RE cells (track 2) followed by western blotting with U90 monospecific antibody. This shows that a 90K homologue of the U90 is seen in RE cells which also accumulates following HSV infection. Molecular weight markers are illustrated to the left of the blot. Figure 45a kindly carried out by Dr JCM Macnab

cells generated from a single pregnant rat), fractionated and the U90 homologue isolated from both the cytoplasmic and membrane fraction of each sample using the FPLC in the manner previously described. The flow through samples of each of the four conditions were analyzed by western blotting using the monospecific U90 antibody (Figure 46). From these experiments it could be concluded that a U90 homologue is present in RE cells, and that in both Bn5T and RE cells the U90 and the U90 homologue is situated mainly in the membrane but could also be detected in the cytoplasmic fraction. Secondly, following HSV-2 infection of either cell type, the overall quantity of U90 (or its homologue in RE cells) was increased and that in a similar manner to the Bn5T cells the vast majority of the U90 homologue was isolated in the cytoplasmic fraction.

5 The Half Life Of U90 Is Different In Transformed And Control Cells

The U90 in RE cells as already discussed, has been shown to react with both TBS in immunoprecipitation reactions, (ie the native form of the protein is recognised by the antibody) and the monospecific polyclonal antibody in western blots (ie the denatured from of the protein is recognised). In these circumstances the U90 was visualized by Coomassie blue staining and horse radish peroxidase conjugated second antibody respectively, but as it could not be visualized by [^{35}S] L-methionine radiolabelling over a period of 17 hours the half life of the U90 in RE cells and tumour cells was investigated. This was done by carrying out pulse labelling experiments in Bn5T and RE cells using a large excess of [^{35}S] L-methionine.

The tumour specific U90 had previously been shown to have a half life of around 28 hours in Bn5T cells (Hewitt, 1988). To establish the half life of the U90 isolated from RE cells, after a minimum of 2 hours methionine starvation, 2X10^6 RE cells and 2X10^6 Bn5T cells were each radiolabelled over a period of 5 minutes with 2.5mCi of [^{35}S] L-methionine/ml of medium (50 times more radiolabel than normal) This large amount of radiolabel was required due to the very short radiolabelling period and the half life of the U90 polypeptide in RE cells. These cells were harvested on ice, extracted immediately, immunoprecipitated with TBS then analyzed by SDS page as quickly as possible. A similar experiment was also carried out, however, after radiolabelling for 5 minutes, the [^{35}S] L-methionine was removed, the cells washed three times with PBS and chased with cold methionine containing medium for 1 hour. These cells were also harvested, immunoprecipitated with TBS, run on the same gel in the shortest time possible and visualized by autoradiography.

Comparison of the Bn5T cells and the RE cells shows that the U90 and its homologue do incorporate radiolabel under the conditions outlined. When

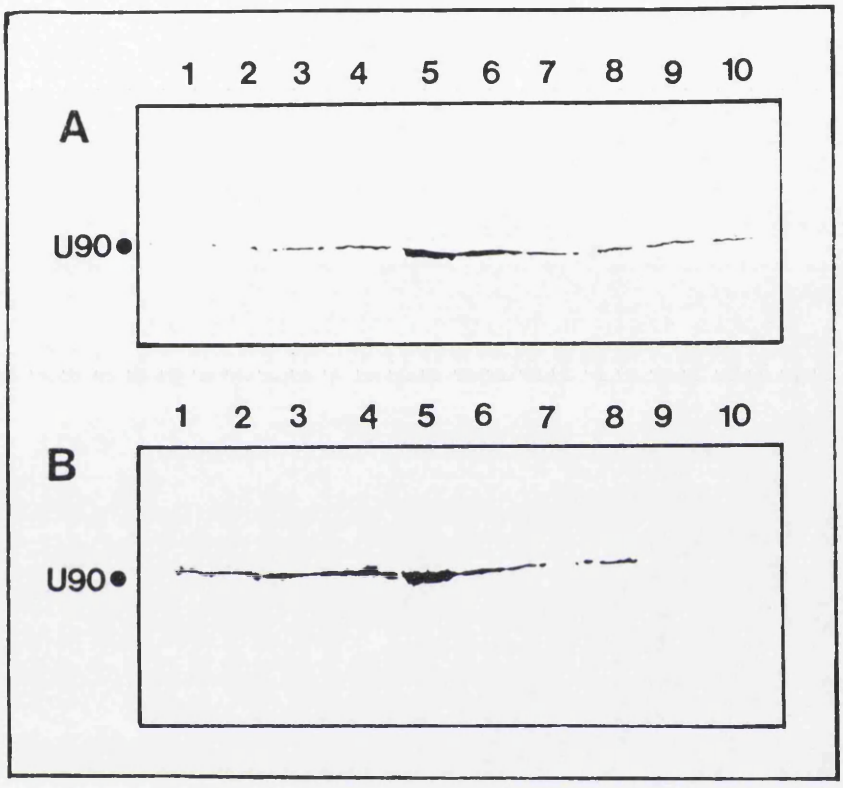


FIGURE 46 A & B: Western blot with U90 monospecific antibody showing the U90 homologue purified from $2X10^9$ control RE cells (A) and HSV-2 infected RE cells (B) using the U90 purification scheme as described in Figure 35. Tracks 1-4 show the flow through samples of the cytoplasmic fraction of control (A) and HSV-2 infected (B) RE cells, tracks 7-10 show the flow through samples of the membrane fraction of control (A) and HSV-2 infected (B) RE cells. U90 isolated from the cytoplasmic fraction (tracks 5) and membrane fraction (tracks 6) of HSV-2 infected Bn5T cells are shown as controls.

however, these samples were chased for one hour, the U90 and its homologue behaved in a different manner (Figure 47). In the Bn5T cells, the U90 was seen to loose relatively little radiolabel over a period of one hour, whereas when the autoradiographs were scanned the U90 homologue in RE cells had lost in excess of half of its incorporated radiolabel after one hour (Figure 48). This showed that the U90 homologue isolated from the control RE cells and the U90 isolated from the transformed Bn5T cells had a dramatically different half life. A large number of non-specific polypeptides can be seen in Figure 47. We and others find this if very large amounts of radioactivity are used (A Cross, personal communication).

These results were later repeated by Dr Joan Macnab and Mr David McNab and analyzed by an alternative method using a Molecular Dynamics Phosphor Imager. The radiolabel incorporated in the U90 and U90 homologue were quantitated using the volume integration function within the ImageQuant programme. The value obtained for each time point was then plotted against time using the Apple Macintosh Cricket Graph package version 1.3.2 and the equation of the line using exponential decay was obtained. From this the half life of the U90 homologue was calculated to be 29.8 minutes from the Phosphor Imager data, and 36 minutes from the previous experiment scanned in Figure 48. An average value of 32.9 +/- 3.1 minutes for the half life of the U90 homologue was obtained which is dramatically shorter than the half life of the U90 which was estimated to be 13 hours (Grassie et al, 1993).

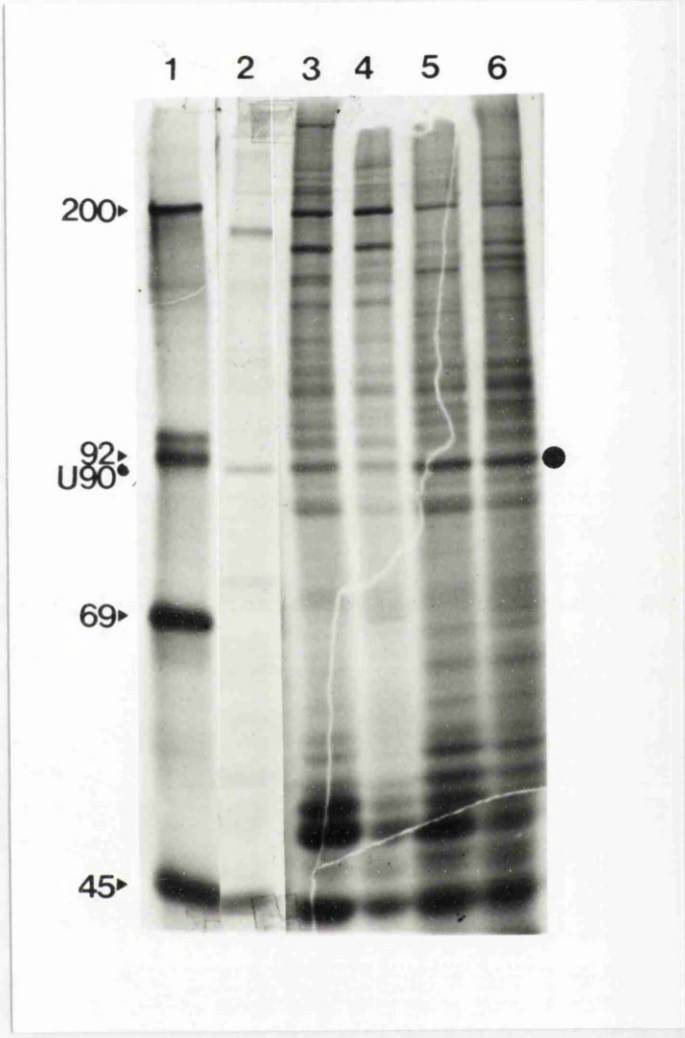


FIGURE 47 : Autoradiograph of IP reactions with TBS of RE cells (tracks 3 & 4) and Bn5T cells (tracks 2, 5 & 6) using $2X10^6$ cells radiolabelled with 5mCi [^{35}S] L-methionine for 5 minutes and harvested either immediately (tracks 3 & 5) or chased with excess cold methionine for 60 minutes (tracks 4 & 6). Prior to radiolabelling cells were starved of methionine for 2 hours. Track 2 shows an IP reaction of $4X10^6$ cpm of Bn5T cells extracted using RIPA buffer. In tracks 3-6, cells were harvested, extracted with RIPA buffer and IP reaction carried out immediately. Molecular weight markers can seen in track 1 and their values in kilodaltons indicated to the left of the gel. Increased non-specific background protein in tracks 3-6 is thought to be as a consequence of the large amount of radiolabel required to incorporate [^{35}S] L-methionine into the U90 homologue in control cells.

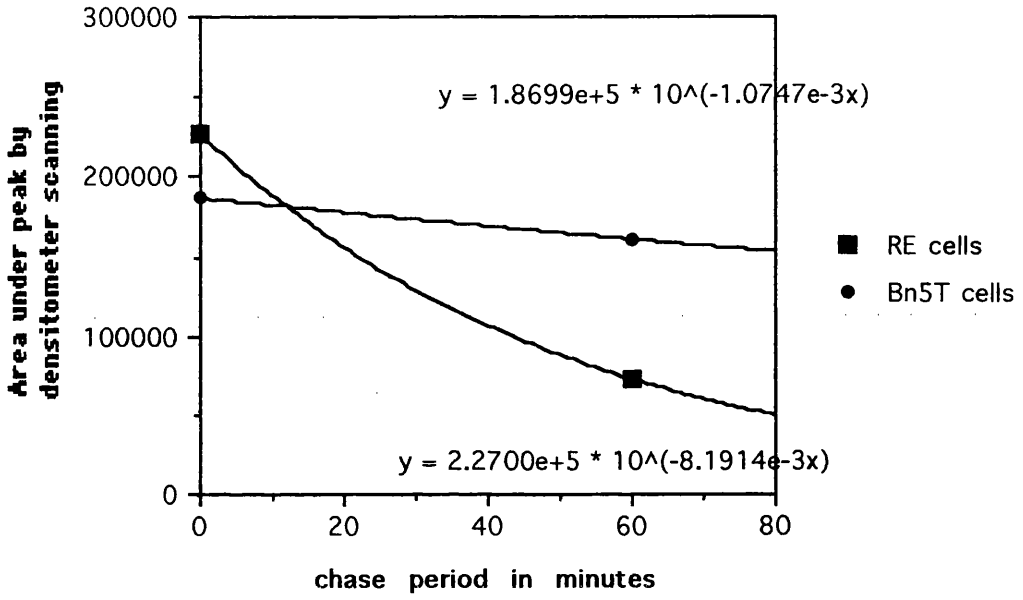


FIGURE 48 : Densitometer scan of U90 IP from control RE and Bn5T cells after being radiolabelled for 5 minutes with 5mCi of [^{35}S] L-methionine and chased for 0 or 60 minutes with medium containing excess cold methionine as described for Figure 47. Data corresponds to the area under the peak when the U90 band on the autoradiograph was quantitated by scanning densitometery, as described previously.

The equations of the curves (as determined assuming exponential decay using the Apple Macintosh programme Cricket graph version 1.3.2) are illustrated adjacent to the respective curves, which allows the half life of the U90 homologue in RE cells to be calculated as 36 minutes. From the graph it can be seen that the radiolabel present in the Bn5T U90 after a 0 and 60 minutes chase is almost unchanged when compared to the U90 homologue in RE cells.

DISCUSSION OF SECTION 6 : CHARACTERIZATION AND PURIFICATION OF U90 FROM PRIMARY RE CELLS.

To date work investigating the cell encoded polypeptides of molecular weights 200,000, 90,000 (U90 and L90), 40,000 and 32,000 detected in radiolabelled transformed cells (Bn5T) could find no corresponding proteins to be immunoprecipitated from radiolabelled control (RE) cells (Macnab et al, 1985a; Hewitt 1988; Hewitt et al, 1991).

Extensive studies in RE cells as summarized in section 6.1 agreed with previous studies, as no treatment of the control cells resulted in the visualization of the U90 by autoradiography. However, it was discovered that if such an IP reaction was visualized by Coomassie blue staining following SDS page, a number of similar protein bands could be seen in both RE and Bn5T cells. By aligning the relevant autorad track containing Bn5T cells with the stained Bn5T track, the band corresponding to the U90 was identified on the stained gel. Comparison of the RE and Bn5T stained tracks led to the discovery that a similar molecular weight band was present in the RE cells. As the IP reactions were carried out using Pansorbin (Calbiochem, UK) as opposed to purified protein A, other background polypeptides were visualized (Pansorbin is a suspension of *S.aureus* Cowan 1 strain which expresses a coat of protein A). The possibility consequently existed that the 90K band of interest was a component of the bacteria instead of the U90. This was unlikely to be the case, as previous experiments had seen this band to increase in Bn5T cells following HSV-2 infection and was therefore most likely to be the U90. Despite this, no further investigations could be completed at this point until an antibody was available which could be used in western blotting experiments.

Studies of the potential presence of the U90 in RE cells were therefore halted until the U90 purification was developed and subsequently a monospecific antibody produced (Mr. D. McNab).

A series of experiments were carried out to investigate if this 90K band seen by staining of IP reactions of both RE and Bn5T polypeptides was in fact U90. A large number of primary RE cells were made from one pregnant rat and the strategy used to purify the U90 from Bn5T cells was applied in an identical manner to the primary RE cells. The RE cells were infected with HSV-2 in anticipation that the U90 in RE cells, if it existed, would behave in a similar manner to the U90 in Bn5T cells (ie with HSV-2 infection resulting in an increase in U90 in the cytoplasm of the cell). HSV infection was therefore used to maximize the chance of visualizing the U90 in RE cells as it was anticipated that it might be present only in very small amounts under normal conditions.

Experiments using the monospecific U90 antibody in conjunction with the purification demonstrated that not only did the control RE cells express the U90, but they expressed it in a highly similar manner to the Bn5T cells. In the uninfected RE cells the U90 could be detected in the membrane fraction with some U90 also seen in the cytoplasm. Following HSV-2 infection, the U90 was seen to accumulate to higher levels in the cytoplasm with only a small proportion of the total U90 being found in the membrane fraction, as seen by western blotting using the monospecific antibody. These observations taken together with the preliminary experiments visualizing immunoprecipitated U90 by Coomassie blue staining, and the IP reactions of the control and HSV-2 infected RE cells with TBS followed by western blotting (using the monospecific antibody), indicated that it was unlikely that a conformational difference in the U90 in RE cells prevented the protein from being visualized by radiolabelling and IP.

These experiments suggested that a protein very similar to the U90 was present in control RE cells but for some reason could not be visualized by [^{35}S] methionine. Two possible contrasting explanations were proposed - either the metabolic rate of the U90 in RE cells was so slow that it did not incorporate any radiolabel over a period of 17 hours, or the polypeptide lost the radiolabel so quickly by protein degradation that it could not be visualized by autoradiography. Both experiments were investigated.

RE cells were radiolabelled over a period of 18, 22, 42, 48 and 72 hours then harvested and IP reaction carried out as before. No band could be seen which corresponded to the U90. When the RE cells were radiolabelled with a vast excess of radiolabel, harvested and the IP reaction completed in as short a time as possible, a band of similar molecular weight to the U90 could be visualized. When the cells were chased with methionine containing medium following radiolabelling, the 90K band identified in the RE cells was seen to lose label far more rapidly than the U90 in Bn5T cells. It is possible that the 90K band identified in the RE cells is not a U90 homologue (due to the high level of back-ground in this immunoprecipitation reaction), however, this experiment has been independently repeated by Dr JCM Macnab and Mr David McNab who also found a 90K polypeptide to be immunoprecipitated in RE cells by TBS using the conditions defined previously. Densitometer scanning of the 90K bands (after IP) enabled an estimate to be made of the half lives of the two forms of U90. This was done assuming exponential decay of the radiolabel from the protein, and the equation of the line calculated using the computer software Cricket graph version 1.3.2 as indicated previously. From this equation the half life of the U90

homologue in RE cells was found to be in the order of 36 minutes, which is considerably less than the 13 hours half life found for the U90 in Bn5T cells.

The half life of the U90 in Bn5T cells from the pulse-chase experiments was calculated to be in the order of 4 hours, which was much less than expected. This is most likely to be a consequence of the short chase period (relative to the previously reported half life of U90 in this thesis and Hewitt 1988, in Bn5T cells) and as any experimental error occurring in the densitometer reading would be considerably amplified. Due to the cost of this experiment, a minimal approach to time points had to be taken, and therefore the predicted half life of the U90 in RE cells can only be considered an estimate.

This experiment however was later repeated by Dr Joan Macnab and Mr David McNab using a Phosphor Imager. With chase periods of 15, 30 and 45 minutes, they found the half life of the U90 homologue to be 29.8 minutes. Taken together with the previous results, it can be concluded that the U90 homologue has a half life of 32.9 +/- 3.1 minutes. These pulse-chase experiments clearly show that the half life of the U90 in Bn5T cells is extended by a considerable period when compared to the U90 homologue present in RE cells.

It should also be noted that the U90 seen by western blotting of RE cells, migrates very slightly slower than the transformed U90. This could be explained by a post-translational modification (eg phosphorylation, glycosylation, pamitoylation, myristoylation or sulphation), in the control U90 which is absent in the U90 isolated from transformed cells. All of the above modifications with the exception of sulphation have previously been shown to be absent in the U90 in the transformed cell using appropriate radiolabelling techniques (Macnab, unpublished results). Whether sulphation accounts for the different half lives of the two forms of U90 is unknown. Exactly how an alteration in the half life of the U90 could affect the cell cannot be predicted until the function of the U90 has been established. It is probable that U90 plays an important role in the cell since it is highly conserved (as it is present in human and rodent cells in addition to cells of different lineages).

It is interesting to note that when compared to control cells, a similar alteration in the half life of the tumour specific protein p53 occurs in transformed cells. In the normal cell, p53 has a half life of between 6 and 20 minutes, but in the transformed cell this is increased to between 6 and 25 hours (Oren et al, 1981; Reich et al, 1983; Rogel et al, 1985). It has been proposed that this increase in half life is a consequence of the formation of protein complexes which stabilize the protein (Linzer & Levine 1979; Linzer et al, 1980), but whether a similar mechanism is involved with the increased half life of U90 in transformed cells cannot be predicted.

It can be concluded from this section that the RE cells express a 90K polypeptide which is immunologically indistinguishable from the U90 and has the same properties as the U90 such that it can be purified in an identical manner. The expression of this 90K protein is also increased following HSV infection in the same manner as the U90. It is therefore concluded that RE cells express the U90 polypeptide, but in a form which has a considerably shorter half life compared to the U90 isolated from Bn5T cells. As the U90 is highly conserved it is probable that the U90 plays an important role in the cell. Previous work visualized the U90 by radio-immunoprecipitation in rat embryos up to 14 days gestation, but after this point the U90 could not be detected (Macnab, unpublished results). This observation and the fact that the U90 is conserved in different cell types, suggests the function of the U90 in the cell maybe implicated with cell regulation or differentiation. The exact function of the U90 however remains to be disclosed.

CHAPTER 5: CONCLUSIONS AND FUTURE PROSPECTS

1. Conclusions

The U90 polypeptide was originally identified as a cellular protein which was expressed in transformed cells and accumulated on HSV infection as indicated by activity against this polypeptide found in tumour bearing sera and antisera raised against HSV infected cells (Macnab et al, 1985). From experiments described in this thesis it can now be stated that the U90 is in fact a normal cellular polypeptide which is expressed in a number of different cell types originating from mouse, rat and human cells. The protein is also found in cells of different lineages including adrenal, epithelial, fibroblastic and lymphoid cells indicating that it is a highly conserved protein and as such is likely to play an important role in the cell. These cells were established in culture, a factor which has to date been found to be essential for the U90 to be visualized by radiolabelling.

By visualizing U90 in Bn5T cells using radiolabelling techniques, HSV types 1 and 2 were found to increase U90 in the infected cells by up to eight fold at 3 hours post infection (falling to between 2-5 fold at 17 hours post-infection). This increase was a consequence of an increase in the total amount of U90 in the cell and not a result of a significant increase in protein stability or increased rate of radiolabel incorporation. The use of temperature sensitive (ts) mutants indicated that an early event in HSV infection was implicated with U90 induction where Vmw 175 played an important role in this induction. However, it is still not established if Vmw175 acts in a direct or indirect manner. Pulse label experiments indicated that U90 accumulated prior to the onset of viral DNA synthesis with levels falling after the start of DNA synthesis.

HSV infection also was found to alter the sub-cellular location of the U90 from being located in the membrane to being located in the cytoplasmic fraction. This may occur as a result of the increased U90 present in the cell, with a "bottle neck" occurring at this point in the association of the U90 with the membrane. This may be a result of a limiting number of membrane proteins with which the U90 may complex, or simply a consequence of the increased number of U90 molecules. Irrespective of which supposition is correct, the accumulation of the U90 in a soluble form in the cytoplasmic fraction facilitated the partial purification of the U90 to be carried out with much greater ease.

In addition to HSV infection resulting in the accumulation of the U90 polypeptide, HSV-1, but not HSV-2 infection was found to increase the total amount of GRP94 in the cell. In contrast to the induction of U90 by HSV infection, immediate early viral expression is insufficient for the induction of

GRP94. This increase in GRP94 was not detected as a consequence of transcriptional activation but rather due to an increase either at the level of translation or protein stability (perhaps due to post-translational modification).

Using the information obtained in the characterization studies the U90 was partially purified using a combination of selective protein precipitation, FPLC analysis and gel electrophoresis. This enabled partial amino acid sequence data to be obtained despite the discovery that the U90 was naturally blocked. By comparing the partial amino acid sequence data to the databases it was established that the U90 was in fact a novel tumour associated polypeptide displaying no obvious homology with any of the proteins already sequenced and entered in the databases (assuming that the sequence data obtained was correct).

The purification of the U90 polypeptide also allowed the production of a mono-specific polyclonal antibody raised against the U90 protein which recognised the U90 in western blots. This facilitated the disclosure that control RE cells did in fact express a 90K polypeptide similar to the U90 as seen by western blotting, despite the fact that the U90 could not be visualized by radio-immunoprecipitation from RE cells. By using the U90 monospecific antibody it was concluded that the 90K protein present in RE cells behaved in a similar manner to the U90 isolated from Bn5T cells, ie it was found to be associated with the membrane and it accumulated in the cytoplasm following HSV infection. Application of the U90 purification strategy developed in Bn5T cells to HSV infected RE cells, resulted in the 90K polypeptide being isolated in the same fractions after purification as the U90 purified from Bn5T cells further confirming that this 90K polypeptide was possibly a homologue of U90. Pulse chase experiments identified that the U90 found in the Bn5T cells had a dramatically increased half life as compared to the half life of the U90 homologue in the control RE cells. It appears that this difference in half life distinguishes the U90 homologue isolated from primary cells and U90 isolated from the Bn5T tumour cell line.

In attempts to identify the function of the U90 more extensive sequence data was sought by trying to either isolate the cDNA encoding the U90 by cDNA library screening, or by generating genomic or cDNA sequence by the polymerase chain reaction to amplify the U90 DNA or mRNA followed by cloning and sequencing of these clones. Unfortunately time limitations and the absence of a highly homologous probe prevented these experiments from being completed.

It can be concluded that the U90 is a highly conserved cellular polypeptide and as such is likely to play an important role in the cell. The U90 has been previously reported to be visualized by radiolabelling in RE cells prior

to 14 days gestation (Macnab, unpublished) suggesting a possible role in growth control or differentiation. The aberrant expression of the U90 could therefore be strongly implicated in transformation and tumourigenesis. It can now be reported that a U90 homologue is expressed in control cells but has a reduced half life (ie a high turnover). Furthermore, HSV infection increases the expression of the U90 in control cells, but such an infection alone cannot convert the U90 homologue found in "normal" cells to that of the U90 with an increased half life (and a slow turnover) as is found in the transformed cell. Since the U90 is increased prior to the onset of viral replication (most probably by transcriptional activation) it may be that on rare occasions the host cell can survive and continue to replicate in an abnormal manner. If this was to occur, such an increase in U90 expression by HSV infection may be one of the many changes which leads to a transformed phenotype.

2. FUTURE PROSPECTS

Despite the progress which has been made in the characterization of the U90 polypeptide, some aspects of this work still needs to be clarified. This includes the question as to whether Vmw175 is responsible for the increase in the U90 and if it is, is this increase a consequence of transcriptional activation, as might be expected for Vmw175. Alternatively, an abnormal Vmw175 may induce a conformational change as previously proposed for Hsp90 (Russell et al, 1987).

By obtaining accurate cDNA sequence for U90 to generate a highly homologous probe, (by PCR or sequencing a U90 cDNA clone) northern blotting could be used to answer the question of transcriptional activation. Further sequence data could also be used to search for functional domains within the U90 and hence indicate the function of this protein.

Comparison of sequence data generated from Bn5T and RE cells could also be used to investigate if any differences occur at the DNA level which might explain the alteration in the half life of the protein in normal and transformed cells. Additionally, it would be intriguing to discover if the U90 in transformed cells was similarly regulated in rat embryos prior to 14 days gestation by culturing primary RE cells generated from embryos of 13 days gestation and younger.

To answer the question of the role of U90 in transformation, the cDNA will need to be cloned. Transfection studies using the cloned U90 may determine if the altered half life of the U90 is fundamental for some stage in the transformation of cells or is a consequence of transformation.

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