Identification of a 40KD protein increased by HSV-2 infection.

by

Jean-François LUCASSON

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

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Unless stated otherwise, the work described in this thesis was carried out by the author.

Abbreviations.

The following abbreviations are used in this thesis.

AA	amino acid
Ad	adenovirus
AEV	avian erythroblastosis virus
Aldolase A	fructose 1-6 diphosphate aldolase A
alpha TIF	alpha transinducing factor
Bis	N-N'-methylene bisacrylamide
Bistris	2[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol
BHK	baby hamster kidney
BLV	bovine leukemia virus
b.p.	base pair
BPV	bovine papilloma virus
cAMP	cyclic adenosine monophosphate
CAPS	3-[cyclohexylamino]-1-propane-sulfonic acid
cAspAT	cytoplasmic aspartate aminotransferase
CEF	chick embryo fibroblast
CIN	cervical intra epithelial neoplasia
"COLUMN 40"	40KD polypeptide retained on the Mono Q column at pH.9.5 (buffer C) and eluted by 7.5% buffer D.
c-onc	cellular oncogene
CRPV	cotton tail rabbit papillomavirus
DATD	N,N'-diallyltartramide
DNA	deoxyribonucleic acid
DMP	N-dimethyl-N'-phenylthiourea
ds	double stranded
DPT	N-N'diphenylthiourea
DW	distilled water
E	early gene
EBV	Epstein-Barr virus
EBNA	Epstein-Barr nuclear antigen
EDTA	ethylenediamide tetra acetic acid di-sodium salt
EGF	epidermal growth factor
FGF	fibroblast growth factor
FLV	feline leukemia virus

FPLC	fast protein liquid chromatography
FPLC:40	40KD polypeptide eluting in the void volume of the Mono Q at pH.8 (buffer A)
g	gramme
G	$10m/s^2$
GAP	GTPase activating protein
GDP/GTP	guanosine di/triphosphate
h.	hour
HAT	hypoxanthine, aminopterin and thymidine
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCMV	human cytomegalovirus
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HPLC	high performance liquid chromatography
HPV	human papilloma virus
HRP	horseradish peroxidase
HSP	heat shock protein
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HT	hypoxanthine and thymidine
HTLV-1	humam T lymphotropic virus 1
IE	immediate early HSV genes
i.p.	immunoprecipitation/immunoprecipitated
k.b.	kilobase
KD	kilodalton (1 Dalton is $1/12^{th}$ of the mass of a mole of the atom ^{12}C).
L	late (gene)
LAT	latency associated transcripts
LMP	latent membrane protein
LTR	long terminal repeat
Mab	monoclonal antibody
mAspAT	mitochondrial aspartate aminotransferase
mDBP	major single stranded DNA binding protein
min.	minute
mRNA	messenger ribonucleic acid
MTR	morphological transformation region
m.u.	map unit
MW	molecular weight
MDHV	Marek disease herpes virus

TG7A	Mouse monoclonal antibody raised by Dr N. LaThangue against DNA binding proteins of BHK cells clone C13 infected by HSV-2 strain 333 (Macnab et al., 1985; LaThangue and Latchman, 1988).

MMTV	murine mammary tumor virus
NPC	naso-pharygeal carcinoma
npt	non permissive temperature
ORF	open reading frame
ori	origin of replication
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PEG	polyethylene glycol
PGK	phosphoglycerate kinase
p.i.	post infection
pH.	hydrogen potential
pI	isoelectric point
PK	protein kinase
pKa	cologarithm of the equilibrium constant
PVDF	polyvinyl difluoride
RE	rat embryo
RB	retinoblastoma gene
RNA	ribonucleic acid
r.p.m.	revolution per minute
RR	ribonucleotide reductase
R_L	long repeat segment
R_S	short repeat segment
RSV	Rous sarcoma virus
RT	room temperature
s	Svedberg
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SV	simian virus
TBS	tumour bearing serum
TBS:40	40KD polypeptide i.p. by TBS
TBS:90	90KD polypeptide i.p. by TBS
TG7A	
TG7A:40	40KD polypeptide i.p. by TG7A
TG7A:90	90KD polypeptide i.p. by TG7A
TBuS	tris buffered saline.
TCA	trichloracetic acid
TEMED	N,N,N',N'-tetramethylenediamine
TES	2-2{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] amino}ethanesulfonic acid
TFA	trifluoroacetic acid

TTBuS	tris buffered saline with 0.5% Tween 20

"Void Volume" is used as a synonym of flow through in this thesis.

TK	thymidine kinase
TPA	12-O-tetradecanoyl-phorbol-14 acetate
tris	tris(hydroxymethyl)aminomethane
ts	temperature sensitive (mutant)
TS	tris saline
UL, U_L	unique long segment
US, U_S	unique short segment
v	volume
UV	ultra violet
UTP	uridine triphosphate
"VOID VOLUME 40"	40KD polypeptide eluting in the void volume of the Mono Q column at pH.9.5 (buffer C)
VZV	varicella zoster virus
Vmw	virus specific polypeptide of apparent molecular weight (X)
v-onc	viral oncogene
w	weight
wt	wild type
uCi	microCurie
ug	microgramme
ul	microlitre

THREE AND ONE LETTER AMINO ACID CODES

AMINO ACID	THREE LETTERS CODE	ONE LETTER CODE
alanine	Ala	A
arginine	Arg	R
asparagine	Asn	N
aspartic acid	Asp	D
cysteine	Cys	C
glutamic acid	Glu	E
glutamine	Gln	Q
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
tryptophane	Trp	W
tyrosine	Tyr	Y
valine	Val	V

SUMMARY.

Herpes simplex virus (HSV) has been implicated in the etiology of human cancer, but its role in the transformation process is not well understood.

A set of cellular polypeptides of 200KD, 90KD (a doublet U90 and L90) and 40KD (TBS:40) was previously detected by immunoprecipitation (i.p.) with tumour bearing serum in the Bn5T cell line. The Bn5T cell line is derived from rat embryo fibroblast transformed by a fragment of the HSV type 2 (HSV-2). These polypeptides were detected in cell lines transformed by other agents and were not detectable in control rat embryo (RE) cells (Macnab et al., 1985).

The aim of the project was to purify and obtain amino acid sequence for the TBS:40. The 40KD polypeptide was characterized by its digestion pattern with the enzyme Staph. aureus V8 protease. The U90 and the TBS:40 were increased upon infection with HSV (Macnab et al., 1992) but this was not known at the start of this thesis. The 40KD protein was purified from Bn5T cells and notfrom infected cells.

Attempts to raise antibodies in mice and rabbits were carried out. In rabbit there was no immunological response. To raise monoclonal antibodies (Mab) twelve mice were immunized against Bn5T tumour cells. The 90KD polypeptide i.p. by mouse antisera and the U90 i.p. by TBS were similar, in respect of the Staph. aureus V8 protease peptide map. By contrast the 40KD polypeptide i.p. by the serum of the mice and the TBS:40 had different peptide maps. Therefore, in this instance, the immune system of the rat and the mouse recognized different 40KD proteins. Unfortunately attempts to raise Mabs against the 90KD and the 40KD polypeptides failed. Although the 40KD polypeptide did initially raise Mabs, these were unfortunately subsequently lost.

The TBS:40 was purified by biochemical methods. In these experiments TBS was found to i.p. more than one polypeptide. It was decided to purify and identify each polypeptide in turn and lastly to test the effect of HSV-2 infection on its expression.

Ammonium sulphate fractionation separated two 40KD proteins i.p. by TBS. One was mainly insoluble, the other was soluble in a 70% saturated ammonium sulphate solution. The Staph. aureus V8 peptide map of the TBS:40 and the 40KD protein soluble in a 70% saturated ammonium sulphate solution were undistinguishable. Therefore it was decided to purify the soluble 40KD protein.

The 40KD protein was further purified by anion exchange chromatography at pH. 8. The 40KD protein eluted in the void volume. The pH. of the void volume was increased to pH.9.5 and anion exchange chromatography at pH. 9.5 separated two 40KD polypeptides, one eluted in the void volume and was called the "VOID VOLUME 40"; the other was eluted from the columm and was called the "COLUMN 40".

Both had a Staph. aureus V8 peptide maps different from the TBS:40 peptide map suggesting that the TBS:40 peptide map was produced when both proteins interact with each other. The "COLUMN 40" was i.p. by TBS but not the "VOID VOLUME 40", these results suggested that the "VOID VOLUME 40" is i.p. as part of a complex.

The amino acid sequences obtained from the "VOID VOLUME 40" matched the sequence of the mitochondrial aspartate aminotransferase (mAspAT). The "VOID VOLUME 40" was also immunologically related to the mAspAT in a Western blotting experiment. Expression of polypeptides immunologically related to the mAspAT were increased upon infection with HSV-2.

Two peptides obtained by digestion of the "COLUMN 40" were successfully sequenced. The sequence of one matched the sequence of the rat fructose 1-6 diphosphate aldolase, and the sequence of the second peptide matched the sequence of the rat phosphoglycerate kinase-1. The experiment was repeated and the sequence data obtained suggested that the "COLUMN 40" was also related to the mAspAT were obtained in the second experiment. Further experiments must be set up to confirm this result.

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PART 1
INTRODUCTION.

1.1. HERPESVIRUSES.

1.1.1. CLASSIFICATION.

The human herpesviruses belong to the family or Genus Herpesviruses. The family herpesvirus comprises at least 80 members infecting a wide range of animals, mainly mammals, including man. They are large (150-200 nm), icosahedral, enveloped viruses containing double stranded (ds) DNA. Their replicative cycle is intranuclear and all can infect their host latently. They are classified according their biological properties into alpha, beta and gamma herpesviruses.

Alpha herpesviruses have experimentaly a variable host range *in vivo* and *in vitro* and a short replicative cycle. They spread rapidly with efficient destruction of infected cells and they establish latency primarily in neural ganglia, . Examples of alpha herpesviruses are herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus (VZV), equine herpesvirus 1 and bovine herpesvirus 2.

Beta herpesviruses have a narrow host range and a long reproductive cycle. The viruses spread slowly in tissue culture and the cells frequently become enlarged and a carrier culture can be established, i.e. the cytopathology is so slow that sufficient regeneration occurs to maintain the culture alive. Latent infections can be established in secretory glands, lymphoreticular cells and kidneys. Examples of beta herpesviruses : Human cytomegalovirus (HCMV), murine herpesvirus 1 and probably human herpes virus 7 (Frenkel *et al.*, 1990).

Gamma herpesviruses host range is restricted *in vivo* to the family or order to which the natural host belongs. The

viruses are specific either for B or T lymphocytes, but the infection is frequently abortive with either establishment of a latent infection, or cell death without production of a complete virion. Latent virus is frequently demonstrated in lymphoid tissues. Examples of gamma herpesviruses : Epstein-Barr virus and gallid herpesvirus 2.

1.1.2. HUMAN HERPESVIRUSES.

Seven human herpesviruses have been isolated. Human infections are widespread, producing usually no clinical manifestation or a mild disease, except for the recently isolated RK virus which is not well known.

Herpes simplex virus 1	Human herpesvirus 1
Herpes simplex virus 2	Human herpesvirus 2
Varicella zoster virus	Human herpesvirus 3
Epstein-Barr virus	Human herpesvirus 4
Cytomegalovirus	Human herpesvirus 5
Herpesvirus 6	Human herpesvirus 6
RK virus	Human herpesvirus 7

1.1.2.1. HERPES SIMPLEX VIRUS 1 AND 2.

Primary-infection occurs very early in life with HSV-1, and may occur in over 90% of the population causing a variety of conditions from the mild to the fatal. Acute herpetic gingivostomatitis is the most common clinical manifestation of primary infection with HSV-1, it usually occurs in young children (1-3 years of age). In some individuals, reactivations occur, the most frequent clinical manifestation is also acute gingivostomatitis.

The clinical manifestations of HSV-2 are undistiguishable from those caused by HSV-1, but HSV-2 has a tropism for the genitalia, it frequently is the agent of genital herpes and neo-natal herpes infections.

In vitro infection of permissive cells results in the lysis of cells within 24-48 hours and generation of progeny virus.

1.1.2.2.VARICELLA ZOSTERVIRUS (VZV).

Varicella is a mild , very infectious disease of children. It is also called chicken pox and is characterized by a vesicular eruption of the skin and mucous membranes. VZV infection can be lethal for immunocompromised individuals.

Herpes zoster is the reactivation of VZV in a specific ganglion. Shingles occur in one fifth of the population reaching the age of 75, and is frequent in immunosuppressed patients. The clinical lesions are indistinguishable from those of chicken pox but are localized in the dermatome innervated by the sensory ganglion where reactivation occurs and are painful. Scarring and severe pain can remain as sequellae.

1.1.2.3. CYTOMEGALOVIRUS (HCMV).

HCMV is an beta herpesvirus. Acquired infection is usually inapparent. Congenital infection may result in the death of the foetus in utero or produce a severe disease of the foetus. Prematurity, malformation and somatic and mental retardation are potential sequellae. Inapparent intra uterine infection seems to occur frequently.

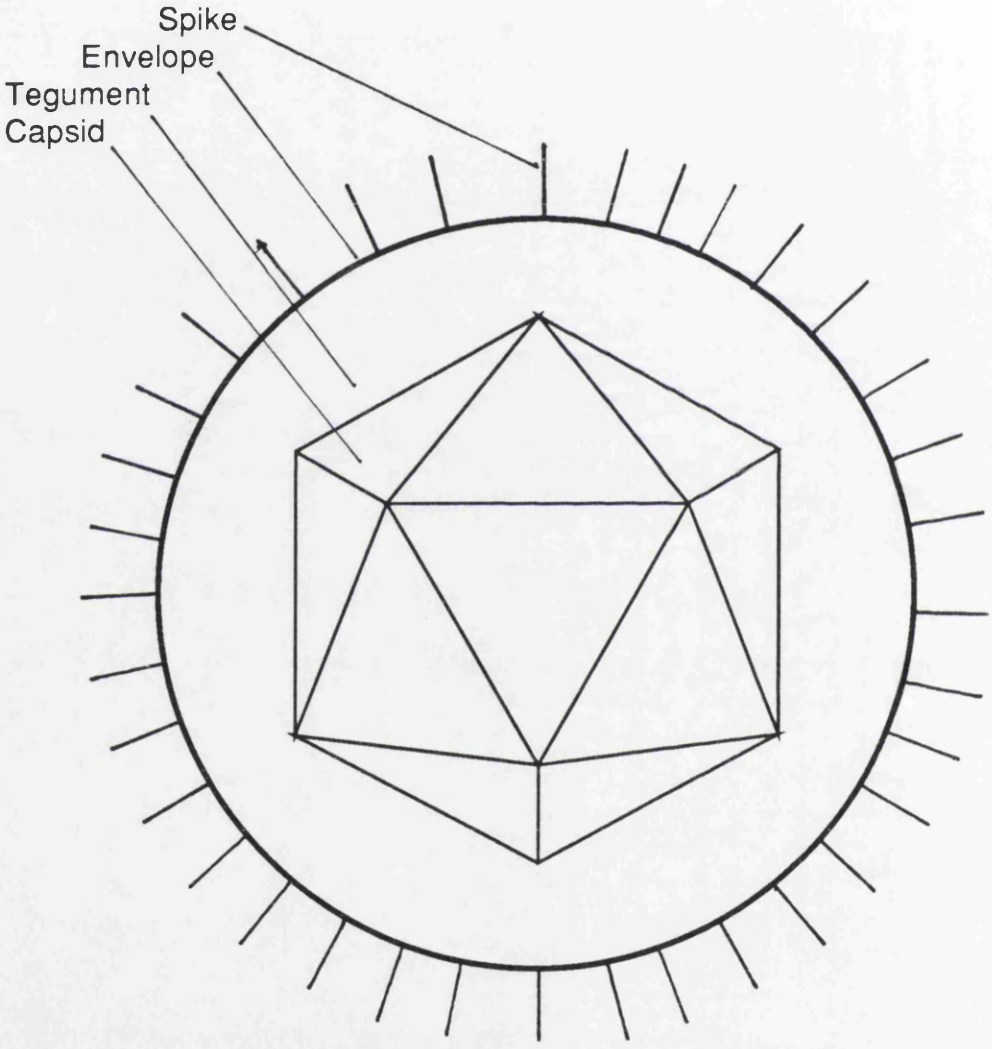
1.1.2.4. EPSTEIN-BARR VIRUS (EBV).

EBV is a gamma herpes virus. Most childhood infections are inapparent. In young adults EBV causes infectious mononucleosis. EBV has been involved in the etiology of Burkitt's lymphoma (BL) and naso-pharyngeal carcinoma (NPC). Primary infections of pregnant women can result in foetal damage. In immunocompromised patients, EBV reactivation may trigger the production of B cell lymphomas (Reviewed by Griffiths, 1990).

1.1.2.5. HUMAN HERPESVIRUS 6 (HHV-6).

HHV-6 was first isolated from a patient with lymphoproliferative disorders (Salahuddin et al., 1986). It is found associated with exanthem subitum (Yamanishi et al., 1988) and interstitial pneumonitis (Carrigan, et al., 1991). The seroprevalence in the normal population is over

Figure 1.1. Structure of HSV.



80 % (Levy et al., 1990). HHV-6 DNA can be detected by PCR from healthy adult from peripheral mononuclear cells (Kondo et al., 1991).

1.1.2.6. RK virus (HHV-7).

A seventh human herpes virus, called RK virus, has recently been isolated from CD4+ T cells of a healthy subject (RK). Its DNA shows partial homologies with HHV-6 DNA in blotting experiments (Frenkel et al., 1990). It has not been connected with any disease yet.

The properties of herpes simplex viruses will be discussed in more detail.

1.1.3. STRUCTURE OF THE VIRION.
(Figure 1.1.)

1.1.3.1. ENVELOPE.

The HSV envelope is a lipid membrane which forms the outer margin of the virus particle. It is derived from the nuclear membrane of cells (Darlington and Moss, 1968). HSV-1 encodes at least eight glycoproteins, designated gB to gJ, which are on the surface of the virion. The glycoproteins gB, gC and gD were shown to be components of the envelope spikes (Stannard et al., 1987).

1.1.3.2. TEGUMENT.

The tegument is an amorphous layer between the capsid and the envelope (Roizman and Furlong, 1974). It makes up 65% of the volume of the virion (Schrag et al., 1989). It contains among other proteins Vmw 65 the alpha transinducing factor (alpha TIF), and UL 41, the virion host shut off (VHS) protein.

1.1.3.3. NUCLEOCAPSID.

Schrag et al., (1989), proposed the following model for the nucleocapsid. The nucleocapsid is made of three layers. The outer layer is icosahedral and made of 162 capsomeres with a 5:3:2 cubic symetry. The protein VP 5 and VP 23 are the main components of the outer layer. The intermediate layer is also icosahedral, its constitution is not defined. The inner layer contains DNA associated with spermine which allow a dense packaging of DNA and other proteins. However in the latest hypothesis, the DNA and associated proteins fill the outside layer of the capsid entirely. (Rixon, F.J., personal communication).

Three types of capsids are described.

A capsids are intranuclear, lack DNA and are not enveloped. They are made of five polypeptides, VP 5 (155KD), VP 19C (53KD), VP 23 (36KD), VP 24 (24KD) and a component of 12 KD. These capsids do not seem to contain DNA.

B capsids are also intranuclear, They do not contain DNA, but they contain two more proteins, VP 21 (45KD) and VP 22a (38KD) which are also involved in DNA packaging.

C capsids are obtained by chemicaly stripping the envelope and the tegument off the mature virion. They contain the same proteins as capsid B except VP 22a.

1.1.4. THE LYTIC CYCLE OF HSV.

1.1.4.1. ADSORPTION AND PENETRATION.

Adsorption of HSV to the cell surface occurs maximally within 30 minutes (min.) of addition of the virus to the cells. HSV-1 binds to the cell surface through an interaction with heparin like cell associated glycosaminoglycans (Wu Dunn and Spear, 1989). The sequence of the events is thought to be the following. The viral envelope fuses with the plasma membrane (Morgan et al., 1968; Fuller and Spear, 1987) and releases the viral nucleocapsid into the cytoplasm of the cell (Vahlne et al., 1979). It was reported that HSV-1 penetration of the cells is mediated by basic fibroblast growth factor (Baird et al., 1990), but this result was not confirmed in our department

(L. Wood, personal communication).

It is unclear which viral proteins are involved in binding to the cells. Of the identified glycoproteins, only gB (Sarmiento et al., 1979; Little et al., 1981), gD (Ligas and Johnson, 1988) and gH (Weller et al., 1983; McGeoch and Davison, 1986; Gompel and Minson, 1986; Desia et al. 1988) are essential for infectivity. Glycosylation was shown unnecessary for adsorption (Campadelli-Fiume, 1982). Mutants in gB and gD retain the ability to adsorb to the surface but fail to synthesise viral polypeptides (Ligas and Johnson, 1988). Only gH may be essential for adsorption (Buckmaster et al., 1984).

HSV-1 infection interferes with the superinfection by other HSV-1 strains but not by HSV-2. These results suggested that HSV-1 and HSV-2 have different receptors on the cell surface (Vahlne et al., 1979; Addison et al., 1984).

HSV-1 adsorption to the cellular receptor was found to be selectively inhibited by neomycin and the polyamino acid polylysine whereas HSV-2 infection is unaffected (Langeland et al., 1987, 1988). The DNA region of HSV responsible for the different sensitivity of HSV-1 and HSV-2 to polylysine and neomycin was mapped by two different groups (Langeland et al., 1990; Campadelli-Fiume et al., 1990) .

Using intertypic recombinants, Langeland et al., (1990) mapped the proteins affecting the binding of the virion between coordinate 0.580 and 0.687 m.u.. This region contains two partial and eight complete genes including the glycoprotein gC gene, and three proteins with potential transmembrane sequences (McGeoch et al., 1988a). The adsorption of a HSV-1 mutant in gC was prevented by polylysine and neomycin showing that HSV-1 is not responsible for the sensitivity of HSV-1 to these chemicals. However gC-1 is involved in the adsorption of HSV-1 to the cell surface because the kinetics of adsorption of the gC-1 virus is slower than the wild type and because adsorption can be blocked by Mabs specific to gC-1.

Campadelli-Fiume et al., (1990) defined the genome fragment which encodes the lack of sensitivity of HSV-2 to neomycin and polylysine. It is a Sal 1 DNA fragment encoding the entire gC UL44 open reading frame (ORF), and part of the

UL43 and UL45 ORFs. HSV-1 and HSV-2 gC- mutants adsorption was blocked by neomycin and polylysine. They concluded that HSV-2 could mediate adsorption to the cells by two mechanisms. One pathway blocked by neomycin and polylysine, a second pathway gC-2 dependent, unaffected by neomycin and polylysine, which was found effective with BHK cells but not with Vero cells and Hep2 cells.

1.1.4.2. UNCOATING OF VIRAL GENOME.

Following penetration the viral capsids are translocated to the nucleus, where, after uncoating, the viral DNA then enters the nucleus at a nuclear pore. Release of the viral DNA into the nucleoplasm requires a viral function. A temperature sensitive (ts) mutant in UL36 (McGeoch et al., 1988a) (m.u. 0.501-0.503) cannot release the DNA in the nucleoplasm at non permissive temperature (npt)(Knipe et al., 1981; Batterson et al., 1983).

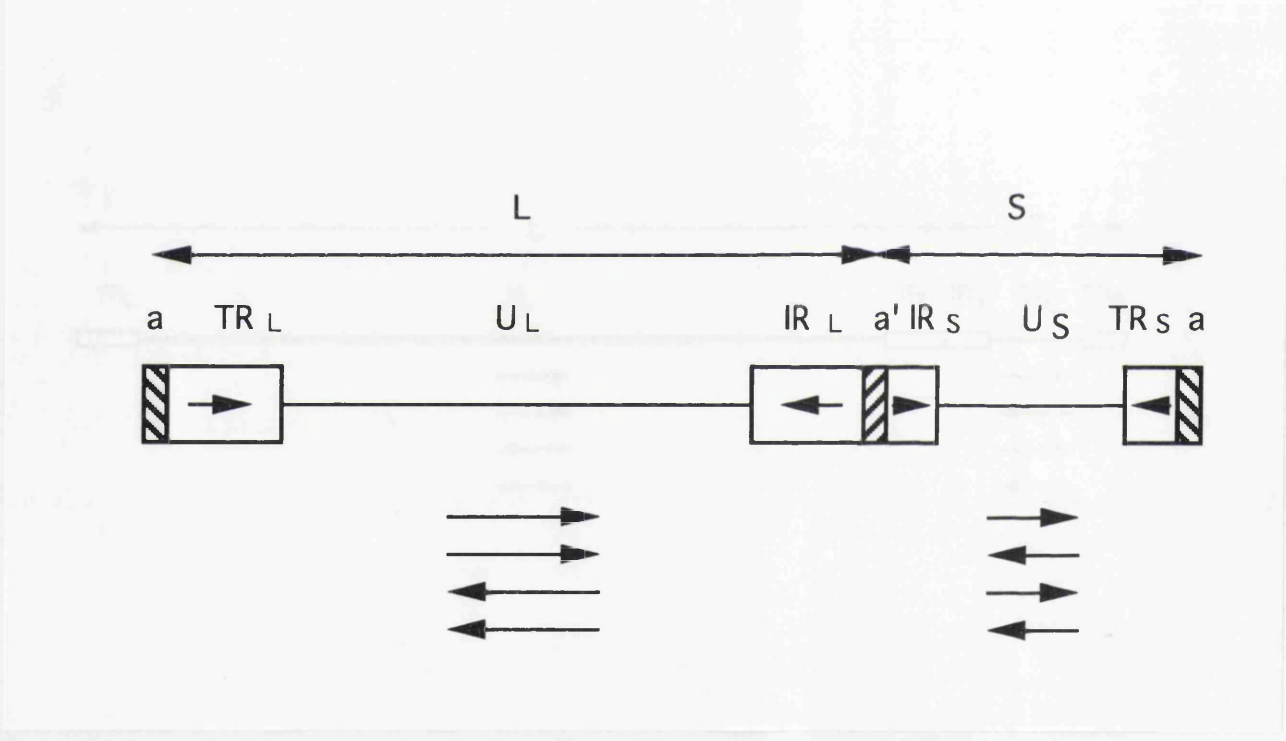
1.1.4.3. TRANSCRIPTION, TRANSLATION AND REPLICATION.

Virus DNA replication and transcription of virus DNA occurs in the nucleus. Immuno-fluorescence microscopy has identified replication compartments in the nucleus where virus DNA is synthesized at the same site where DNA polymerase (UL30) is visualized (Quinlan et al., 1984). These processes are described in detail below.

1.1.4.4. ASSEMBLY AND RELEASE.

Herpesvirus capsids are assembled in the cell nucleus (Morgan et al. 1954). Virus DNA is packaged into preformed capsids. In late infection, reduplicated membranes and thick patches appear particularly in nuclear membranes. It is likely that these patches represent aggregation of viral membrane proteins. There is general agreement that the inner lamellae are the site of initial envelopmemt (Reviewed by Roizman and Sears, 1990). Capsids containing fragments less than the standard genome length are retained in the nucleus (Vlazny et al. 1982). The exact mechanism of the release of

Figure 1.2. Organization of the HSV genome.



the virus is unknown, but cytoplasmic transit is associated with maturation of the glycoproteins. This supports a role for the Golgi apparatus (Reviewed by Campadelli-Fiume and Serafini-Cessi, 1985).

1.1.5. GENOME STRUCTURES.

Genome structures have been studied most extensively in HSV-1. Intertypic recombination and complementation data (Timbury and Subak-Sharpe, 1973; Marsden et al., 1978), and DNA/DNA hybridisation studies (Davison and Wilkie, 1983) showed that HSV-1 and HSV-2 genomes are essentially colinear with viral genes mapping at equivalent positions on both genomes.

1.1.5.1. CHEMICAL COMPOSITION.

The HSV-1 genome is a dsDNA whose molecular weight is 95.1×10^6 Daltons (Becker et al., 1968). The G+C composition is 68.3% (McGeoch et al., 1988a). The entire nucleotide sequence of HSV-1 strain 17 has been determined. It contains 152,260 bp but this number may vary according the number of "a" sequences and short tandem reiterations (McGeoch et al., 1988a).

1.1.5.2. LONG AND SHORT SEGMENT.

Electron microscopy studies of structures produced by intra-molecular hybridization of single stranded (ss) DNA have shown that the HSV genome is made of two covalently linked components, long (L) and short (S) (Sheldrick and Berthelot 1974), which can invert relative to each other creating four genomic isomers functionally equivalent and present in equimolar amounts (Hayward et al., 1975; Clements et al., 1976; Delius and Clements 1976 Wilkie and Cortini, 1976). Each component is made of an unique segment flanked by two repeat sequences inverted relative to each other. The unique long segment (U_L) is 110 Kbp, and the internal and terminal long repeats (R_L or IR_L and TR_L) are each 9.2 Kbp. The unique short (U_S) segment is 13 Kbp long and is also flanked by internal and terminal short repeats (Rs or IRs and TRs) of 6.6 Kbp.. (Figure 1.2).

An additional gene UL26.5 is entirely contained within the coding sequences of UL26 (Liu and Roizman, 1991).

1.1.5.3.THE "a" SEQUENCE.

The "a" sequences are short direct repeats observed at the termini of the HSV genome (Grafston et al., 1974) At least one copy of the "a" sequence is also located at the junction of the long and short component (Wadsworth et al. 1976; Wagner and Summer, 1978; Davison and Wilkie, 1981). The HSV-1 "a" sequence 250-500 bp varies depending on the strain , because of the different numbers of repeat elements. (Davison and Wilkie 1981; Mocarski and Roizman, 1981; Varmuza and Smiley 1985). Each terminal "a" sequence has a overhanging residue with a free 3' OH group (Mocarski and Roizman, 1982).

The "a" sequences mediate the circularization of the genome (Davison and Wilkie, 1983b), cleavage and packaging of the DNA (Stow et al., 1983, Varmuza and Smiley 1985) and isomerization of the genome. (Mocarski et al., 1980, Mocarski and Roizman, 1981; Varmuza and Smiley 1985).

The "a" sequence contains the promoter for a gene involved in HSV neurovirulence (Ackermann et al., 1986a; Chou and Roizman, 1986; Chou et al., 1990; Taha et al., 1988 and 1989; McLean et al., 1991; McGeoch et al., 1991).

1.1.5.4. SHORT TANDEM REITERATIONS.

Short tandem reiterations are found in U_S, in R_S and in R_L. They range from five to 54 bp long (Rixon et al., 1984; McGeoch et al., 1985) and may increase the level of genetic exchange between the repeat regions (Rixon et al., 1984). It is notable that one set in the R_S has homology with sequences of the Ig class switch recombination sites (Gomez Marquez et al., 1985).

1.1.5.5.ORGANIZATION OF THE GENES.

HSV-1 genome encodes 72 genes (McGeoch et al., 1989), found in both orientation and encoding 70 distinct proteins. A gene whose product is designated ICP 34.5 has been identified in R_L between the "a" sequence and the immediate early gene 1 (IE-1)(Chou and Roizman, 1986; McLean et al.,

TABLE 1.1

The HSV-1 genes encoding proteins.

(Fareed, 1992)

Gene	No. of residues	M_r [1]	Properties or functions	Status[4]	Reference[5]
RL1	263	43500[2]	ICP34.5 protein;	ne	Ackerman *et al.*, 1986; Chou and Roizman,
	248	28184[3]	Neurovirulence factor		1986, 1990; Chou *et al.*, 1990; MacLean, A.R. *et al.*, 1991; McGeoch *et al.*, 1991; Dolan *et al.*, 1992
RL2	(19)		IE transcriptional		
	(222)		regulatory protein	ne	
	(534)		(IE110; IE-1)		
	775 Total 78452				
UL1	224	24932	Hydrophobic N terminus; proposed virion glycoprotein L	e	Hutchinson *et al.*, 1992
UL2	334	36326	DNA repair enzyme; Uracil DNA glycosylase	ne	Mullaney *et al.*, 1989
UL3	235	25607	Hydrophobic N terminus	ne	Baines and Roizman, 1991
UL4	199		Unknown	ne	Baines and Roizman, 1991
UL5	882	98710	DNA replication; possibly responsible for the DNA helicase activity	e	
UL6	676	74087	Virion protein; possible role in DNA packaging	e	
UL7	296	33057	Unknown	-	
UL8	750	79921	DNA replication	e	
UL9	851	94246	DNA replication; OBP	e	
UL10	473	51389	Multiply hydrophobic; possible membrane-inserted protein	ne	Baines and Roizman, 1991; MacLean, C. A. *et al.*, 1991
UL11	096	10486	Myristylated virion protein	ne	MacLean, C.A. *et al.*, 1989; MacLean, C.A. *et al.*, 1992
UL12	626	67503	Deoxyribonuclease; possible role in DNA packaging or processing	ne	Weller *et al.*, 1990
UL13	518	57193	Predicted protein kinase	ne	L Coulter, personal communication
UL14	215	23454	Unknown	-	
UL15	(343)		Possible role in packaging	-	Dolan *et al.*,1991
	(392)		nascent DNA into capsids		
	735 Total 80918				
UL16	373	40440	Unknown	ne	Baines and Roizman, 1991

Gene	No. of residues	M_r [1]	Properties or functions	Status[4]	Reference[5]
UL17	703	74577	Unknown	-	
UL18	318	34268	Virion capsid protein; VP23	-	Rixon *et al.*, 1990
UL19	1374	149075	Major capsid protein	e	
UL20	222	24229	Multiply hydrophobic; possible role in viral egress	ne*	Baines *et al.*, 1991
UL21	535	57638	Unknown	-	
UL22	838	90361	Virion glycoprotein H	e	
UL23	376	40918	Thymidine kinase	ne	
UL24	269	29474	Unknown	ne	
UL25	580	62666	Virion protein; possible role in the formation of full capsids & virus entry into cells	e	Preston, V. G. 1990
UL26	635	62466	Capsid protein?; Protease; role in DNA packaging	e	Liu and Roizman,1991a, b;Preston, V.G. *et al.*, 1992
UL26.5	329		Substrate of the UL26 gene product; located entirely within the UL26 ORF	-	Liu and Roizman,1991a
UL27	904	100287	Virion glycoprotein B	e	
UL28	785	85573	Probably structural; possible role in the formation of mature capsids	e	Addison *et al.*, 1990
UL29	1196	128342	DNA replication; MDBP	e	
UL30	1235	136413	DNA polymerase	e	
UL31	306	33951	Unknown	-	
UL32	596	63946	Locus of immune cytolysis resistance mutation; structural protein	e	
UL33	130	14436	Structural protein; involved in DNA packaging	e	Al-Kobaisi *et al.*, 1991
UL34	275	29788	Virion protein; hydrophobic C terminus; Probable substrate of the viral protein kinase	-	Purves *et al.*, 1991
UL35	112	12095	Possible capsid protein	-	McNabb and Courtney, 1992
UL36	3164	335841	Virion protein; large tegument protein	e	
UL37	1123	120549	Unknown	-	

Gene	No. of residues	M_r [1]	Properties or functions	Status[4]	Reference[5]
UL38	465	50260	Virion protein; required for capsid assembly; VP19C	e	Pertuiset *et al.*, 1989; Rixon *et al.*, 1990
UL39	1137	124043	Large subunit of ribonucleotide reductase	ne*	
UL40	340	38017	Small subunit of ribonucleotide reductase	e	
UL41	489	54914	Virion host shut-off protein	ne	Fenwick and Everett, 1990
UL42	488	51156	DNA replication; DBP (65K)	e	
UL43	434	44905	Multiply hydrophobic	ne	MacLean, C. *et al.*, 1991
UL44	511	54995	Virion glycoprotein C; possible role in virus adsorption to cells	ne	Herold et al., 1991
UL45	172	18178	Hydrophobic N terminus	ne	Visalli and Brandt, 1991
UL46	718	78239	Unknown; May modulate activity of UL48 protein	ne	Barker and Roizman, 1990
UL47	693	73812	Possible tegument protein	ne	Barker and Roizman, 1990; McLean *et al.*, 1990
UL48	490	54342	Major tegument protein; activator of IE genes	-	
UL49	301	32252	Virion protein; VP22	-	Elliott and Meredith, 1992
UL49.5 or UL49A	091		membrane inserted protein	e	Barker and Roizman, 1992; Barnett *et al.*, 1992
UL50	371	39125	Deoxyuridine triphosphatase	ne	
UL51	244	25468	Unknown	ne	Barker and Roizman, 1990
UL52	1058	114416	DNA replication; possibly responsible for the DNA primase activity	e	
UL53	338	37570	Multiply hydrophobic; syn locus; proposed virion glycoprotein K	-	Hutchinson *et al.*, 1992; Ramaswamy and Holland, 1992
UL54	512	55249	IE transcriptional regulatory protein (IE63; IE-2)	e	
UL55	186	20491	Unknown	ne	
UL56	197	21182	Unknown	ne	
IE175	1298	132835	IE transcriptional regulatory protein(IE175; IE-3)	e	

Gene	No. of residues	M_r [1]	Properties or functions	Status[4]	Reference[5]
US1	420	46521	IE protein (IE68; IE-4)	ne	
US2	291	32468	Unknown	ne*	
US3	481	52831	Protein kinase	ne	
US4	238	25236	Virion glycoprotein G	ne	
US5	092	09555	Putative glycoprotein	e	
US6	394	43344	Virion glycoprotein D	e	
US7	390	41366	Virion glycoprotein I	ne	
US8	550	59090	Virion glycoprotein E	ne	
US9	090	10026	Tegument phosphoprotein	ne	
US10	312	34053	Virion protein	ne	
US11	161	17756	Unknown; Localized in nucleolus	ne	
US12	088	09792	IE protein (IE12; IE-5)	ne	

Table 1. Properties of HSV-1 encoded proteins

1. M_r : Molecular weight for unprocessed polypeptide chain.
2. Apparent molecular weight of ICP34.5 protein in HSV-1 strain F.
3. Molecular weight of ICP34.5 protein in HSV-1 strain 17^+.
4. e, essential; ne, nonessential; * , necessity depends on culture conditions or temperature.
5. References not mentioned are cited in the original papers of McGeoch *et al.* (1988b) and McGeoch (1989).

1991). This gene was shown to be important for the neurovirulence in mice (McLean et al., 1991)(See section 1.1.5.3). In latently infected cells, latency associated transcripts are transcribed downstream of IE-1 (See section 1.1.11.2) (Stevens et al., 1987; Rock et al., 1987; Spivack and Fraser, 1987). A novel HSV gene designated UL49A has recently been located between the coding region of UL49 and UL50. It encodes a putative membrane protein (Barnett et al., (1992).

HSV genes are densely arranged, show some gene overlap and only rarely have introns (e.g. Vmw 110) (McGeoch, 1987). Several genes (IE110, UL2, 23, 24, 39, 44, 50, 55, 56, US1-4 and US7-12) seem to be dispensible for virus growth at least in actively dividing cells (Reviewed by McGeoch et al., 1988a; McGeoch, 1989).

1.1.6. REPLICATION OF VIRAL DNA.

DNA replication is semi-conservative. At 37°C DNA replication starts in the nucleus of BHK cells at 3h post infection. It reaches a maximum at 9-11 hours p.i., and it is completed by 16h p.i. (Rixon, 1977). DNA in the nucleus, is thought to be quickly converted to a circular form, because there is a decrease in the number of terminal fragments detectable when compared with virion DNA (Jacob et al., 1979; Poffemberger and Roizman, 1985; McGeoch, 1987). Studies with murine cytomegalovirus suggested that circularization may be the result of direct ligation of the ends (Marks and Spector, 1988).

Late in infection, DNA is in a very rapid sedimentable form. There is a decrease of terminal fragments. This is in favour of a circular molecule, and a rolling circle mechanism, yielding head to tail concatemeric DNA genomes (Jacob et al.,1979). This theory has been supported by an *in vitro* experiment using a preformed replication fork and nuclear extracts of HSV-1 infected cells. The products of the DNA synthesis were concatemeric molecules as demonstrated by alkaline gel electrophoresis and electron microscopy (Rabkin and Hanlon, 1990).

1.1.6.1. ORIGINS OF REPLICATION.

There are three origins of replication in HSV.

There is one origin of replication in the middle of the UL,termed ori_L,(Gray and Kaerner, 1984; Quinn and McGeoch, 1985), and one in the middle of the short repeat region, termed ori_s (Stow, 1982). The location and the sequence of the ori_s was detemined using plasmid and deletion analysis. The origin of replication ori_s is located between the divergently transcribed IE-3 and IE-4/5 genes. The cis-acting sequences are present within a 90 b.p. region. A prominent feature of the origin region is an almost perfect palindrome sequence 45 b.p. long containing 18 A or T residues at its center (Stow and McMonagle, 1983). The cis-acting sequence contains two binding sites for the origin binding protein, UL9, both sites are required for origin activity (Weir et al., 1989, Weir and Stow, 1990).

The DNA sequence of ori_s of HSV-2 HG52 is closely similar to HSV-1 ori_s (Whitton and Clements, 1984).

The ori_L is located between the divergently transcribed genes for the major DNA binding protein and the DNA polymerase (Gray and Kaerner, 1984; Quinn and McGeoch, 1985). It is constituted by a long perfect palindrome with 144 b.p.. The arms are similar to ori_s (Weller et al., 1985).

1.1.6.2. CLEAVAGE AND PACKAGING SIGNALS.

Cleavage and packaging are tightly linked (Deiss and Fraenkel, 1986; Stow et al., 1986). The "a" sequence contains signals for both cleavage and packaging (Stow et al., 1986; Varmuza and Smiley, 1985). Two cis-acting sequences within the "a" sequence appear to be essential for both cleavage and packaging (Deiss et al., 1986; Deiss and Frenkel, 1986). The cleavage signal has been identified as a 179 bp fragment across a "a-a" junction (Nasseri and Mocarski, 1988). The structure of the signals involved in processing and packaging have been partially conserved among herpes viruses. The mechanism is therefore likely to share common features (Davison, 1984; Albrecht, 1985; Spaete and Mocarski, 1985; Hammerschmidt et al., 1988; Marks and

The gene UL26 encodes a protease. This protease processes the virion protein VP22a, the product of the gene UL26.5, truncating the carboxy terminus (Preston, V.G. et al., 1992).

Spector, 1988).

The viral proteins required for processing and packaging DNA have been defined using mutants capable of replicating DNA but unable to process and package DNA. Insertion mutants in the alkaline exonuclease gene (UL12) produced wild type levels of DNA synthesis and late proteins. Electron microscopy studies showed that a large number of empty capsids accumulated in infected cells, This indicated that UL12 may play a role in DNA packaging (Weller et al., 1990). The other genes implicated are UL6, UL26, UL28, UL32 and UL33 (Weller et al., 1983, 1987; Preston, V.G. et al., 1983; Matz et al., 1983; Sherman and Bachenheimer, 1987, 1988; Rixon et al., 1988; Addison et al., 1990; Al Kobaisi et al., 1991). The role of the individual genes is not yet clear. The product of gene UL26 is required for the formation of viral capsid (Preston, V.G. et al., 1983), and is transiently associated with it (Rixon et al., 1988). This suggests a link between formation of the capsid and DNA packaging.

1.1.7. HSV TRANSCRIPTION.

Upon infection of permissive cells, three sets of HSV genes are expressed sequentially. They are termed immediate early (IE) or alpha, early (E) or beta and late (L) or gamma genes (Honess and Roizman, 1974; Clements, J.B. et al., 1977).

1.1.7.1. IE GENES AND GENES PRODUCTS.

IE genes are expressed immediately after viral infection, and no de novo protein synthesis is required (Honess and Roizman, 1974; Kozak and Roizman, 1974; Clements J.B. et al., 1977, Jones and Roizman, 1979). All the IE genes with the exception of IE-2 are located near the repeat regions of the genome. IE genes 1 and 3 are situated within the long and short repeat regions respectively resulting in the presence of two copies of each (Watson et al., 1979; Anderson et al., 1980; Mackem and Roizman, 1980; Marsden et al., 1982; Rixon et al., 1982). All IE genes with the exception of IE-5 encode phosphoproteins. IE genes products,

except IE-5, are found in the nucleus of the infected cells (Pereira et al., 1977; Hay and Hay, 1980). IE genes products appear to be transcriptional modulators whose activity is essential for the expression of later classes of viral genes (O'Hare and Hayward, 1985a and b; Everett, 1986).

In addition to the five IE genes encoded by HSV, the gene encoding the large sub-unit of the ribonucleotide reductase (Vmw 136) of HSV-1 exhibits characteristics of an IE gene. It is expressed very early in infection, in the presence of cycloheximide and in the absence of IE transcription (Preston C.M. et al., 1979a; De Luca et al., 1985). Moreover a TAATGARAT element has been demonstrated in the promoter (Wymer et al., 1989).

1.1.7.1.1. Vmw 175, IE3, ICP4.

Vmw 175 is essential for virus growth in vitro; a deletion mutant in IE-3 failed to grow in normal cells (De Luca et al., 1985). Temperature sensitive and deletion mutants in the IE-3 gene fail to synthesize DNA or to express early and late genes at npt. Temperature sensitive mutants in Vmw 175 overexpress IE transcripts at npt (Preston C.M. et al., 1979b; Dixon and Schaffer, 1980), suggesting that Vmw 175 autoregulates IE genes. In transfection assays Vmw 175 can repress gene expression induced by its own promoter (O'Hare and Hayward, 1985b), and other IE promoter elements can be repressed by Vmw 175 under certain conditions (De Luca and Schaffer, 1985; Gelman and Silvestein, 1987a).

At early time of infection Vnw 175 is distributed diffusely in the nucleus after viral DNA replication occurs, Vmw 175 is distributed in replication compartments within the nucleus (Reviewed by Knipe, 1989).

Vmw 175 binds to the consensus sequence 3'-ATCGTCnnnnYCGRC-5'(R=purine, Y=pyrimidine, which is present at the transcription start site of its own gene (Faber and Wilcox, 1986).

Using insertion mutants the functional domains of the protein were defined. The domain between residues 275 and

490 is important for both repression and transactivation, the domains between residues 840 and 1000 are more important for transactivation than repression and the nuclear signal is located between residues 682-774 (Paterson and Everett, 1988).

Vmw 175 binds to the consensus sequence in promoter regions of IE-1, IE-3 and glycoprotein D (Faber and Wilcox, 1986; Kristie and Roizman, 1986; Muller, 1987), but has also been reported to bind to several promoter regions lacking the consensus (Kristie and Roizman, 1986). The role of Vmw175 binding to the consensus sequence is, however, not clear .

It was thought that this interaction mediated autoregulation (Gelman and Silverstein, 1987b; Muller, 1987), but experiments with HSV-1 infected cells did not support this hypothesis. Mutations in the Vmw 175 binding site on the Vmw 110 promoter element reduced the ability of Vmw 175 to repress IE-1 expression in transfection assays, but had no effect on the level of Vmw 110 expression during normal HSV-1 infection (Everett and Orr, 1990).

1.1.7.1.2.Vmw 110, IE-1, ICP0.

Vmw 110 is essential for viral growth <u>in vitro.</u> At early and late time during the viral replication cycle, Vmw 110 is localized diffusely in the nucleus (Knipe and Smith, 1986). Vmw 110 is a phosphorylated protein, it transactivates or increases the transcription of many genes both viral and cellular <u>in vitro</u> (Everett, 1984a; O'Hare and Hayward, 1985a and b; Quinlan and Knipe, 1985; Gelman and Silverstein, 1985; Mavromara-Nazos <u>et al.</u>, 1986). The growth of Vmw 110 deletion mutants is impaired at low multiplicity of infection (m.o.i.) but not at high m.o.i. (Stow and Stow, 1986, 1989).

Vmw 110 is a potent transactivator of gene expression in transfected cells, either by itself or in cooperation with Vmw 175 (Everett, 1984b; O'Hare and Hayward, 1985b; Quinlan and Knipe, 1985; Gelman and Silverstein, 1985). There is, however, little evidence that Vmw 110 can promote gene expression on its own in lytically infected cells, since as

discussed in the previous section, in cells infected with ts mutants in the IE-3 gene, there is very low expression of beta gene products. Vmw 110 increases the amount of transcripts of the gene it transactivates, but the mechanism of action is unknown (Reviewed by Knipe 1989).

1.1.7.1.3. Vmw 63, IE-2, ICP27.

Vmw 63 is a complex regulatory gene, with positive and negative regulatory effects in infected and transfected cells. In infected cells Vmw 63 coperates with Vmw 110 and Vmw 175 (McCarthy et al., 1989). Vmw 63 is essential for viral growth in vitro. Null mutants were replication incompetent, they induced the synthesis of greatly reduced level of DNA and were characterized by the overexpression of early proteins, reduced levels of leaky late and absence of detectable true late proteins (McCarthy et al., 1989).

In transfection assays Vmw 63 modulates the activity of Vmw 175 and Vmw 110 (Everett, 1986; Sekulovitch, 1988; Su and Knipe, 1989). However Vmw 63 seems to be capable of independent transactivation of the gB promoter (Rice and Knipe, 1988).

1.1.7.1.4. Vmw 68, IE-4, ICP 22.

A virus mutant with a deletion in the Vmw 68 genes grows normaly on certain human and monkey cells (Post and Roizman, 1981). This virus does not grow well in rodent cells and the level of the transcription of the late genes is reduced in HEL cells (Sears et al., 1985a).

1.1.7.1.5.Vmw 12, IE5, ICP 47.

In contrast to all other IE gene products, Vmw 12 is not phosphorylated and localizes in the cytoplasm (Preston, C.M. et al., 1979b, Marsden et al., 1982). Vmw 12 is non essential for growth in tissue culture (Umene, 1986, Longnecker and Roizman, 1987; Brown and Harland, 1987).

1.1.7.2.PROTEINS REQUIRED FOR DNA REPLICATION.

The genes required for DNA replication were identified using virus mutants. The results were confirmed in transfection assays. Seven proteins encoded by the genes UL30, UL29, UL42, UL9, UL5, UL8 and UL52 are necessary and sufficient to support the replication of origin containing plasmids (Challberg, 1986). These genes were cloned in Baculovirus expression vectors using their predicted sequences and the proteins purified to homogeneity (McGeoch et al., 1988b; Olivo et al., 1989). Antibodies have been raised against synthetic peptides deduced from the predicted DNA sequences (Quinn and McGeoch, 1985; Olivo et al., 1989). These developments allow the determination of the function of each protein separately, as well as their localization in the cell during the replication of the viral DNA (Olivo et al., 1989).

1.1.7.2.1.UL30, viral DNA polymerase.

The virus DNA polymerase was distinguished from the cellular DNA polymerase by its sensitivity to inhibitors, by the salt concentration required for its maximum activity and by its immunologic reactivity (Purifoy et al. 1977). It has been purified from infected cell extracts, migrates with an apparent MW of 140,000 on SDS gels (Powell and Purifoy, 1977; Knopf 1979).

UL30 and UL42 are closely linked, and purify together. Calculation from the Stokes radius and the sedimentation coefficient yielded a MW of 190,000 which is in agrement with a predicted MW of 187,000 for UL30+UL42 (Crute and Lehman, 1989).

In addition to its DNA polymerase activity on single or double stranded DNA, UL30 gene product has a 3'-5' exonuclease (proof reading) activity (Knopf, 1979; O'Donnel et al., 1987; Marcy et al., 1989). UL30 has also a ribonuclease H activity (Crute and Lehman,1989).

Experiments with purified proteins showed that UL30 is the active enzyme and the addition of UL42 to UL30 enhances its activity (Gallo et al., 1989) by increasing its processivity (Gottlieb et al., 1990).

1.1.7.2.2. UL29, major single stranded DNA binding protein. (mDBP).

UL29 is the major ssDNA binding protein. UL29 binds ss and dsDNA although the binding is five times more effective to ssDNA . No specific binding sequence is known (Bayliss et al., 1975; Conley et al., 1981; Reviewed by Knipe, 1989). During the viral DNA replication the protein localizes in the replication compartments in association with viral DNA (Quinlan et al., 1984).

UL29 is essential for DNA synthesis in vitro, since ts mutants fail to synthetize DNA at npt (Weller et al., 1983).

The functional domains of UL29 were defined by mapping mutants. Four classes of mutants were defined showing that the domains required for localization to the nucleus and DNA binding as demonstrated with affinity columns were different. One of the protein mutants lacking the N-terminus bound to ssDNA and localized in the nucleus but the virus mutants failed to replicate showing that the role of UL29 in replication is more than just binding to DNA. The N-terminal one fifth of UL29 shares sequence homologies with rat proliferating cell nuclear antigen (PCNA) also termed cyclin (Matsumo et al., 1987). Synthesis of PCNA is tightly associated with the cell cycle, occuring immediately before DNA synthesis. Therefore it is conceivable that the N-terminus of UL29 has some nuclear function (Gao and Knipe, 1989).

UL29 may also have a role in transcriptional regulation of the IE-3 gene (Godowski and Knipe, 1986) and late gene expression (Gao and Knipe, 1991).

1.1.7.2.3. UL42, double stranded DNA binding protein.

UL42 is an HSV-1 DNA binding protein, it has a molecular weight of 65,000 Daltons and binds dsDNA in a sequence independent manner. Its homologue in HSV-2 is a 55 KD protein. It is different from the 65 KD trans inducing factor (Marsden et al., 1987). It purifies in close association with DNA POL (UL30) (Gallo et al., 1988). It stimulates but is not essential for DNA POL activity (Gallo

UL8 may be required for the efficient entry of UL5 and UL52 in the nucleus (Calder and Stow, 1990).

et al., 1989)(See section 1.1.7.2.1.). It was thought that UL29 was required for the DNA POL activity of UL30 + UL42, but recent work using more highly purified proteins did not find such a requirement (Gottlieb et al., 1990)

1.1.7.2.4. UL9, origin binding protein.

UL9 is the origin binding protein. Protein-DNA complexes containing Oris and the origin binding activity were immunoprecipitated only by the anti-UL9 antiserum (Olivo et al., 1988); the binding of UL9 to the Oris sequence was confirmed by gel retardation assays (Weir et al., 1989). UL9 binds to a 11 bases sequence (site I) within Oris. UL9 binds also to a second 11 base sequence in inverted orientation in the right arm of the palindrome (site II), but with a lower affinity. However conservation of site II is required for efficient initiation of HSV-DNA replication as shown by deletion analysis (Weir and Stow, 1990) and the inefficient replication of VZV Oris which lacks site II, in response to HSV helper functions (Stow and Davison, 1986)

1.1.7.2.5. UL5, UL8 and UL52.

The HSV-1 helicase-primase complex consists of the products of the UL5, UL8 and UL52 genes. These three proteins exhibits ATPase, DNA dependent GTPase, DNA helicase and DNA primase activities in HSV-1 infected cells (Crute et al., 1988, 1989; Dodson et al., 1989). These three proteins were expressed in insect cells using baculovirus vectors. DNA dependent ATPase and DNA helicase activities were detected in cells triply infected with viruses expressing UL5, UL8 and UL52 but also in cells where only UL5 and UL52 were expressed (Calder and Stow, 1990).

1.1.7.3. VIRAL GLYCOPROTEINS

HSV encodes at least eight glycoproteins, namely gB, gC, gD, gE, gG, gH, gI and the gene product of US5, gJ (Spear, 1976; Marsden et al., 1978, 1984; Baucke and Spear, 1979; Buckmaster et al., 1984; Roizman et al., 1984; Gompels and Minson, 1986; Frame et al., 1986; Ackerman et al., 1986b; Longnecker et al., 1987; Johnson and Feenstra, 1987; McGeoch, 1987; Gao and Spear, 1990).

From experiments with mutants, the glycoprotein gC is

gK : The product of the gene UL53 was known to be involved in the syncytial phenotype(Ruyechan et al., 1979; Bond and Person, 1984; Pogue-Geile et al., 1984) and was recently shown to be glycosilated. The gene UL53 was translated in vitro using a rabbit reticulocyte lysate and the products analyzed on SDS gels. When canine pancreatic microsomal membranes were added, the UL53 gene product was glycosilated as showm by the increase in mmolecular weight and the sensitivity to glycosidases (Ramaswamy and Holland, 1992). Furthermore, the UL53 gene product was labelled with [^{3}H] glucosamine in vitro (Hutchinson et al., 1992a).

gL : The product of the gene UL1 was involved in the syncytial phenotype (Little and Schaffer, 1981) but its exact role has only recently be discovered. Peptide antisera to the predicted sequence of the UL1 gene immunoprecipitated two species of 30KD and 40KD from HSV-1 (KOS) infected cells as well as two proteins of 100KD and 110 KD which were identified as gH. Immunoprecipitation followed by Western blotting experiment showed that gH and gL were forming a complex. The 30KD species is processed to the 40KD species as shown by pulse chase experiments. This processing does not occur in the absence of g H and require only gH. Report that the protein was glycosylated were confirmed by the sensitivity of the 30KD and 40KD species to endoglycosidase F. Formation of a complex between gH and gL is required for their localization to the cell surface (Hutchison et al., 1992).

not essential for infectivity (Heine et al., 1974; Holland et al., 1984; Draper et al., 1984; Homa et al., 1986). gE, gG, gI and the product of the gene US5 was shown to be dispensable for virus growth (Longnecker and Roizman, 1986, 1987; Longnecker et al., 1987; Weber et al., 1987; Harland and Brown, 1988). Only gB, gD and gH are required for infectivity in vitro (Sarmiento et al., 1979; Little et al., 1981; Ligas and Johnson, 1988; Weller et al., 1983; Gompels and Minson 1986; Desia et al., 1988).

The properties of the individual glycoproteins are the following.

The glycoprotein gB, gD and gH are required for virus penetration of the cell in vitro.

The glycoprotein gC of HSV-1 (gC1) binds C3b (Friedman et al., 1984). The glycoproteins gC1 and gC2 are involved in adsorption of the virus to the cell membranes (Langeland et al., 1990; Campadelli-Fiume et al., 1990; see section 1.1.4.1.).

The glycoprotein gD prevents re-infection by HSV. gD is present on the plasma membrane of infected cells. Experiments show that HSV-1 and HSV-2 attach but fail to penetrate in cells expressing gD (Campadelli-Fiume et al., 1988).

The glycoprotein gE binds polymeric IgG (Dubin et al., 1990) but gI is required in conjunction with gE to bind monomeric IgG. This is probably a mechanism to protects the virus from the immune responses (Dubin et al., 1990).

The glycoprotein gG (Marsden et al 1984) provides antigenic specificity of HSV and elicits an antibody response which allows for the distinction between HSV-1 and HSV-2 (Whitley, 1990). The genes encoding gG1 and gG2 have diverged more than any equivalent genes HSV-1/HSV-2 studied (McGeoch et al., 1985, 1987; Reviewed by Marsden, 1987).

1.1.7.4. CAPSID PROTEINS

There are 7 major capsid proteins.

Protein	gene	MW	Functions
VP5	UL19	155 KD	It is the major capsid protein, being the constituent of both pentons and hexons. (Schrag et al., 1989; Newcomb et al., 1991).
VP19C	UL38	53 KD	It is a DNA binding protein (Braun et al. 1984). It is probably an internal protein important for morphogenesis (Pertuiset et al., 1989).
VP22a	UL26.5	40 KD	It is associated only with empty capsids. It is involved in DNA packaging (Preston, V.G. et al., 1983; Rixon et al., 1988).
VP23	UL18	36 KD	It may form the trimeric structure that links adjacent hexons (Schrag et al., 1989).
VP21		45 KD	Not known.
VP24	UL26	24 KD	Protease? (Preston V.G. et al., 1992).
VP26	UL35	12 KD	Not known.

(Rixon, F.J., personal communication).

1.1.7.5. ENZYMES.

1.1.7.5.1. Ribonucleotide reductase (RR).

Ribonucleotide reductase is an enzyme required for the synthesis of deoxy-ribonucleotides. The viral enzyme can be distinguished from the cellular enzyme by its immunological (Bacchetti et al., 1984) and biochemical properties (Huszar et al., 1983). The enzyme consists of two subunits which

(Cohen et al., 1972)
(Averett et al 1985)

together give the RR activity namely RR1 + RR2 of MWs 136 and 38 KD and is active as a dimer. (Dutia, 1985)

Using mutants in the RR genes, it has been shown that the HSV-1 RR activity is dispensable for virus growth and DNA replication in exponentially growing cells in vitro at 34°C, but it is required for optimal growth in resting cells or at high temperature (39.5°C). (Goldstein and Weller, 1988a and b; Preston V.G., et al., 1988). Therefore a cellular factor is capable of complementing HSV RR but only in certain conditions.

A 9 AA peptide of the small subunit of the RR specifically inhibits the viral enzyme interfering with the interaction between the two subunits (Dutia et al., 1986; Darling et al., 1990) These findings can form the basis of the design of an antiviral drug, based on a synthetic peptide which abrogates replication. The potentiality of RR as an antiviral target is supported by the observation that the virulence of RR mutants in mice is highly attenuated (Cameron et al., 1988).

In vivo a deletion mutant in RR failed to grow in the mouse eye model and failed to establish reactivable latency (Jacobson et al., 1989). Mutant viruses in RR, however, inoculated into the skin of guinea pigs produced cutaneous lesions as severe as those produced by wild type strains and in vivo replication was demonstrated for one of the mutants suggesting that the virus encoded enzyme is non-essential for replication in the guinea pig (Turk et al., 1989).

1.1.7.5.2. Thymidine kinase.

The virus encoded thymidine kinase (TK) activity (Kit and Dubs, 1963) was mapped in HSV-1 (Wiggler et al.,1977; Halliburton et al., 1980; Reyes et al., 1982) gene UL 23 (McGeoch et al., 1988a). HSV TK is dispensable for growth in growing cells in tissue culture (Dubbs and Kit, 1974) but not in resting cells (Jamieson et al., 1974). In vivo HSV TK mutants have a decreased neurovirulence (Marcialis et al., 1975; Field and Wildy, 1978; reviewed by Tenser et al., 1991). HSV TK is important for reactivation of latency in vivo (Reviewed by Tenser, 1991). The possible explanation is

discussed in the latency section 1.1.11.3.1.

The specificity of HSV TK is wider than cellular TK. Virus TK phosphorylates thymidine (Dubs and Kit, 1964), deoxycytidine (Jamieson and Subak-Sharpe, 1974) and a variety of nucleoside analogues (Elion et al., 1977). This property was used to specifically engineer antiviral drugs such as Acyclovir* effective in the infected cell (Elion et al., 1977; Fyfe et al., 1978).

1.1.7.5.3. dUTPase.

HSV induced UTPase catalyses the hydrolysis of dUTP to dUMP and pyrophosphate (Wohlrab and Franke, 1980; Caradonna and Cheng 1981; Williams 1984). The gene encoding the dUTPase activity has been mapped on the HSV-1 genome (Preston V.G. and Fisher, 1984) to a region corresponding to the UL50 gene (McGeoch et al., 1988a). Viral dUTPase is not essential for growth.

1.1.7.5.4. Uracil DNA glycosylase.

Uracil DNA glycosylase is an enzyme involved in DNA repair. It removes uracil residues from DNA. This gene is not required for growth in tissue culture (Mullaney et al., 1989). This gene has been mapped on HSV-1 to a position corresponding to the UL2 gene (Worrad and Caradonna, 1988; Mullaney et al., 1989).

1.1.7.5.5. Alkaline exonuclease.

HSV-1 and HSV-2 induce an alkaline exonuclease activity in infected cells (Keir and Gold, 1963; Morrison and Keir, 1968; Hay et al., 1971). The evidence that this activity is virally encoded is the following. An HSV-1 ts mutant in alkaline exonuclease activity has been isolated (Franke et al., 1978). Micro-injection of HSV DNA fragments in *Xenopus laevis* oocytes resulted in the production of alkaline exonuclease activity (Preston, C.M. and Cordingley, 1982). The activity has been mapped on the HSV-1 genome (Preston and Cordingley, 1982; Costa et al., 1983; Wathen and Hay

1984; Banks et al., 1985) to a position corresponding to the gene UL12 (McGeoch et al., 1988).

Early experiments with a ts mutant suggested that the exonuclease activity was essential for virus replication (Moss et al., 1986). But an insertion mutant in UL12 has shown that the activity is dispensable for virus growth as a small amount of infectious virus is produced. UL12 is dispensable for viral DNA and late protein synthesis. The UL12 gene product may be involved in processing the viral DNA into capsids (Weller et al., 1990).

1.1.7.5.6. Protein kinase.

HSV induces a protein kinase activity following infection of cells (Blue and Stobbs, 1981; Purves et al., 1986; McGeoch and Davison, 1986). Several genes can encode a protein kinase activity.

By comparison of eukaryotic protein kinases with the published sequences of HSV-1 (McGeoch et al., 1985), HSV-2 (McGeoch et al., 1987) and VZV (Davison and Scott, 1986), a protein kinase activity was predicted for the gene US3 (McGeoch and Davison, 1986; McGeoch et al., 1987). This was confirmed by immunological studies using an antiserum raised against a synthetic peptide from the predicted DNA sequence (Frame et al., 1987). This activity is dispensable for viral growth in tissue culture (Frame et al., 1987; Purves et al., 1987).

Other potential HSV protein kinases are the product of UL13 (Smith and Smith, 1989) and the amino terminus of the large subunit of the ribonucleotide reductase of HSV-2 but not of HSV-1 (Chung et al., 1989; see section 1.2.10.2.).

1.1.8. REGULATION OF HSV TRANSCRIPTION.

1.1.8.1. IE GENE REGULATION.

Transcription of immediate early genes is induced by a viral protein Vmw 65/alpha TIF, which is also an essential structural protein (Ace et al., 1988). Each viral particle contains 500-1000 copies of Vmw 65 packaged in the tegument.

Vmw 65 translocates to the cell nucleus independently of the virus DNA (Batterson and Roizman, 1983). Vmw65 interacts with the consensus sequence 3'-TAATGARAT-5' (R = purine) which is found in the far upstream region of all IE genes but not E and L genes (Mackem and Roizman 1982; Preston, C.M. 1984)

Vmw 65 does not interact with the viral DNA directly but it induces the formation of a specific complex with one or more host proteins on TAATGARAT elements (Preston, C.M. et al., 1988). One of the cellular proteins appears to be the ubiquitous octamer binding protein Oct-1, also called NF III (Pruizn et al., 1986; OBP 100 (Baumruker et al., 1986); OTF 1 (Gerster and Roeder, 1988); TRF (O'Hare and Goding, 1988) and alpha 1 (Kristie and Roizman, 1988).

The activation domain of Vmw 65 has been located to the C terminus. It is not required for immediate early complex formation. The C terminus contains a high proportion of acid residues and has been shown to bind directly and specifically to TFIID in vitro. Mutations in this region which inactivate the polypeptide in vivo eliminate TFIID binding in vitro (Stringer et al., 1990).

In addition to the TAATGARAT sequence other cis-acting regulation signals have been identified. The promoter sequences regulating the IE-3 genes were found to contain a TATA homology located approximately -25bp from the RNA start site, there are also multiple GC rich region (Sp1 binding sites) (Whitton et al., 1983) similar to those of the simian virus 40 (SV40)(Jones and Tijan, 1985), and a GA rich element which enhances the responsiveness of the TAATGARAT elements (Bzik and Preston, 1986).

It was long held that IE gene expression peaked in the early stage of infection. A gene was defined as IE if it was expressed without prior viral protein synthesis or functional Vmw 175. But in fact where studies were carried out IE genes expression persists during the course of infection (Reviewed by Everett and Orr, 1991). Vmw110 was found to accumulate throughout the viral infection. A mutation preventing Vmw 175 binding to the promoter of Vmw 110 resulted in an increased expression of Vmw 110 in a transient assay, but has no noticeable effect on Vmw 110

expression in lytic infection (Everett and Orr, 1991).

1.1.8.2. E GENE REGULATION.

Prior synthesis of IE gene products is required for early gene expression (Honess and Roizman, 1974; Clements et al., 1977).

The kinetics of expression of early genes are variable. gD is expressed as an early gene but is maximally produced only at the onset of viral DNA replication (Gibson and Spear, 1983; Johnson et al., 1986). Such genes have been described as beta-gamma or early-late genes (Wagner, 1985; Harris-Hamilton and Bachenheimer, 1985).

Promoters of the early genes show a considerable diversity. No specific viral sequences involved in transactivation have been detected in the promoters of the early gene already studied. All regions have an identifiable "TATA" box 28-30 bases upstream of the mRNAs end. Most have recognizable variants of "CAAT" at about 60 and again 90 bases and many have distinguishable GC rich elements between 90 and 120 bases (Wagner, 1985).

The promoter regulatory region of the TK gene was found to contain a TATA box, two GC rich elements, and a CAAT box. (McKnight et al., 1981; McKnight and Kingsbury, 1982; Jones and Tijan, 1985). Analysis of the sequences sufficient for regulated expression of gD found a TATA box and two GC rich elements (Everett, 1983).

This diversity may explain the different rate of expression observed among early genes (Honess and Roizman, 1974; O'Hare and Hayward, 1985a; Harris-Hamilton and Bachenheimer, 1985). Increase of transcription of early/late genes may be the result of an increase in template copy number rather than a requirement for viral DNA synthesis (Johnson and Everett, 1986a).

1.1.8.3. L GENE REGULATION.

Late gene products appear 3 hours post-infecton but are greatly increased following initiation of DNA replication. Presence of a functional Vmw 175 (Watson and Clements, 1980)

and DNA synthesis are required for late gene expression as shown by experiments with mutants ts in DNA synthesis in addition to experiments with inhibitors of DNA synthesis (Swaanstrom and Wagner, 1974; Honess and Roizman , 1974; Powell et al., 1975; Marsden et al., 1978; Jones and Roizman, 1979; Holland et al., 1980; Conley et al., 1981; Pederson et al., 1981).

Late genes are subdivided into "leaky late" and "true late" genes. "Leaky late" gene expression is detectable in the absence of DNA synthesis but requires DNA synthesis for maximum expression. An example of a "Leaky late" gene is the UL19 product, Vmw 155. True late genes have an absolute requirement for DNA synthesis for expression. An example of true late gene is the US 11 gene product (Wagner, 1985; Roizman and Batterson, 1985; Johnson et al., 1986).

Using plasmids which contains a functional origin of replication ori_s, it appears that a late gene promoter consists only of a proximal TATA box and a cap site region (Johnson and Everett, 1986b).

It is unclear how DNA synthesis increases late gene expression. An increase in the number of templates cannot be the only explanation because this would affect early gene expression also. Vmw 63 and Vmw 68 may be involved since ts mutants in these genes failed to synthesize some late genes (Sacks et al., 1985; Sears et al., 1985).

1.1.9. EFFECT OF HSV INFECTION ON CELL METABOLISM.

In order to provide a suitable environment for the efficient replication of its DNA, HSV causes a disruption of the metabolic processes of the host. Cellular DNA and RNA synthesis is largely inhibited, protein synthesis decreases rapidly, within 2-4 h postinfection (Reviewed by Fenwick, 1984). The shut off of host functions by HSV-1 occurs in 2 stages.

Early shut off does not require protein synthesis. Infection in the presence of actinomycin D or with UV irradiated virus still induces a rapid shut off of host protein synthesis suggesting that it is controlled by a

virion component (Sydiskis and Roizman, 1967; Fenwick and Walter, 1979; Fenwick et al., 1979; Scheck and Bachenheimer, 1985). HSV-2 is generally more efficient at host shut off mechanism than HSV-1 (Powell and Courtney, 1975; Peireira et al., 1977; Fenwick et al., 1979; Scheck and Bachenheimer 1985).

The virion component involved has been identified as the product of gene UL 41 (Read and Frenkel, 1983; Kwong et al., 1988). Transfer of a competent gene into a virus deficient in early shut off creates the shut off function (Fenwick and Everett, 1990). Recently HSV strain HG 52 has been found to encode a truncated UL41 product which most likely accounts for its poor shut off (Everett and Fenwick, 1990).

Late shut off requires de novo virus protein synthesis after infection (Honess and Roizman, 1974; Nishioka and Silverstein, 1978; Silverstein and Engelhardt, 1979).

1.1.10 ACTIVATION OF CELLULAR GENES BY HSV.

Some cellular RNAs are induced upon infection by HSV-1. The mechanism involved may require IE genes expression (Patel et al., 1986), or parallel activation of IE genes by Vmw 65, or they may simply be activated by adsorption of the virus to the cell surface (Kemp et al., 1986).

HSV IE gene products can activate certain cellular genes in transfection experiments. The rabbit beta globin gene promoter responds to virus activation when in a plasmid. This activation may be explained by the presence of G rich sequences in the promoter of the beta globin gene. These sequences are similar to constitutive elements in the promoters of HSV-1 genes (Everett, 1983). The beta globin gene promoter can be activated when integrated into the host chromosome of a biochemically transformed cell line. Transfection of the transformed cells with plasmids expressing Vmw175 and/or Vmw110 also activated the promoter (Everett, 1985). However virus infection fails to activate the endogenous beta globin gene of rabbit kidney cells. The promoter may be unavailable for viral trans-activation because of the densely packed chromatin configuration. Many

TG7A is a Mab raised by Dr N. LaThangue against the DNA binding proteins of BHK cells infected with HSV-2 strain 333 (Macnab et al., 1985; LaThangue and Latchman, 1988).

other genes may not be activated for the same reason.

HSV-1 infection can activate proto-oncogenes. HSV-1 inoculated by scarification in the eye of mouse was reported to activate the expression of c-fos, c-jun and Oct-1 (Valgiy Nagiy et al., 1991).

Induction of heat shock proteins follows infection of chick embryo fibroblast by HSV-1 tsk at non permissive temperature (Notorianni and Preston, C.M. 1982). This cellular response has been shown to be the result of the presence of an abnormal Vmw 175, rather than the overproduction of IE proteins (Russell et al., 1987a). This effect may be important in vivo, since natural isolates frequently show ts mutation in the gene coding Vmw 175 (Knipe et al., 1981; Post et al., 1981).

Infection of human fibroblasts with HSV-2 induces the synthesis in a growth regulated manner of a minor cellular stress protein of 57 KD (LaThangue et al., 1984). Induction of this gene is at the level of transcription (Patel et al., 1986).

Polypeptides of MW 90KD, 40KD and 32KD were recognized in transformed cell lines by two types of sera. Sera to HSV-2 infected BHK cells and sera of rats bearing tumours induced by injection of Bn5T cells. (Macnab et al., 1985). These results suggest that the activation of cellular genes in HSV-2 infected cells and in several transformed cell lines shares a common mechanism.

A monoclonal antibody (Mab) TG7A, was used to identify a set of tumour specific proteins of MWs 200KD, 90KD (a doublet, U90 and L90) 40KD and 32KD (Macnab et al., 1985. These peptides were also precipitated by TBS. Later studies from LaThangue and Latchman (1988) showed that Mab TG7A reacted with a protein of 90KD which increased with HSV-2 infection and a protein of 40KD which increased with HSV-1 infection. However, the results of J.C.M. Macnab (personal communication) do not confirm those of LaThangue and Latchman. Using TBS Macnab et al., (1992) and Grassie et al., (in preparation) did not find this difference but showed that the U90 protein and the 40KD protein were both increased by HSV-1 and HSV-2. This suggests that Mab TG7A

The absence of viral termini can be due to circularization, concatemerization or integration via the U_L or U_S regions of the genome.

and TBS recognize similar but not identical proteins.

1.1.11 LATENT INFECTION WITH HSV.

1.1.11.1. NATURAL HISTORY.

HSV-1 and HSV-2 share with other herpesviruses the ability to latently infect its host following a primary infection. During the latent state there are no overt signs of viral replication, cell damage or disease and no infectious virus can be detected. Sometimes the viral genome may be reactivated, viral replication occurs and a clinical disease may result. The stimuli triggering reactivation in humans include menstruation, psychological and physical stresses such as ultra-violet light, meningococcal and pneumococcal infections and nerve section.

The site of HSV latency is the neuron of the sensory ganglia (Cook et al., 1974; McLennan and Darby, 1980; Kennedy et al., 1983; Reviewed by Stevens, 1989). Latency in other cells has been reported. Latent HSV genomes have also been detected

in human brain tissues (Sequera et al., 1978; Fraser et al., 1981) and from corneas arising from patients with herpes simplex keratitis but not from the normal human corneas (Cook et al., 1987).

←——The viral genome appears to be in an endless state (Rock and Fraser, 1983, 1985). HSV DNA is associated with nucleosomes in a structure similar to cellular chromatin (Deshmane and Fraser, 1989). HSV DNA is not methylated in vivo (Dressler et al., 1987), but may be methylated in vitro (Youssoufian et al., 1982). Demethylating agents enhance reactivation of latent infection established in vivo (Whitby et al., 1987; Stephanopoulos et al., 1988) or in vitro (Kondo et al., 1990).

1.1.11.2. VIRAL GENE EXPRESSION.

1.1.11.2.1. Latency associated transcripts.

The expression of the latent HSV genome is very limited. The detection of HSV RNA has been reported in the neurons of latently infected mice, rabbits and humans. These transcripts are termed latency associated transcripts (LAT) and are located in the nuclei. They are transcribed from the long terminal repeat, from the complementary strand encoding Vmw 110, but 3' of its gene and partially overlapping it (Stevens et al., 1987; Rock et al., 1987; Spivack and Fraser, 1987; Croen et al., 1987, 1991; Krause et al., 1988; Wagner et al., 1988a; Steiner et al., 1989). These RNAs comprise a major 2 kilobases (kb) transcript with one or two minor transcripts of 1.5 and 1.4 kb spliced from within the major transcript. These minor transcripts are characteristic of neurons (Spivack and Fraser, 1987; Wagner et al., 1988b). Transcription has also been detected 5' and 3' of the major transcript in trigeminal ganglia of latently infected mice (Mitchell et al., 1990). There is evidence that latency associated transcripts are processed from a larger 8 kbp transcript. It starts from a TATA box 700 bp upstream the 5' end of the major LAT and terminates downstream of the LAT at a polyadenylation signal (Dobson et al., 1989).

The two major transcripts contain an open reading frame (ORF) (Wechsler et al., 1988), but are not polyadenylated and it is thought that no protein is expressed (Wagner et al., 1988a). Moreover these ORFs are not conserved in different HSV strains (McGeoch et al., 1991).

1.1.11.2.2. The role of the LATs

The role of the LATs is unclear. The latency associated transcripts are not required for the establishment of latency in vivo (Leib et al., 1989). They are not required for the reactivation of the latent HSV genome in vitro (Javier et al., 1988; Block et al., 1990), but LATs may facilitate reactivation in some models since reactivation is delayed (Steiner et al., 1988) or occurs at reduced frequency (Leib et al., 1989). In one case LATs have been

Latent HSV genomes have also been detected in tumour derived from rat cells (Park and Macnab, 1983),

found to facilitate reactivation *in vivo* but not *in vitro* (Hill *et al.*, 1990).

There is strong evidence that LAT is a stable intron. The arguments are the following. The 5' end of LAT is an excellent match for the vertebrate splice donnor sequence. HSV DNA encompassing the LAT was inserted into the beta-galactosidase gene of an expression vector and another DNA fragment encompassing the LAT and Vmw 110 were inserted into an expression vector. In both cases LAT of the expected size (two kilobases) as well as beta galactosidase and Vmw 110 mRNA were detected by Northern blotting experiments. Analysis by PCR of the spliced products revealed that the LAT was spliced from the beta galactosidase fusion transcript at the consensus splice donnor and acceptor site. In a transient system cells were transfected with three plasmids expressing the LAT, Vmw110 and luciferase driven by the HSV-1 TK gene. Expression of LAT prevented transactivation of the HSV-1 TK gene by Vmw110. The author concluded that LAT modulates reactivation events by inhibiting Vmw110 gene expression possibly via an antisense message, although this is difficult to envisage given the phenotype of LAT- mutants (Farrell *et al*, 1991).

1.1.11.3. VIRAL GENES INVOLVED IN LATENCY.

The viral genes required for the establishment, maintenance and the reactivation from latency have been studied in animal models and in *in vitro* models.

1.1.11.3.1. *In vivo* models.

Latent HSV infection can be established in the dorsal root ganglia of the mouse, the rabbit and the guinea pig (Reviewed by Stevens, 1989). Outside the neural tissues, a latent HSV infection can be established in the mouse footpad (Al Saadi *et al.*, 1983; G.B. Clements and Subak Sharpe, 1988; G.B. Clements and F. Jamieson 1989), in the mouse eye (Shimeld *et al.*, 1990 a and b; Claoue *et al.*, 1990), and in the rabbit cornea (Cook *et al.*, 1987).

The following genes were found dispensable for the establishment of latency *in vivo* : IE Vmw 68 (Sears *et al.*, 1985 a and b), IE Vmw 12, glycoprotein G and E (Meignier *et al.*, 1988), IE Vmw 110 (G.B. Clements and Stow, 1989). Replication is not required for the establishment of latency (Katz *et al.* 1990; Steiner *et al.*, 1990).

The role of gene expression has been further studied with the HSV-1 mutant in1814. The HSV-1 mutant in1814 contains an insertion inactivating alpha TIF destroying its transinducing properties and therefore the level of IE gene expression is reduced. (Ace *et al.*, 1989), it does not replicate in the mice trigeminal ganglia, but is capable of establishing latency *in vivo* (Steiner *et al.* 1990). Investigation of its gene expression suggests that the level of viral IE genes has a key role in determining the outcome of HSV infection *in vivo* (Valgiy-Nagy *et al.*, 1991).

Two enzymes ribonucleotide reductase and thymidine kinase were found necessary for reactivation from latency *in vivo*. A HSV-1 mutant lacking most of the large subunit of the ribonucleotide reductase failed to establish a reactivable latent infection (Jacobson *et al.*, 1989). A genetically engineered HSV-1 TK deletion mutant established latency in mice but failed to reactivate by explant culture (Efstathiou *et al.* 1989). This result is consistent with the hypothesis that HSV requires deoxypyrimydine kinase activity under conditions where the host cell *de novo* metabolism is low (Jamieson *et al.*, 1974). However not all experiments support the importance of the TK gene in latency *in vivo* (Reviewed by Tenser, 1991).

1.1.11.3.2. *In vitro* models.

Several methods have been devised to establish a latent HSV infection in cells cultured *in vitro.* They are described in more detail.

A latent infection can be established by treatment of HSV-1 infected cells by chemicals inhibitors such as (E)-5-(2-bromovinyl)-2'- deoxyuridine, with or without human leukocyte interferon. After removal of the chemicals latency

is maintained by incubation at 40.5°C the virus is reactivated by decreasing the temperature to 37°C (Wigdahl et al., 1984)

A latent infection is established when a culture of primary sympathetic neurons is infected at a low multiplicity by HSV-1 (Wilcox and Johnson 1987). The viral genome is reactivated by deprivation of neural growth factor (Wilcox and Johnson, 1988).

Human foetal lung cells can be latently infected by transient incubation at 42°C. The cells are inoculated with HSV-2 HG52 at 37°C for 1 hour. The infected cells are incubated 6 days at 42°C, then the temperature is decreased to 37°C and the virus remains latent. The virus is reactivated by superinfection with HCMV or with a HSV-1 ts mutant at npt (Russell and Preston, 1986).

The viral genes involved in the establishment of latency were defined in this model by using HSV-1 mutants. Virus mutants in Vmw 175 and Vmw 110 were able to establish a latent infection and were recovered by superinfection with complementary mutants, suggesting that very little gene expression is required for establishment of latency (Russell et al., 1987b). This point was confirmed by the establishment of latency with the HSV-1 mutant in1814 whose alphaTIF/Vmw 65 is non functional. Mutant in1814 established a latent infection after incubation at 42°C but also at 37°C. The authors concluded that the absence of transactivation by Vmw 65 predisposes to latency and supports the concept that gene expression is not required for the establishment of latency (Harris and Preston, 1991).

The importance of Vmw110 for reactivation was demonstrated by the following experiments. A HSV-1 mutant in Vmw 110 was unable to reactivate latent HSV-2 (Russell et al., 1987b). Latent HSV-2 could be reactivated by an adenovirus recombinant expressing Vmw 110. The region of Vmw110 required for reactivation was found to be the second exon (Harris et al. 1989).

1.2. ONCOGENES.

1.2.1. INTRODUCTION.

1.2.1.1. THE MULTISTAGE NATURE OF CANCER.

Several viruses are involved in the etiology of cancer (Klein 1985), but viruses would be only one of the factors, because naturally occuring cancer is viewed as a multifactorial disease (Reviewed by Weinberg, 1988). Other factors can be another virus, a chemical, ionizing radiation or a genetic event. (Reviewed by Weinberg, 1989). The development of cancer is also a stepwise process. The cells are altered stepwise, and with each step, the probability of evolving ultimately into a malignant neoplastic population is increased (Reviewed by Farber, 1984).

1.2.1.2. EXAMPLES OF VIRUSES INVOLVED IN THE ETIOLOGY OF CANCER.

Retroviridae

- Avian leukemia and sarcoma virus (ALV,ASV)
- Feline leukemia virus (FLV)
- Bovine leukemia virus (BLV)
- Murine mammary tumour virus (MMTV)

Hepadnaviridae

- Hepatitis B virus (HBV)
- Ground squirrel hepatitis virus
- Woodchuck hepatitis virus

Papovaviridae

- Human Papilloma virus (HPV)
- Bovine papillomavirus (BPV)
- Cotton tail rabbit papillomavirus (CRPV)

Herpesviridae

- Marek Disease Herpesvirus (MDHV)

Epstein-Barr virus (EBV)
Herpes simplex viruses 1 and 2 (HSV-1 and 2)

1.2.1.3. THE DISCOVERY OF THE TRANSFORMING GENES OF CHEMICALLY INDUCED AND NATURALLY OCCURING TUMORS.

It was first realized that the transformed phenotype of chemically induced tumours could be induced by transfection of the naked DNA of the tumour by Shih et al., (1979). The transforming DNA sequences were then isolated from a human bladder carcinoma (Shih and Weinberg, 1982). The genes responsible for transformation were called oncogenes. Oncogenes have been shown to be involved in the genesis of several tumours and they are also the transforming genes of RNA viruses. The properties of some oncogenes are summarized at the end of the introduction.

RNA and DNA viruses transform cells by different means. RNA viruses transduce and modify or deregulate cellular genes. The normal cellular counterpart of the oncogene is called a proto-oncogene, it is a gene involved in cell growth and proliferation. In contrast the genome of DNA viruses encodes virus coded oncogenes.

1.2.2. RETROVIRUSES.

1.2.2.1. CLASSIFICATION.

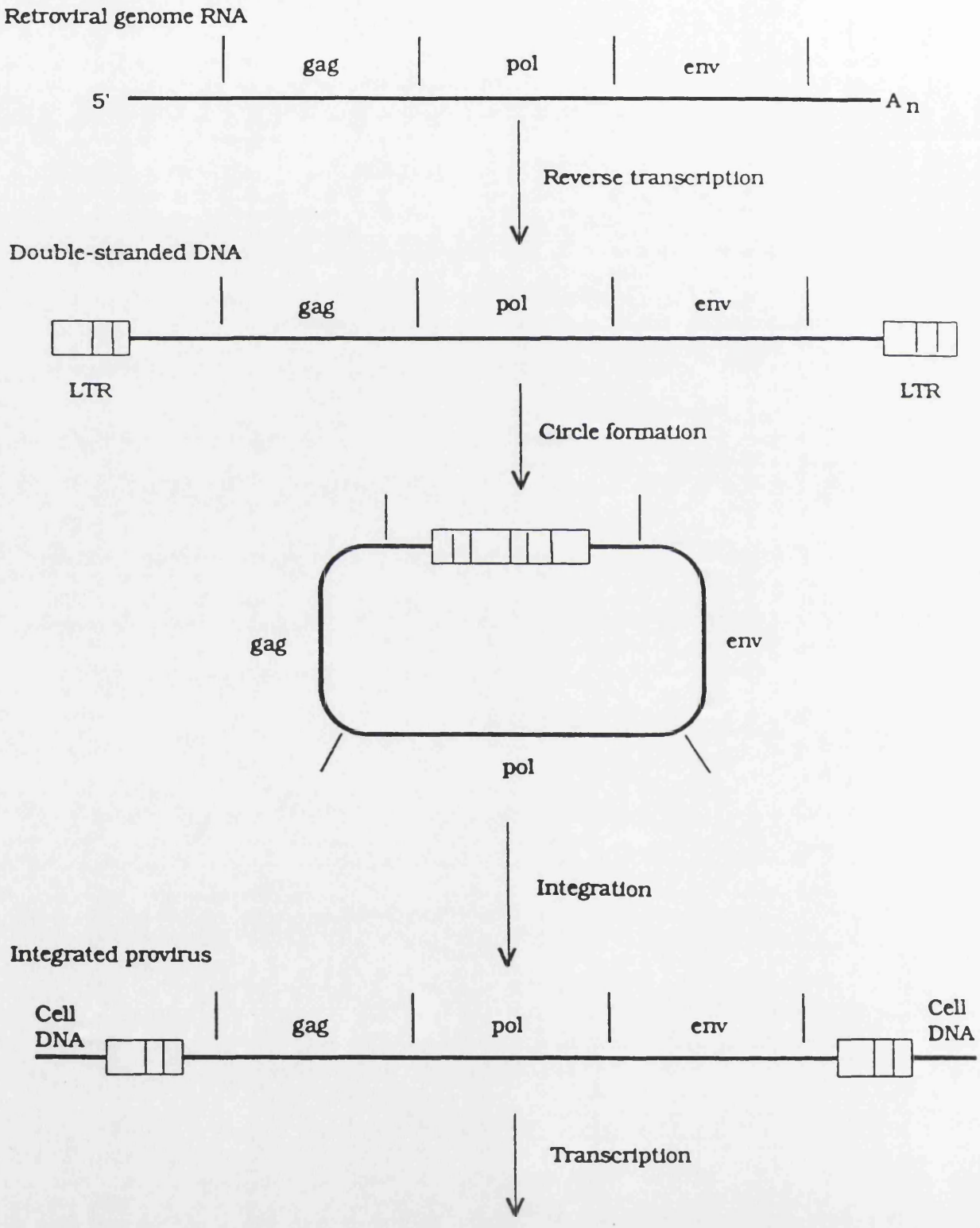
Retroviruses are classified into three major subfamilies, the lentiviruses the spumaviruses and the oncoviruses which contain the oncogenic retroviruses.

Oncoviruses are subdivided into type B, C and D in accord with their particle morphology; the vast majority of oncoviruses have the type C morphology. Type C contains the animal and human leukemogenic viruses. Among them is the HTLV group, HTLV-1 is associated with adult T cell leukemia. An example of type B oncovirus is MMTV and of type D is Squirrel monkey virus.

1.2.2.2. REPLICATION.

Figure 1.3. Retroviruses.

Integration of the retroviral genome in the cellular genome. (Hewitt, 1988).



Viral mRNA and genomes.

1.2.2.2.1. General features.

Retroviruses are enveloped RNA viruses. The core contains the viral genome and the virion associated enzyme the reverse transcriptase. The viral RNA genome is diploïd. It consists in two identical positive-sense ssRNAs that possess a capped 5' structure and a polyadenylated 3' end. The glycoproteins of the envelope serve as receptors for the virus to bind to target cells. After binding, the core is released into the cytoplasm. The reverse transcriptase transcribes the viral RNA genome into a double stranded DNA copy, called the provirus, which migrates to the nucleus and integrates covalently into the chromosomal DNA. Integration is a random process and is mediated by the reverse transcriptase which can function like an integrase. The retrovirus replication may be halted at this stage and the virus remains latent in the cells .

If transcription is activated, new genomic RNA and viral mRNA encoding proteins are transcribed from the provirus. Viral glycoproteins are inserted into the plasma membrane. The viral internal core is assembled, and the virus is released by budding from the cell wall . The release of RNA viruses is not usually cytopathic and the infected cells can divide. Furthermore, the viral infection can persist for all the life of the host (Reviewed by Coffin, 1990).

1.2.2.2.2. Genomic organisation.

The viral genome encodes three genes (*gag*, *pol*, *env*). The gene *gag* codes for internal antigens, *pol* codes for the reverse transcriptase and the IN protein needed for the integration of the viral DNA in the cellular DNA and *env* codes for envelope glycoproteins. At each end of the integrated pro-virus is a long terminal repeat which contains a strong promoter. It contains enhancers elements, a transcriptional control signal a "CAAT" box, an initiation site for RNA polymerase II, a "TATA" box and a

polyadenylation signal (Reviewed by Coffin, 1990).

1.2.2.3. ONCOGENIC TRANSFORMATION.

The mode of transformation is described below.

1.2.2.3.1. Transformation by transduction and mutation.

Leukemia does not necessarily follow the infection of a chicken by avian leukemia virus. But if a tumour appears, acutely transforming viruses may be isolated from tumour tissues. These viruses are capable of reproducing rapidly the same type of tumour in its host.

Solid tumours are caused by RNA viruses such as Rous sarcoma virus (RSV) which was found to contain a gene, the v-*src* gene, responsible for the transforming properties of the virus. The origin of the v-*src* gene is the cellular counterpart c-*src* which is altered mutationally, incorporated in the virus genome by recombination and under the control of the strong viral LTR promotor.

The origin of viral oncogenes (v-*onc*) is the normal cellular genes (c-*onc*) involved in replication and differentiation, i.e v-*oncs* are the equivalent of a cellular gene transduced into the viral genome. They lack introns and are derived by reverse transcription of processed mRNA. They contain mutations which activate their oncogenic potential by modifying their growth properties (v-*src*) or are expressed as a fusion protein with the *gag* gene product (*gagErbA*).

The transforming gene of Rous sarcoma virus, v-*src*, isolated from chicken sarcoma is a mutated form of the normal c-*src* gene (Takeya and Hanafusa, 1982). The oncogene v-*ras* of Harvey murine sarcoma virus has a human homologue. The oncogenic capacity is activated by a point mutation in bladder carcinoma resulting in the incorporation of valine instead of glycine as the twelfth amino acid (AA) of p21/*ras* (Reddy et al., 1982).

Several oncoviruses have been discovered which have lost some viral sequences. They must be complemented by non

mutant viruses for replication and spread into the host.

1.2.2.3.2. Transformation by insertion.

These retroviruses are inserted adjacent to a target cellular gene. They disrupt the regulation of the expression of the cellular proto-oncogene. The oncogene c-*myc* is deregulated by the sequences contained in the LTR. In in vitro experiments they immortalize the cells.

Avian leukosis virus (ALV) integrates within or adjacent to c-*myc* genes in bursal lymphoma (Hayward et al., 1981). The level of c-*myc* transcription is increased (Linial and Groundine, 1985) and chimeric transcripts of c-*myc* can be detected.

1.2.2.3.3. Transformation by HTLV1.

It is now established that HTLV-1 is an etiologic agent of adult T cell leukemia particularly in Japan (Reviewed by Sarnagadhran et al., 1985).

The organisation of the HTLV1 genome is different from the other oncoviruses. It contains in addition to the *gag*, *pol* and *env* genes a Px region. The Px region encodes two genes *tax* and *rex*. The product of the *tax* gene, p40, is a transcriptional activator, it interacts with cellular proteins to upregulate the transcription of the provirus. p40 also stimulates the expression of interleukin 2 and receptors for interleukin 2. *Rex* is a nuclear oncoprotein which controls HTLV gene expression.

1.2.3. FUNCTIONS OF THE ONCOGENE PRODUCTS.

Oncogenes can be classified as cytoplasmic or nuclear. Cytoplasmic and nuclear oncogenes can cooperate to transform cells in culture.

The oncogene *myc* codes for a nuclear protein, which can immortalize primary cells in culture. Normal cells have a limited capability to divide in vitro. Transfection with a nuclear oncogene gives them the capability to divide

perpetually. Immortalized cells are also able to grow in low concentrations of serum. Serum contains growth factors which promote cell growth and attachment and spreading of cells on a solid support.

Cytoplasmic oncogenes e.g. H-ras generally transform cells which have been immortalized. Cytoplasmic oncogenes have usually no effect on primary cells on their own unless they are overexpressed e.g. *ras* overexpressed by the control sequences of Moloney murine sarcoma virus or simian virus 40 (SV40) can transform passage three Chinese hamster lung cells, early passage rat cells from rat embryo and muscle and skin from 2-week-old Wistar rats (Spandidos and Wilkie, 1984).

Transformed cells differ from normal cells by several aspects.

Transformed cells fail to regulate their entry into and out of the cell cycle in response to factors such as population density and serum. Proliferation of transformed cells is not limited by contact inhibition. Cells can grow on top of each other and do not stop dividing at the monolayer stage in vitro. Proliferation is not limited by serum concentration, the cells deplete the nutrients and die rather than stop dividing.

Transformed cells grow in suspension thus in the absence of a solid support. The ability of transformed cell to grow in soft agar appears to be the parameter that correlates most consistently with the ability to form tumours in animals.

1.2.3.1. CYTOPLASMIC ONCOGENES.

The products of cellular proto-oncogenes are part of signalling pathways. Phosphorylation is critical for their functions. (Reviewed by Cantley et al., 1991).

1.2.3.1.1.Growth factors: *sis*.

The oncogene of simian sarcoma virus (v-*sis*) is a

truncated form of the cellular gene encoding the beta chain of platelet derived growth factor (PDGF) (Waterfield et al., 1983). PDGF stimulates cell proliferation as part of the wound healing process. Therefore v-*sis* could stimulate cell proliferation by abnormaly activating PDGF receptors (Waterfield et al., 1983).

1.2.3.1.2. Tyrosine kinase growth factor receptors: *ErbB*.

ErbA and *ErbB* are the oncogenes of the avian erythroblastosis virus (AEV). *ErbB* encodes sequences similar to the epidermal growth factor (EGF) receptor. The v-*erbB* gene product lacks the external ligand binding domain for the EGF. It retains, however, the phosphorylation site and the protein kinase domain of the normal receptor which is presumed to be in a permanently active state (Downward et al., 1984). These defective viruses induce rapid erythroblastosis (Graf and Stehlin, 1982).

AEVs can also induce neoplasms with a long latency. Half of the tumours contain apparently intact proviruses. They are inserted upstream of c-*ErbB*, These proviruses disrupt the structure of the gene and may influence the level of its expression (Nilsen et al., 1985, Raines et al., 1988)

1.2.3.1.3. Non receptor membrane bound tyrosine kinase: *src*.

The *src* gene of Rous sarcoma virus encodes a phosphoprotein pp60^{src} which has a tyrosine specific kinase activity (Hunter and Sefton, 1980). The following experiments suggest that this activity is required for the transforming properties.

Transformation by RSV is accompanied by an increase of phosphotyrosine (Sefton et al., 1980).

Mutations in the *src* gene that inactivate the protein kinase activity of pp60^{src} abolish transformation activity (Kamps and Sefton, 1986).

The protein sequence can be divided into four domains of activity.

Myristic acid is covalently attached to the N-terminus. The 17 N-teminus AAs are required for myristilation. Myristilation enables the protein to bind to the cytoplasmic side of the plasma membrane (Krueger et al., 1983) and is required for the transforming activity (Wilson et al, 1989).

The modulatory region contains two serine residues which can be phosphorylated by cAMP protein kinase or protein kinase C (PKC) Purchio et al., 1985; Patschinsky et al., 1986; Gould et al., 1985).

Mutations within the catalytic domain result in both temperature sensitive and transformation deficient phenotypes (Reviewed by Parson and Weber, 1989). Tyrosine 416 is the site of autophosphorylation (Patschinsky et al., 1986; Smart et al., 1981). Mutation of Tyr 416 suppresses the transformation induced by alteration of Tyr 527 (Piwnicka-Worms et al., 1987).

The carboxy-terminus of c-*src* has regulatory functions, it contains 19 AAs missing from the viral oncogene (reviewed by Parson and Weber, 1989). The major site of in vivo phosphorylation of $pp60^{src}$ is on tyrosine 527. Phosphorylation of this residue inhibits and down regulates the tyrosine kinase activity of $pp60^{src}$ in vivo (Courtneidge, 1987; Cooper and King, 1986). Binding to the polyoma middle T antigen prevents phosphorylation of Tyrosine 527 of $pp60^{src}$ thus activating the tyrosine kinase activity.

1.2.3.1.4. Cytoplasmic mediators: the *ras* family.

The *ras* oncogenes were discovered in two murine retroviruses. Cellular homologs Ha-*ras* and Ki-*ras* are found

at two separate chromosomal loci. They are part of a family containing 5 oncogenes. Mutant *ras* sequences are also detected in some human tumours (Reddy et al., 1982; Wigler et al., 1984).

The *ras* gene product is a GTPase: GTPase activity is lower in $p21^{c\text{-}ras}$. The decrease in activity is the result of point mutations at AA residues 12, 59 and 61 (Reviewed by McCormick, 1989).

Clues for the activity of *ras* came from the study of the yeast Saccharomyces cerevisiae which has two *ras* genes which stimulate adenylate cyclase, and also by analogy with the G proteins to which the *ras* p21 proteins have structural and biochemical homologies (Gilman, 1987).

G proteins have GTPase activity. They are converted to GTP bound active forms by membrane receptors and transmit signals to the inside of the cells, the GDP bound proteins are the inactive forms. They can interact with adenylate cyclase as a second message (Gilman, 1987).

Normally the GTPase activating protein (GAP) inactivates the normal p21/*ras* protein. If p21/*ras* is mutated this inactivation does not take place. Therefore once activated the mutated *ras* protein remains in an active state. (Trahey and Mc Cormick, 1987; review by Mc Cormick, 1989).

1.2.3.2. NUCLEAR ONCOGENES.

Nuclear proto-oncogenes interact with specific DNA sequences to modulate transcription. Nuclear oncogenes usually immortalize primary cells in vitro.

1.2.3.2.1. Intracelluar hormone receptors: p75*gag/ErbA*.

p75*gag/erbA* is a mutated form of the receptor for the thyroid hormone triiodothyronine (Sap et al., 1986). It is part of a family of hormone receptors which bind to specific DNA sequences and promote hormonal sythesis. Members of this family include the glucocorticoid receptor, the oestrogen receptor and the progesterone receptor. DNA

binding is mediated by zinc finger elements. The motif of a zinc finger element comprises of four cysteine residues linked to a zinc atom. Specific binding to DNA sequences depends on the AA sequence within the motif (Umesomo and Evans, 1989).

The oncogene v-*ErbA* was found fused with the viral *gag* gene and contained mutations which prevented its product binding to triiodothyronine. (Munoz et al., 1988) but not to DNA (Boucher et al., 1988). P75*gag/ErbA* prevents thyroid hormone induced transcriptional activation by c-*ErbA* in a competitive manner (Damm et al., 1989; Sap et al., 1989).

ErbA co-operates with *ErbB* to transform cells. *ErbB* alone can transform erythroid cells, but *ErbA* is required for stable transformation, *ErbA* can also cooperate with other cytoplasmic oncogenes e.g. v-*src* and v-Ha-*ras* (Kahn et al., 1986)

1.2.3.2.2. The oncogenes *fos/jun*.

The oncogene *fos* was first isolated from FBJ murine sarcoma virus (Curran and Teich, 1982). The *fos* gene product is a nuclear phosphoprotein of 55-62 KD. Its expression by agents inducing cell differentiation or proliferation is usually transient.

Molecular studies showed that the oncogenic conversion of c-*fos* is the result of increased abundance of *fos* mRNA, either by increased transcription or by stabilisation (Revewed by Verma, 1986). Mutants in Fos sequences C terminal to AA 338 fail to shut-off c-*fos* transcription after serum induced activation (Wilson and Treisman, 1988)

The oncogene *jun* was defined originally as the oncogene of avian sarcomavirus ASV 17. It is part of a multi-gene family including *jun*B and *jun*D. The product of its cellular homolog p39-*jun* is similar to the mammalian transcription factor AP-1 (Angel et al., 1988) AP-1 has a transactivating activity and is stimulated by phorbol esters such as 12-O-tetradecanoyl-phorbol-14 acetate (TPA) (Chiu et al., 1987) and Ha-*ras* which stimulates the phosphorylation of the

activation domain of c-*jun* (Binetruy et al., 1991).

Fos and p39/*jun* form a complex which binds to the DNA sequence TGACTA to modulate transcription (Schontal et al. 1988; Wilson and Treisman, 1988). Both belong to a class of DNA binding protein which shares a conserved structural motif the "Leucine zipper". A "Leucine zippers" is a periodic array of leucine residues on two proteins which interact with each other. This interaction enables the two proteins to bind to specific DNA sequences (Landschutz et al. 1988).

1.2.3.2.3. The oncogene c-*myc*.

The c-*myc* gene is part of a multigene family. It is expressed in normal cells in which it responds to growth control and is an immediate early gene. It can be activated by a retrovirus inserted upstream of c-*myc* coding sequences. In Burkitt's lymphoma it is activated by a translocation. It can also be activated by amplification as in murine T-cell lymphoma (Reviewed by Alitalo and Schwab, 1986).

The human c-*myc* gene gives rise to two proteins which have DNA binding properties in vitro (Persson et al., 1984). Their transactivating activity is inhibited by TGF beta (Pietenpol et al., 1990).

1.2.4. DNA VIRUSES: INTRODUCTION.

There are three main groups of oncogenic DNA viruses, the adenoviruses, the papova-papillomaviruses and the herpesviruses. The genome of DNA viruses encode the oncogenes which are viral genes transcribed early in infection. However, in contrast to some other herpesviruses no oncogenes have been discovered for HSV.

1.2.5. ADENOVIRUSES.

Adenoviruses are small non enveloped DNA viruses. The MW of the DNA is 20×10^6 and the diameter is 70 to 90nm.

Adenoviruses are ubiquitous in the animal kingdom, infecting birds and mammals. Human adenoviruses are not tumourigenic in the natural host. Neither adenovirus DNA nor adenovirus proteins have ever been found in human tumour.

Human adenoviruses can transform non permissive rodent cells, in vitro. They can be divided according their tumourigenic and transforming properties.

Acutely oncogenic e.g. Ad 12, 18, 31.

Transforming e.g. Ad 2, 5.

Not associated with tumours, all other types except above (1-44).

Adenovirus DNA is integrated randomly into the chromosomal DNA of the transformed cell. The 8-12% left hand end of the adenovirus genome is conserved in all transformed cells. This region contains the sequences sufficient for the transformation of primary cells in culture (Gallimore et al., 1974; Sambrook et al., 1974).

This region of the DNA corresponds to the early region E1 which consists of two transcriptional units E1A and E1B.

E1A encodes two different mRNA, 12s and 13s. Their main products are two structurally related phosphoproteins MW 23,000 and 26,000, which are transcriptional activators (Winberg and Schenk, 1984). E1A can transactivate certain cellular genes like heat shock protein (HSP)70 (Kao and Nevins, 1983) and beta tubulin (Stein and Ziff, 1984). E1A does not activate trancription directly but does so associated with cofactors ATF, E2F, TF3D and AP1 and may also be associated with some TATA box binding factors. E1A immortalizes primary cells in vitro. The expression of major histocompatibility complex (MHC) is decreased in rat cells transformed by Ad12 E1A, giving them a mean to escape lysis by cytotoxic lymphocytes (Bernards et al., 1983; Schrier et al., 1983).

E1B encodes two major RNA species of 22s and 13s which encode two unrelated polypeptides of MW 17,000 and 55,000. The 55 KD protein is nuclear and cytoplasmic, it forms a

complex with the anti-oncogene p53 (Sarnow et al., 1982). The 17KD protein is localized in the nucleus and the nuclear envelope. E1B activates the expression of E1A. E1A immortalized cells express E1A mRNA at substantially reduced levels as compared to transformants that contain both E1A and E1B (Jochemsen et al., 1987). E1B is required for complete transformation of rodent cells but cannot transform the cells in the absence of E1A. (Gallimore et al., 1974; Van den Elsen et al., 1984).

1.2.6. HEPATITIS B VIRUS.

HBV is an enveloped virus, its diameter is 42 nm. The DNA is circular and is partially stranded with a deletion in the internal strand, its molecular weight is $2x10^6$ KD. It codes for a reverse transcriptase. This virus cannot be cultured in vitro, studies have been carried out using cloned genomes.

The arguments supporting the involvement of HBV in the etiology of hepatocellular carcinoma (HCC) are the following.

Members of the family hepadnaviridae, mainly woodchuck hepatitis virus (Popper et al., 1987) but also ground squirrel hepatitis virus, produce tumours in their natural host.

An epidemiological study of 22707 patients in Taiwan showed that the 10% of the individuals who were of HBV carrier status had a 223 fold higher risk of developing carcinoma than the HBV negative controls (Beasley et al., 1981).

The results of the molecular biology studies did not give a clear explanation of the oncogenicity but recently several mechanisms have been suggested.

HBV DNA has been found integrated to the cellular DNA (Koshy et al., 1983; Ziemer et al., 1985) or as an episome in HCC tissues but but integration is also found in tissues surrounding the neoplasm. HBV DNA has been found in a

position where it disrupts the cyclin gene, and it was speculated that the HBV enhancer element could have been responsible of the increased steady state level of cyclin A mRNA obseved in this tumour (Wang, E. et al., 1989).

A possible explanation is also the transcription of host genes by HBV promoter sequences, but HBV is integrated randomly in the chromosomal DNA. The integrated HBV DNA always contains the S gene. Transcription initiated by viral sequences can continue in the host DNA (Freytag von Loringhoven et al. 1985).

The X gene may be involved in transformation. It encodes a protein which is a transactivator (Koshy and Hofschneider, 1989). Recently, transgenic mice were derived by microinjection of a HBV DNA fragment encoding the HBx gene. Male mice died earlier than female, on autopsy all males had liver tumours, many of which were diagnosed as HCC. This suggest strongly that the X gene of HBV is a virus encoded oncogene (Chang-Min Kim et al., 1991).

If mass immunization were practical, a reduction of the incidence of HCC in the populations with a high carrier rate of HBV, would be a strong argument in favour of the involvment of HBV in the etiology of HCC.

1.2.7.PAPOVA-PAPILLOMAVIRUS.

The family Papovaviridae is divided in 2 genera, Polyomaviruses and Papillomaviruses. They are small non enveloped viruses, containing double stranded DNA. The diameter of polyomaviruses is 45 nm, and the MW of its DNA is $3.5x10^6$. Papillomaviruses are slightly larger, the values are 55 nm and $8x10^6$ respectively. SV40 and polyomavirus are not tumourigenic in their natural host in the wild, but they are oncogenic in hamsters and mice respectively in the laboratory

1.2.7.1. SV40.

The early region of SV40 codes for two proteins, known

as the large T and the small T antigens.

The large T antigen is a complex multidomain phosphoprotein. It plays a direct role in viral DNA replication. It binds to viral DNA at the origin of replication, DNA binding is mediated by zinc finger domains which recognize the sequence GAGGC and it has an ATP dependent helicase activity. It interacts with DNA polymerase alpha and triggers the initiation of DNA replication. The SV40 large T antigen regulates its own synthesis and its activity is regulated by phosphorylation.

The SV40 large T antigen can both immortalize and transform rat embryo cells (Livingston and Bradley, 1987). SV40 large T can activate certain cellular genes, and has a mutagenic activity. The SV40 large T binds to the central region of the anti-oncogene p53. The affinity of the binding is lower in permissive than in non permissive cells (Lane et al., 1982). Both p53 and DNA polymerase alpha bind competitively with the large T antigen and the relative affinity of the two proteins may be a determinant of the host cell permissiveness (Murakami et al., 1986). The large T antigen also binds to another antioncogene, Rb/p105 in the underphosphorylated form. The large T interfers with the function of p53 and Rb/p105 which are both involved in the control of cell proliferation.

A small proportion of the large T antigen is associated with the cell membrane. Purified large T antigen has a tyrosine kinase activity, and can catalyze the phosphorylation of multiple proteins (Livingston and Bradley, 1987).

The SV40 large T antigen seems to combine the properties of a nuclear oncogene (immortalizing) and a cytoplasmic (transforming) oncogene.

The small T antigen co-operates with the large T to transform cells and is involved in the dissolution of actin cables.

1.2.7.2. POLYOMAVIRUS.

Transformation by polyomaviruses requires the cooperation of three distinct oncogenes, which are encoded by the early region of polyomavirus. In virus transformed and tumour cells, they are integrated into the cell DNA and are continually expressed (Cuzin et al., 1984).

The large T antigen is a nuclear phosphoprotein, It immortalizes primary cells and gives serum independence. Cells immortalized by the large T antigen can be transformed by an oncogene such as activated *ras* or the middle T antigen. The Large T antigen is a multidomain protein. One domain has DNA binding activity and is required for virus DNA replication and is different from the immortalizing domain (Strauss et al., 1989).

The middle T antigen is a membrane protein. It is sufficient to transform established cells (Treisman et al., 1981). The protein is associated with the cell membranes. The N-terminus of the middle T binds to the carboxy teminus of c-*src*, and activates c-*src* by phosphorylation of Tyr 416. Binding of c-*src* is indispensable for the transformation activity of middle T (Markland and Smith, 1987).

The small t antigen affects the cytoskeleton and cooperates with the large and the middle T antigen for full transformation (Rassoulzadegan et al. 1982).

1.2.7.3. PAPILLOMA VIRUSES.

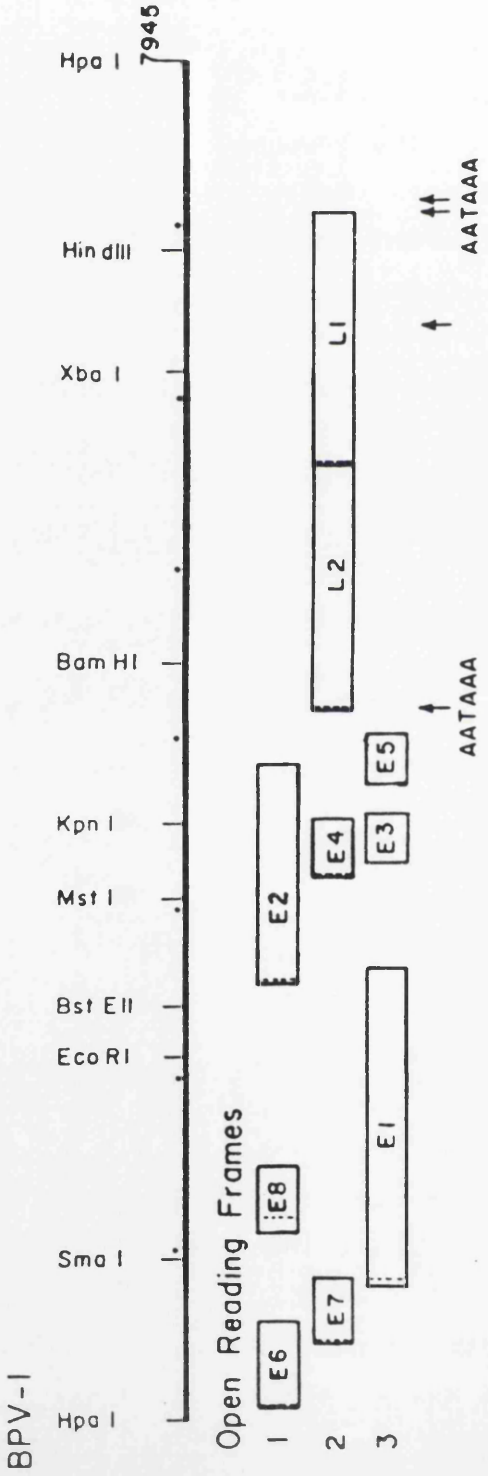
1.2.7.3.1. Animal papillomaviruses.

Papillomavirus are ubiquitous in the animal kingdom and are associated with cell proliferation (Warts).

In the rabbit, the cotton tail rabbit papillomavirus (CRPV) causes warts which within 6-9 months progress to carcinoma in which the CRPV is integrated into the cellular DNA. (Mac Vay et al., 1982).

In cattle, bovine papillomavirus type 4 (BPV-4) causes alimentary tract warts which progress to carcinomas. The DNA in this case is not retained after the initial

Figure 1.4. Genetic organization of BPV-1.
(Broker and Botcham, 1986).



proliferating event. A co-carcinogen in the west of Scotland may be bracken which is indigenous and may act by depressing the immune response or by action of the co-carcinogen quercitin (Campo and Jarret, 1987).

The role of the early genes have been studied in BPV-1 which is the agent of benign skin papillomas in cattle (Reviewed by Horwitz et al., 1989). There are 8 early and 2 late ORFs (Figure 1.4).

E1 is essential for replication.

E2 codes a DNA binding protein. It regulates E5 from BPV1 (Horwitz et al., 1989).

E5 codes for a protein of 44 AA residues. It is the transforming protein of BPV-1 (Iftner et al., 1989). The protein probably transforms by induction of DNA synthesis. The carboxy terminus expresses mutagenic activity (Horwitz et al., 1989).

E6 codes for a 15.5 kD protein found both in nuclear and membrane fractions of the transformed cell.

1.2.7.3.2. Human papillomaviruses.

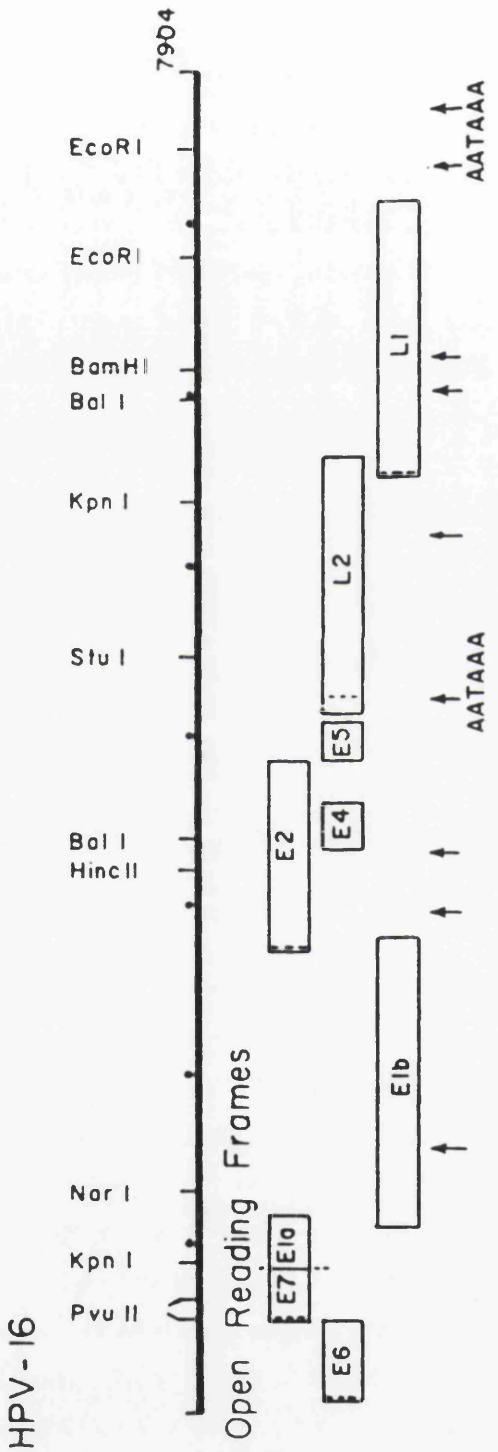
Epidemiology

In the human, papillomaviruses are associated with benign and malignant diseases. The most persuasive study is that of epidermo dysplasia verruciformis in which many papilloma subtypes but principally type 5 and 8 are present. The disease which also has a genetic component progresses from premalignant lesion to malignant skin lesions (carcinoma) following exposure to the cofactor U.V. in sunlight.

HPV subtypes are associated with both premalignant and malignant lesions in the female genital tract. Carcinoma of the cervix is a progressive disease known as cervical intra epithelial neoplasia (CIN) and graded CIN I to CIN III according the severity of the lesions. When the basal membrane is breached, then invasive cancer develops.

Original and limited studies showed HPV type 6 and 11 (Gissmann, et al., 1982) to be characteristic of CIN

Figure 1.5. Genetic organization of HPV-16.
(Broker and Botcham, 1986).



whereas HPV 16 (Durst et al., 1983) and 18 (Boshart et al., 1984) were associated with cervical cancer. However carefully controled studies from Scotland (Macnab et al., 1986) with both internal et external controls showed that HPV-16 DNA was present in 84% of the tumour tissues but was also detected in 73% of internal histologically normal tissues, and in 11% in the healthy control women. Moreover Murdoch et al., (1988) in the same geographical region showed that 63 % CIN and 48 % of internal histologically normal control tissues contained HPV-16 DNA.

These studies support in vitro molecular evidence which shows that the E6 and E7 ORFs of HPV 16 can immortalize cells in vitro. These immortalized cells are not oncogenic But the presence of HPV 16 E6 and E7 in cervical tissues is not associated with the progression of the oncogenic phenotype (Parry et al., 1990).

Early genes

The HPV 16 genome (shown in figure 1.5) codes for seven early ORFs which can be expressed in non-permissive cells plus two late proteins associated with virus assembly and expressed only in differentiated cells. No in vitro system of virus propagation exists because the virion requires differentiating cells to synthesize late proteins and assemble virions. HPV-16 DNA is maintained in monomeric episomal form in a human cervical keratinocyte cell line (Stanley et al., 1989). This cell line can be induced to form a stratified differentiated epithelium by grafting onto nude mice. This experimental system appears to permit the completion of the HPV-16 cell cycle in virus containing keratinocytes (Sterling et al., 1990). In vitro the following evidence exists for the function of E1-7 ORF except E3 and E5 for which little is known.

The 3'part of E1 is essential for DNA replication and has homology with the polyoma and SV40 large T, the 5' part of the E1 controls the copy number of extrachromosomal viral DNA at least in C127 cells (Berg et al., 1986).

E2 encodes a DNA binding protein, it is a transcriptional activator which regulates E6 and E7 of HPV 16 (Cripe et al., 1987).

The E4 product is abundant in virus producing cells (Doorben et al., 1986), and is involved in control of late virus functions.

E6 encodes the main transforming protein of HPV-8.

E6 and E7 of HPV 16 are both transforming proteins. Schiller et al. (1990) showed that E6 and E7 are both necessary and sufficient to immortalize primary human keratinocytes. E7 alone, transiently extends the lifespan of the keratinocytes, but E6 is necessary to completely immortalize them. Other workers e.g. Di Paolo et al. (1990) can immortalize a human epithelial cell line with E7 alone. The gene product of E6 binds to p53, and the gene product of E7 binds to p105. The importance of these interactions are demonstrated by the following experiments.

Using proteins expressed in a rabbit reticulocyte lysate, the E6 proteins of different HPVs which bind (HPV-16 and HPV-18) or do not bind to p53 were incubated with p53. P53 remained stable in the presence of increasing amounts of the E6 proteins of HPV-6 and HPV-11. In the presence of E6 proteins of HPV-16 and HPV-18, p53 was degraded in a manner dependent on the concentration of E6 protein. The protein p53 was found to be bound to ubiquitin suggesting that E6 binding enhance the degradation by the ubiquitin-dependent protease system (Scheffner et al., 1990). The association of E6 with p53 is mediated by a 100KD cellular protein (Huibregtse et al, 1991).

Crook et al., (1991a), generated a series of mutations within the E6 ORF. These experiments showed that E6 binding of p53 is not sufficient for E6 enhancement of p53 degradation. This enhancement of p53 degradation is mediated by E6 sequences different from the binding sequence.

Both p53 and RB/p105 proteins are anti-oncogenes (See section 1.2.8).

Role of HPV in cervical cancer

The experiments presented show the importance of E6 and E7 gene products in the origin of cervical cancer, by inactivation of p105/RB and p53.

In premalignant lesions the viral DNA is predominantly extra-chromosomal, in plasmid state. In cancer containing HPV-16 and HPV-18, the DNA has been detected as either an oligomeric episome, a monomeric episome or as an integrated sequence (Durst et al., 1985, Boshart et al., 1984). It has also been isolated from a cancer of the vulva as a head to tail dimeric episome with a deletion in the non-coding region (Kennedy et al., 1986).

When HPV DNA is integrated in the genome of the tumour cell, there is no particular site of integration. But the circular HPV DNA is disrupted within the 3'end of the E1 ORF and the 5'end of the E2 ORF (Schwarz et al., 1985; Pater and Pater ,1985; Baker et al., 1987). The E6 and E7 ORF are not deleted and are transcriptionally functional (Bedell et al., 1987). The loss of the E2 gene product, a DNA binding protein, which can repress the transcription of E6 and E7 (Cripe et al., 1987; Thierry and Yaniv, 1987) deregulates E6 and E7 expression.

Microinjection of E6/E7 antisense RNA interferes with the survival of cells expressing E6 and E7 (Kleinheinz et al., 1989).

The the RB and p53 genes in HPV positive and negative cervical carcinoma cell lines were sequenced (Scheffner et al., 1991; Crook et al., 1991b). Those cell lines containing HPV DNA were found to express wild type p53 and RB while lines lacking HPV DNA contained mutations at both the RB and the p53 genes. This suggests that inactivation of RB/p105 and p53 is important in cervical carcinogenesis and that inactivation can occur through the action of the E6 and E7 proteins.

The data supporting the involvment of HPV in cervical cancer are strong, but other co-factors are required. One of the possible cofactors is herpes simplex virus.

1.2.8. TUMOUR SUPRESSOR GENES.

Studies of RNA viruses show that some of them transform by encoding mutated or altered cell genes. DNA viruses code for an oncogene, their mode of action could involve in some instances the inactivation of normal cell genes termed "anti-oncogenes".

1.2.8.1. p105/RB.

The retinoblastoma gene (RB) controls the normal regulation of cell growth. Manifestation of the disease retinoblastoma involves an inherited genetic defect involving a chromosomal deletion and expression of a mutated abnormal autosomal recessive allele. Reintroduction of the normal Rb gene decreases the growth rate of cultured retinoblastoma cells and inhibits their growth in soft agar (Huang et al., 1988). The Rb gene was found also to be inactivated in breast cancer cell lines (Lee, E.Y. et al., 1988).

The product of the retinoblastoma gene Rb/p105 is a nuclear protein which is reversibly phosphorylated during the cell cycle (Lee, W.H. et al., 1987a and b). It can form complexes with several DNA virus oncogenes.

SV40 large T antigen and adenovirus E1A can both complex to Rb/p105 (Whyte et al., 1988; De Capricio et al., 1989). This interaction with RB/p105 is essential for transformation by adenovirus (Whyte et al., 1989). It occurs through a site on E1A which shows sequence conservation with a region in the transforming proteins of SV40, polyomavirus, and human papillomavirus (HPV-16) (Reviewed by Green, 1989).

The phosphorylation state of Rb/p105 is maximal close to the start of the S phase and low after mitosis and and entry into G1 (De Capricio et al., 1989; Chen, P.L. et al.,

1989; Ludlow et al., 1990). This suggests that only the hypophosphorylated form of RB/p105 suppresses cell proliferation.

The protein Rb/p105 which co-immunoprecipitates with the large T antigen of SV40 is dephosphorylated (Ludlow et al., 1989). The transforming proteins of DNA tumour viruses may act at least in part by complexing with and inactivating the hypophosphorylated protein. E7 proteins of the non oncogenic HPV type 6b and 11 bind RB/p105 with a lower affinity than proteins of oncogenic types HPV 16 and 18 (Gage et al., 1990).

The protein Rb/p105 suppresses cell proliferation not directly, but through interaction with c-fos (Robbins et al., 1990) and c-myc (Pietenpol et al., 1990 a and b). Recent evidence shows that E1A prevents the binding of Rb with the cellular transcription factor DRTF1 (Bandara and La Thangue, 1991).

1.2.8.2. p53.

The product of the gene p53 is a nuclear phosphoprotein which was discovered as a complex with SV40 large T antigen (Lane and Crawford, 1979). It can also form a complex with E1B of adenovirus (Sarnow et al. 1982) and E6 of HPV 16 and 18 (Werness et al., 1990). Complex formation with SV40 LT stabilizes p53. Increased stability is also characteristic of mutants in p53 which are oncogenic. The transforming activity of SV40 large T antigen is linked to the binding of p53 because mutants unable to bind p53 are not oncogenic.

The activity of p53 seems to be regulated both by abundance and by phosphorylation. The level of p53 is very low after mitosis but increases in G1 and p53 is phosphorylated during the S phase of the cell cycle (Bischoff et al. 1990).

The protein p53 seems to be necessary for the proliferation of normal cells (Shohat et al., 1987). Tumours however have either a very low, perhaps undetectable, level of p53 (Mulligan et al. 1990), or have

mutant proteins which can be expressed at very high level (Finlay et al. 1989; Halevy et al. 1990). These p53 mutants can immortalize primary cells, which can in turn be transformed by an oncogene such as *ras* (Parada et al., 1984).

Introduction of the normal p53 gene into tumor cell lines with either low level of p53 or a mutant protein suppresses growth (Baker et al. 1990; Diller et al., 1990).

The mechanisms of action of p53 may be the following (Reviewed by Marshal, 1991).

p53 may inhibit the expression or function of *c-myc*.

p53 may activate genes that suppress cell proliferation.

p53 may control the initiation of the DNA synthesis by interaction with DNA polymerase alpha and other components of the DNA replication complex (reviewed by Marshal, 1991). This is supported by the following facts. Normal p53 blocks the binding of SV40 large T antigen to DNA polymerase alpha (Gannon and Lane, 1987). p53 mutants which do not bind SV40 large T fail to block SV40 DNA replication (Braithwaite et al., 1987; Wang, E.H. et al., 1989).

1.2.9. HERPESVIRUSES.

1.2.9.1. EPSTEIN-BARR VIRUS.

EBV readily infects human B cells in culture transforming them into permanent cell lines in which every cell carries multiple episomal copies of the viral genome (Reviewed by Dambaugh et al., 1986). The EBV genome in naturally infected cells expresses 8 EBV encoded proteins, 6 nuclear antigens (EBNAs) and 2 latent membranes proteins (LMPs). EBV is involved in the etiology of two human tumours, Burkitt's lymphoma and nasopharyngial carcinoma.

Burkitt's lymphoma is a B cell lymphoma found predominantly in Central Africa. It is a childhood disease in which every tumour cell contains episomal copies of the EBV genome. An early age of infection and malaria which

depresses the immune system are probably the co-factors required for the development of the tumour.

The functions of some of the early genes expressed in transformed cells are the following.

EBNA-1 is involved in maintenance of copy number of episomal viral genomes (Yates et al., 1984; Lupton and Levine, 1985).

EBNA-2 plays a role in the expression of the transformed phenotype. It is required for immortalization of B cells in culture and growth in low levels of serum (Dambaugh et al., 1986).

LMP-1 is detected in latently infected proliferating lymphocytes. LMP is a transmembrane protein, analysis of its AA sequence shows 6 possible transmembrane domains (Bankier et al., 1983). It is a phosphorylated protein but does not seem to have a protein kinase activity . It is associated with a component of the cytoskeletal framework : vimentin (Liebowitz et al. , 1987). It has a considerably more rapid turnover than cellular proteins. It converts Rat-1 cells (immortalized cells) to a tumourigenic phenotype (Wang et al., 1985).

Cells from Burkitts'lymphoma contain translocations involving the long arm of chromosome 8 and chromosomes 14, or chromosome 2 and 22. They show translocation of the oncogene c-*myc* normally on chromosome 8 with the immunoglobulin heavy chain gene in chromosome 14 or the immunoglobin light chain genes in chromosome 2 or 22 putting c-*myc* expression under the control of the strong promoter of the immunoglobulin chains.

Nasopharyngeal carcinoma occurs predominantly in southern China. The cells of the carcinoma all contain episomal copies of the EBV DNA genome.

Studies of EBV gene expression was possible once NPC transplanted in nude mice became available. Passage in nude mice eliminates most of the contaminating lymphocytes which may be infected by EBV.

One of the tumours designated C15 contained multiple

episomal copies of EBV. The episomal DNA is not grossly mutated, but the transcription pattern differs from that observed in B lymphocytes. Transcription is strictly regulated, EBNA2-6 are switched off, and therefore EBNA-1 and LMP are the only antigens detected. A family of cytoplasmic mRNA is transcribed in an anti-sense direction to the known ORFs in this region of the genome (Hitt et al., 1989).

1.2.9.2. HERPES SIMPLEX VIRUSES.

There has been a great interest in HSV as a possible agent of cervical carcinoma. However molecular biology studies have not revealed an oncogene or a clear mechanism of oncogenesis.

The role of HSV in the etiology of cervical carcinoma was supported by epidemiological data, which found an increased frequency of HSV-2 positive sera among women with cervical cancer (Rawls, 1983; Melnick et al., 1991) with a seven fold increase of sera positive for HSV-2 and HPV (Rawls, 1983). But the results of two prospective studies do not support a role for HSV-2 in the development of cervical cancer (Vonka et al. 1984, Melnick et al., 1991). But these results may be biased because CIN is treated before progression occurs.

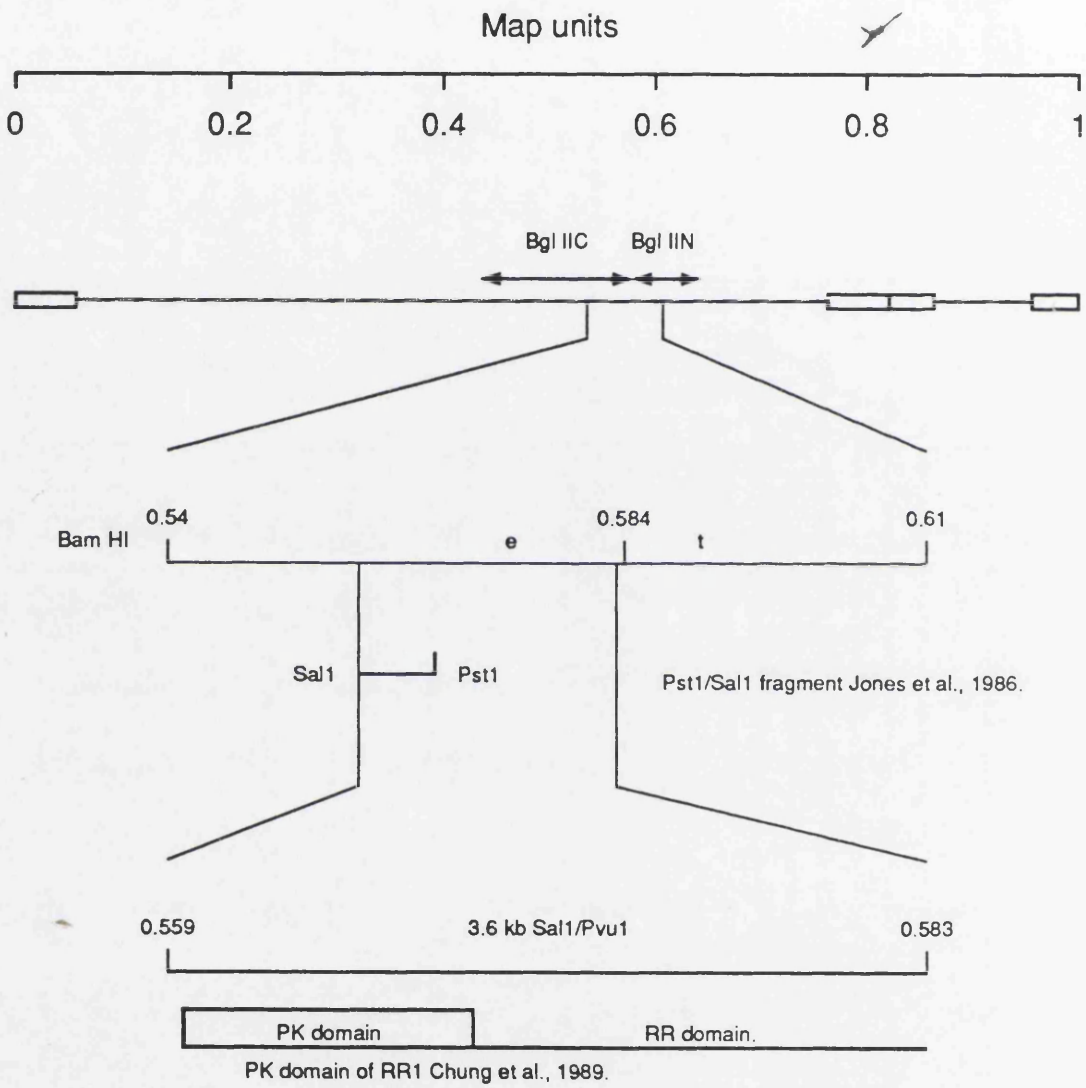
Cervical carcinoma was reproduced in mice, by vaginal application of swabs impregnated with HSV-1 (Wentz et al., 1981), HSV-2 or its DNA (Anthony et al., 1989), and it is inhibited by prior immunization (Wentz et al., 1983; Chen et al., 1986; Skinner et al. 1987).

The transforming properties of HSV have been confirmed by in vitro experiments. Hamster embryo cells can be transformed after exposure to UV inactivated HSV-2 (Duff and Rapp, 1971), and to HSV-1 (Duff and Rapp, 1973). The oncogenic regions of HSV have been defined.

Three regions of the HSV genomes are associated with morphological transformation of cultured cells. One of

Figure 1.6. Genomic organization of HSV-2.

Localization of Bgl IIn and Bgl IIg fragments and sub-fragments. (adapted from Macnab, 1987 and Chung et al., 1988).



these (Camacho and Spear, 1978) present in HSV-1 and known as MTRI (morphological transformation region of HSV-1) maps in XbaIf (0.29 to 0.45 m.u.) and BglIIi (Reyes et al., 1979). The sequences coding for glycoprotein B (gB) can be at least transiently expressed (Camacho and Spear, 1978). In HSV-2 there are two regions of the genome associated with the induction of morphological transformation (Figure 1.6) : these are MTRII (morphological transformation region of HSV-2) mapping (Reyes et al. 1979, Macnab and McDougall, 1980; Galloway and McDougall, 1981; Cameron et al., 1985) in BglIIn (0.58to 0.62 m.u.) and another region referred to as MTR III (the third morphological region of HSV); maps (Jariwalla et al., 1982) in BglIIc (0.54 to 0.58 m.u.). The 64% left hand of BglIIc can immortalize hamster embryo cells, while the 36% right hand is required for tumourigenicity (Jariwalla et al. 1983). A smaller 480 bp sub-fragment of BglIIc was found to be sufficient to transform Rat-2 cells in a focus assay system (Jones et al., 1986).

1.2.10. MECHANISMS OF TRANSFORMATION BY HSV.

1.2.10.1. INTRODUCTION.

HSV-2 DNA sequences are detected in 10% of cervical tumour cells (Park et al., 1983). The BglIIn fragment is the most frequently retained (reviewed by Macnab, 1987). To further identify this region, the insert in one tumour was multiply digested, by restriction enzymes, and restriction sites characteristic of the HSV-2 genome were demonstrated (Park et al., 1983).

Retention of HSV-2 DNA sequences is not necessary for the maintenance of the transformed phenotype in HSV-2 transformed cells (Cameron et al., 1985).

These data suggested that HSV-2 DNA could induce transformation, but would then be eliminated from the transformed cells in a "hit and run mechanism" as suggested originally by Skinner (1976).

The suggested mechanisms by which transformation could occur were reviewed by Macnab, (1987) and summarized to be:

Involvment of the ribonucleotide reductase activity.
Mutagenesis and gene amplification.
Increased or altered expression of cell coded genes.
Activation of endogenous viruses.
Coding capacity of HSV for a protein kinase.
Homologies of HSV-2 and HSV-1 with cellular DNA sequences.

Recent studies strengthen these hypotheses, or suggest new ones, they will be shortly described.

1.2.10.2.MUTAGENESIS.

The mutagenic activity of HSV was tested by inactivation of a gene cloned in the shuttle vector plasmid pZ 189 (Seidmann et al., 1985).

Infection of Cos cells by HSV-1 UV irradiated or not, increases the rate of mutation by two to sevenfold. The mutations are also different (Hwang and Shillitoe, 1990):

1/ The frequency of deletions and complex mutations is higher.

2/ Insertions of cellular genes are observed.

3/ Thirty per cent of the mutations occur at a hot spot with general sequence PPPYYYYPPP (where P = purine and Y = pyrimidine).

Using the same vector, the expression of the viral ribonucleotide reductase does not appear to be the cause of increased mutagenesis in infected cells. UV inactivated DNA is mutagenic in cells which suggests that either a virion component or the HSV DNA itself could be the mutagenic agent (Clarke and Clements, 1991).

1.2.10.3.THE ROLE OF THE RIBONUCLEOTIDE REDUCTASE OF HSV-2.

RR is an enzyme required for the synthesis of deoxy-

ribonucleotides. The viral genes are contained in the MTR II and III regions of HSV-2. The large sub-unit, Vmw 136, is encoded by the Bgl IIc fragment, but the carboxy terminus sequence lies in Bgl IIn (McLauchlan and Clements, 1983). The small subunit, Vmw 38, is encoded by the Bgl IIn fragment. The enzyme is only active if the two sub-units are associated and act as a dimer.

The large subunit of HSV-2 RR, Vmw 136, can be detected in transformed cells by immunoprecipitation with a specific monoclonal antibody (Hayashi et al, 1985). Vmw 136 is also immunoprecipitated in 48% of CIN III, and 57% of genital invasive carcinoma. The protein is expressed even when its gene cannot be detected by Southern blot analysis in which only 5% of CIN III and 16% of genital invasive carcinoma are positive (Di Luca et al., 1990).

Immortalized non tumourigenic Syrian hamster embryo cells transfected by a HSV-2 DNA fragment (0.554 - 0.572 m.u.) encoding the protein kinase activity of Vmw 136 acquire anchorage independent growth potential and neoplastic potential. The importance of Vmw 136 expression was investigated using DNA constructs expressing full length Vmw 136, the protein kinase domain, the non protein kinase domain and a non expressing frameshift mutant. The anchorage independent phenotype was acquired only by the cells transfected by the constructs encoding either full length Vmw 136 or the protein kinase domain. Vmw 136 expression was detected by immunofluorescence in cells transfected by the DNA construct expressing full length Vmw 136 (Smith et al., 1990).

Enzymatic activity of Vmw 136.

Protein kinase activity had been predicted by computer analysis in the amino terminus of the HSV-2, but not of the HSV-1, large subunit of the RR . This protein kinase activity has been immunoprecipitated by specific monoclonal antibodies from either HSV-2 infected cells or from cells

transformed by the cloned fragment of Vmw 136(m.u. 0.559-0583), but not from uninfected Hep2 cells (Chung et al., 1989). Computer analysis indicated the presence of a transmembrane segment, and the protein is myristilated by a cellular enzyme (Chung et al., 1990). This suggests that Vmw 136 encodes an oncogene similar to v-*src* which is a tyrosine kinase and is bound to the cell membrane by myristic acid.

1.2.10.4. GENE AMPLIFICATION.

Gene amplification is induced by a subset of the genes necessary for HSV replication.

Plasmids expressing the viral genes UL30 DNA polymerase, UL29 major DNA binding protein, UL5 DNA helicase, UL8, UL42 DNA binding protein and UL52, but not UL9 origin binding protein were required for SV40 DNA amplification in SV40 transformed syrian hamster (Elona) cells (Heilbronn and Zur Hausen, 1989). Amplification was also obtained by infection with a deletion mutant of HSV-1 KOS for UL9 (Heilbronn et al., 1990).

Amplification by subclones of MTR III.

The minimal transforming fragment of MTR II was mapped to a 486 bp Pst 1-Sal 1 fragment (0.567-0.570 m.u.) utilizing an established rat cell line (Jones et al., 1986). This fragment cloned into a plasmid can induce the replication of the plasmid (Zhu and Jones, 1990). This suggests that this fragment integrated into a cellular chromosome may also trigger replication.

1.2.10.5. COOPERATION WITH OTHER VIRUSES.

DNA of another herpes virus HCMV is found in 5% of cervical cancer biopsies (Fletcher et al., 1986). The IE gene product was expressed in the HCMV insert cloned from this DNA (Fletcher and Macnab, 1989).

Cooperation between HSV and HPV was suggested by Zur Hausen (1982). Both viruses are sexually transmitted and they can be found associated in cervical cancer (Di Luca et al., 1990; reviewed by Macnab, 1987). A model of cooperation was suggested in an in vitro experiment. Cloned HSV-2 BglIIn fragments were unable to transform primary human keratinocytes, but transformed keratinocytes already immortalized by HPV (Di Paolo et al., 1990). The papillomavirus transactivator E2 protein activates expression from the promoter for the large subunit of RR of HSV-2 (Wymer and Aurelian, 1990).

1.2.10.6. TRANSFORMATION BY ALTERATION OF THE ACTIVITY OF CELLULAR PROTEINS.

The following experiment suggests that HSV infection may alter the functions of different cellular proteins involved in the control of cellular division. This could facilitate transformation by analogy with the mechanism of action of other tumourigenic viruses.

The major DNA binding protein, mDBP, is associated with the viral DNA in the replication compartments (Quinlan et al., 1984). Using immunofluorescence techniques, it co-localizes with the following proteins: single stranded DNA binding protein (Waseen and Lane, 1990), proliferating cell nuclear antigen (PCNA) (Kenny et al., 1990), p105/RB and p53. It is however not known if the mDBP binds to p53 or to p105/RB (Wilcox and Lane, 1991) but it cannot be immunoprecipitated (i.p.) (Lane, personal communication).

1.2.10.7. TRANSFORMATION BY ACTIVATION OF CELLULAR GENES.

HSV-1 does not need to enter the cell to activate the expression of cellular genes. The ts 1204 mutant of HSV-1 17 fails to penetrate the cell at npt, (Addison et al., 1984). The mutant ts 1204^{C} induces the expression of a protein of apparent molecular weight 56 KD in human

fibroblast cells but not BHK cells or CV1 cells at npt. Synthesis of p56 is induced by the specific interaction of ts 1204 with the cell surface (Preston, V.G. 1990).

Our studies centre on HSV induced cellular genes (Macnab et al. 1992) in which patients with cervical cancers develop antibodies to a polypeptide of MW 40,000 which can be induced by HSV infection. The relevance of this polypeptide to transformation will be the subject of this thesis. The identification of this (these) polypeptide(s) is the first step in understanding its role.

The sera of rats bearing tumours to HSV-2 transformed cells (TBS) i.p. a set of polypeptides of MW 200KD, 90KD (a doublet), 40KD and 32KD which are expressed in HSV-2 transformed (Bn5T) cells but not in rat embryo cells. Studies with a monoclonal antibody raised against HSV-2 infected cell polypeptide purified by DNA binding affinity chromatography, TG7A, (Macnab et al., 1985; LaThangue and Latchman, 1988) showed that the 90KD polypeptide could be i.p. in cells transformed by RSV and Ad-12 (Macnab et al., 1985). These polypeptides are cellular and induced by viruses.

The pattern of the Staph. aureus V8 digestion of the 90KD polypeptides i.p. by TBS and by the Mab TG7A (TBS:90 and TG7A:90) is undistinguishable. It is not proved but it is reasonable to assume that they are two highly similar polypeptides. The analysis of the same experiment with TBS:40 and TG7A:40 showed that the two polypeptides give different bands but have some in common. The two polypeptides can be similar but different or TBS:40 can consist of several polypeptides one of them identical to TG7A:40 and sharing epitopes with TBS:90.

The aim of this thesis will be to purify and sequence the TBS:40. From the data generated it should then be possible to identify the function of the protein and the relationship to U90 if any.

TABLE 12. Adapted from Fletcher, (1986) and Hunter, (1991).

SUMMARY OF THE PROPERTIES OF SOME ONCOGENES.

onc	Retroviral isolates	v-*onc* origin	v-*onc* protein	virus disease	v-*onc* product activity	v-*onc* product location
src	Rous sarcoma virus	chicken	pp60^{src}	sarcoma	tyrosine kinase	inner side of plasma membrane
	rASV	quail	pp60^{src}	"	"	
fps	Fujinami sarcoma virus	chicken	P130$^{gag\text{-}fps}$	"	"	plasma membrane
fes	Snyder-Theiler feline SV	cat	P85$^{gag\text{-}fes}$	"	"	"
yes	Y73-avian sarcoma virus	chicken	P90$^{gag\text{-}yes}$	"	"	"
fgr	GR-sarcoma virus	cat	P70$^{gag\text{-}actin\text{-}fgr}$	"	"	"
ros	UR2-avian sarcoma virus	chicken	P68$^{gag\text{-}ros}$	"	"	"
abl	Ab-murine leukemia virus	mouse	P90-P160$^{gag\text{-}abl}$	"	"	"
	HZ2-Fes	cat	P98$^{gag\text{-}abl}$	"	"	"

onc	retrovirus isolates	v-*onc* origin	v-*onc* protein	virus disease	v-*onc* product activity	v-*onc* product location
Erb B	avian erythroblastosis virus	chicken	gp65$^{Erb\text{-}b}$	erythroblastosis sarcoma	truncated EGF receptor	membrane
fms	SM-felilne SV	cat	gp120^{fms} gp140^{fms} gp180$^{gag\text{-}fms}$	sarcoma	mononuclear phagocyte CSF-1 receptor	membrane intermediate filaments
sis	simian SV	monkey	p28$^{env\text{-}sis}$	sarcoma	truncated PDGF	membrane
Ha-*ras*	Ha-murine SV	rat	pp21^{ras}	sarcoma erythroleukemia	GTPbinding GTPase activity	membrane
Ki-*ras*	Ki-murine SV	rat	"	sarcoma erythroleukemia	"	"
	Ny-FeSV	cat	"	sarcoma		"
mos	moloney murine SV	mouse	p37$^{env\text{-}mos}$	sarcoma	protein kinase (ser,thr)	cytoplasm
fos	FBJ-MSV NK24-ASV	mouse chicken	pp55^{fos} p100$^{gag\text{-}fos}$	osteosarcoma nephroblastoma	DNA binding complex with *jun*	nucleus

onc	retrovirus isolates	v-*onc* origin	v-*onc* protein	virus disease	v-*Onc* product activity	v-*Onc* product location
Erb-a	AEV-ES4	chicken	P75$^{gag\text{-}Erb\text{-}A}$	erythroblastosis sarcoma	thyroid hormone receptor	cytoplasm
jun	S17-ASV	chicken	P65$^{gag\text{-}jun}$	sarcoma	complexse with *fos*.	nucleus
myc	MC-29	chicken	P110$^{gag\text{-}myc}$ P100$^{gag\text{-}myl\text{-}myc}$ P58^{myc}	sarcoma carcinoma myelocytoma	DNA binding	nucleus
	FeLV-*myc*	cat		granulocytic leukemia		
myb	AMV-BAI/A AMV-E26	chicken	P45^{myb} P135$^{gag\text{-}ets\text{-}myb}$	myeloblastosis myeloblastosis erythroblastosis		nucleus

PART 2

MATERIALS AND METHODS.

2.1. MATERIALS.

2.1.1. ANIMALS

Hooded Lister rats from a closed colony maintained in the Institute of Virology by inbreeding, brother/sister mating for over 20 years were used to prepare Tumour Bearing Sera (TBS) and Rat Embryo (RE) cells .

Female Balb C mice, 3 weeks old were purchased from Bantin and Kingman, Hull, U.K..

2.1.2. CELLS.

RE fibroblast cultures are prepared from embryos of 16 to 17 days gestation. Primary or secondary cultures were used as negative control in the i.p. experiments.

Bn5 is a clone of rat embryo fibroblast transformed by the Bgl IIn fragment of HSV-2 strain HG 52. This fragment codes for the small sub-unit of the ribonucleotide reductase, and the carboxy terminal of the large sub-unit of the ribonucleotide reductase and the host virion shutoff gene, UL41.

BN5T is cell line cultured from tumour induced by injection of Bn5 cells 1n Hooded Lister rats (Cameron et al., 1985).

The myeloma cell line P3-X67 Ag8, was kindly supplied by Dr Ann Cross of this Institute. It secretes IgG. (Köhler and Milstein, 1975).

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

institute, were used in preparation and titration of virus stocks.

2.1.3. VIRUS.

HSV-2 strain HG52 (Timbury 1971) stock was supplied by Mrs M. Murphy working in this Institute.

2.1.4. TISSUE CULTURE MEDIA AND SOLUTIONS

The Dulbecco's modified Eagle's medium (DMEM), BHK21 medium 10X stock (Glasgow modified Eagle's medium) and the supplements, sodium bicarbonate 7.5%, penicillin/streptomycin, glutamine, gentamycin, foetal calf serum, newborn calf serum and horse serum were purchased from Gibco Ltd., Paisley, Scotland, mycostatin, 10U/ml, Squibb and Sons, Hounslow, Middlesex , England and ciprofloxacine from Bayer, Newbury, Berkshire, U.K..

EF2-met , Eagle's minus methionine prepared at the Institute of Virology, Glasgow, was used to label cells with [^{35}S] L-methionine.

Versene: 0,6mM disodium ethylenediaminetetraacetate (EDTA) dissolved in phosphate buffer saline (PBS) containing 0.002% phenol red.

Trypsin: Trypsin (Difco Laboratories, West Molesley, England) was used as a 0,25% (w/v) solution in tris-saline.

Selection medium for hybridomas: 100X HAT stock solution contains 20ml of 10^{-2} M hypoxanthine, 3.2ml 10^{-2} M thymidine, 1.6ml 10^{-3} M aminopterin and 0.6ml of 10^{-2} M glycine. To disolve the aminopterin, 0.5ml of 1M NaOH is added for each 100ml of solution and the solution is titrated back to neutrality with 1M HCl.

2.1.5. CHEMICALS

Analytical or HPLC grade reagents were purchased from the following companies.

Aldrich Chemical Company, Gillingham, Dorset, England: 3-[cyclohexylamino]-1-propane-sulfonic acid (CAPS), N,N'-diallyltartardiamide (DATD), pyridine, vinylpyridine, tributylphosphine, N-heptane, ethylacetate.

Amersham International plc., Amersham, Buckinghamshire, England: [^{35}S]L- methionine >800 Ci/mmol, [^{14}C] labelled molecular weight markers, rainbow marker 3KD-46KD or 14KD-200KD range, with or without [^{14}C] label.

Applied Biosystems Inc.,Foster City, California, U.S.A.: trifluoroacetic acid, acetonitrile.

BDH Chemicals Ltd, England: acetone, ammonium bicarbonate, ammonium sulphate, bromophenol blue, disodium ethylenediamine tetraacetate (EDTA), disodium hydrogen phosphate(Na_2HPO_4), hydrogen peroxide, glycine, 2-mercaptoethanol, potassium chloride (KCl), sodium chloride (NaCl), sodium deoxycholate, sodium dodecyl sulphate (SDS), sodium dihydrogen phosphate (NaH_2PO_4), sodium hydroxide (NaOH), trichloracetic acid tris(hydroxymethyl)aminomethane (tris)

Biorad Laboratories, Richmond, California, USA.: acrylamide, ammonium persulphate, Coomassie brillant blue R-250, horseradish peroxidase (HRP) color development reagent (4-chloro-1-naphtol), N,N-methylene diacrylamide (BIS), protein assay kit (Bradford method), N,N,N',N'-tetramethylenediamine (TEMED)

Boehringer Mannheim, Lewes, East Sussex, U.K.: aldolase A (rabbit).

James Burroughs Ltd., London, England: ethanol.

Koch-Light Laboratories, Haverhill, Suffolk, England:

acrylamide.

May and Baker Ltd., Dagenham Essex. England: acetic acid (glacial), glycerol, hydrochloric acid, methanol.

Pharmacia LKB, Uppsala, Sweden: Pharmalyte pH range 3,5-9,5.

Sigma Chemical Company Ltd., Poole, Dorset, England: 2-aminoethanol, aminopterin, benzamidine, chymotrypsin, glutathione, hypoxanthine, N-chlorosuccinimide, phenanthroline, phenylmethylsulphonyl fluoride (PMSF), thioglycolic acid, thymidine, triton X-100, Staph. aureus V8 protease.

2.1.6. STANDARD BUFFER SOLUTIONS

2.1.6.1. GENERAL USE.

Phosphate buffered saline (PBS): 0.17M Nacl, 3.4mM KCl, 1mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.2

2.1.6.2. CELL LYSIS.

WF buffer: tris acetate pH 7.5 20mM, NaCl 20mM, EDTA 0.1mM, 2-mercaptoethanol 15mM.

2.1.6.3. DOT BLOTS AND WESTERN BLOTS.

Tris buffered saline(TBuS): 20mM tris, 500mM NaCl, adjust to pH. 7.5 with HCl.

TTBuS: Tris buffered saline with 0,1% Tween 20.

Blocking buffer: Tris buffered saline with 1% gelatin for the dot blots experiment or 3% gelatin for the Western blots experiments.

Antibody dilution buffer: TTBuS + 1% gelatin and 0.1%

Na azide.

Electrophoretic transfer of polypeptides to "Problott" or nitrocellulose: CAPS 10 mM is prepared as a 10 times solution, the pH. is adjusted to 11 with 2M NaOH and used with 10% methanol.

2.1.6.4 IMMUNOPRECIPITATION ASSAYS.

RIPA: 0.1%(w/v) SDS, 1%(w/v) sodium deoxycholate, 1%(v/v) triton X-100, 150mM NaCl, 10mM tris-Cl pH7.4.

Tris-saline (TS): 140mM NaCl, 30mM KCl, 280mM Na_2HPO_4, 1mg/ml glucose, 25mM tris-Cl pH7.4.

2.1.6.5. PEPTIDE MAPPING.

Rehydratation buffer: 0.1% (w/v) SDS, 1mM EDTA, 0.125M tris-HCl pH 6.8 or pH. 8.

Enzyme and fill-in buffer have the same composition as the rehydratation buffer but contain 10% and 20% glycerol respectively.

2.1.6.6. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

Electrophoresis tank buffer: 0.1% (w/v) SDS, 53mM glycine, 53mM tris.

Stacking gel buffer: 0.1%(w/v) SDS, 0.125M tris-Cl pH 6.7

Separating gel buffer: 0.1%(w/v) SDS, 0,375M tris-Cl pH 8.9

Electrophoresis sample buffer: 2%(w/v) SDS, 10% (v/v) glycerol, 700mM 2-mercaptoethanol, 50mM tris-Cl pH 6.7, 0.001% (w/v) bromophenol blue.

2.1.7. IMMUNOLOGICAL REAGENTS.

Formalin fixed Staph. aureus type A cells (Pansorbin*) was purchased from Calbiochem, La Jolla, California, USA.

Rabbit anti-goat and sheep anti-mouse IgG, linked to horseradish peroxidase from Sigma Chemical Company ltd, Poole, Dorset, England.

Sheep anti-mouse gamma-globulin from Scottish Antibody Production Unit (SAPU) Law Hospital, Carluke, Scotland.

2.1.8. MISCELLANEOUS MATERIALS.

1.5 ml reaction vials and pipette tips: Starsted Ltd., Leicester, England.

Falcon plastic centrifuge tubes, 15ml and 50ml; Falcon rotating plastic 850 cm2 culture bottles: Becton Dickinson Labware, New Jersey, USA.

Plastic tissue culture flasks, 96 and 24 wells microtiter plates: Nunclon Ltd., Roskilde, Denmark.

Plastic petri dishes, 50mm, 90mm, 140mm: Sterilin Ltd., Feltham, Middlesex, England.

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

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

Problott transfer membranes: Applied Biosystems Inc.: Foster City, California, USA.

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

EN3HANCE: New England Nuclear, Boston, Massachusetts, USA.

XS1 autoradiographic film: Kodak Ltd., London, England.

RPN 213 mouse antibody screening kit. (Single vacuum manifold, membrane bottomed plates), Enhanced chemiluminescence Western blotting detection reagents: Amersham International, Amersham, England.

Nitrocellulose and micro sample filtration manifold (slot blott apparatus): Schleicher & Schuell, Dassel, Germany.

2.1.9. SEPARATION SYSTEMS.

Mini-Protean II electrophoresis cell (Mini-gel Kit) and Mini Trans-Blot Cell: Biorad Laboratories, Richmond, California, USA.

FPLC system, Mono Q HR 5/5 prepacked column (1ml anion exchange Mono Q column), Mono P HR 5/20 prepacked column (chromatofocusing Mono P column), Mono S HR 5/5 prepacked column (1ml cation exchange Mono S column) : Pharmacia LKB, Uppsala, Sweden.

HPLC system AB 130A, 1mm microbore column filled with Brownlee Aquapore RP 300 C8 substituted resin: Applied Biosystem Inc., Foster city, California, USA.

Centrifuges Sorvall RT6000B, RC5B and OTD50: DuPont U.K., Hitchin, Herts, U.K.

Electrophoresis of large gels were carried out using "Institute made" kits using glass plates of the following sizes 160mm x 190 mm, 220mm x 190mm and 265mm x 165mm. Spacers were either 1.5 or 0.75mm thick.

2.2. METHODS

2.2.1. CELL CULTURES

2.2.1.1. RAT EMBRYO CELLS

Rat embryos, 16-19 days old, were separated from the foetal annexes, eviscerated, minced finely and incubated with 150 ml of 0.05% trypsin at 37°C, 30 minutes (min.), with gentle stirring. The supernatant was removed, and 5ml of foetal calf serum was added. The embryos were incubated a second time with trypsin, and the supernatants pooled. The cells were centrifuged at 500G for 5 min., and resuspended in Dulbecco's modified Eagle's medium supplemented with 0.11g of sodium pyruvate supplemented with 5% foetal calf serum, 4mM/l glutamin, 100U/ml of penicillin, 100ug/ml of streptomycin, 10U/ml of mycostatin. Cells were counted in an haemocytometer, and $1x10^8$ cells were seeded in each roller bottle. Cells were incubated at 37°C, with 5% CO_2, until a confluent monolayer was obtained. Cells were passaged by washing the monolayer twice with 20ml of Versene, then 10ml of versene and 10ml of trypsin. When the cells are detached from the surface of the flask, the cells are resuspended in a small volume of media, centrifuged at 500G for 5 min., resuspended in fresh medium, and seeded as required e.g. $2x10^6$ cells per 50mm plate.

2.2.1.2. Bn5T CELLS

Bn5T cells were cultured using the same medium and passaged using the same procedure as for RE cells. Bn5T cells were incubated with 5% CO_2. To obtain the mass of cells required by the project, the cells were grown in 850 cm^2 roller bottle. On confluence the monolayer was washed three times with PBS and the cells scraped into 10ml of PBS, and transfered to 50ml Falcon tubes. A protease inhibitor mixture containing 300mg benzamidine, 999mg

phenanthroline and 340mg phenylmethylsulfonyl fluoride in 10 ml of ethanol was added at a ratio of 1%, and the cells were sedimented for 10 minutes at 3,000g at 4°C. Cells were stored as dry pellet at -70° C.

2.2.1.3. MYELOMA CELLS

Myeloma cells, were thawn from frozen stocks, and grown for at least a week before using for fusion at 37°C and with 5% CO_2. The day before the fusion the cells were counted and seeded at a density of $4x10^7$ in a fresh large flask, with 100ml of fresh media. The medium used was Dulbecco's modified Eagle's medium supplemented with 0.11 g Na pyruvate/l, 4mM/l glutamine, 10% foetal calf serum, 10% horse serum, 100 u/ml of penicillin, 100ug/ml of streptomycin and 0.1mg/ml gentamycin.

2.2.1.4. BHK CELLS.

BHK cells were grown in ETC10: Glasgow modified Eagle's medium supplemented with 10% newborn calf serum, 4mM/l glutamine, 100U/ml of penicillin, 100ug/ml of streptomycin, 10% tryptose phosphate broth and 24uM/ml sodium bicarbonate.
BHK cells were incubated at 37°C in an atmosphere containing 5% CO_2

2.2.2. PRODUCTION OF VIRUS STOCKS.

Stocks of HSV-2 strain HG52 were prepared by infecting subconfluent BHK cells in roller bottles at a m.o.i. of 0.003 plaque forming unit (p.f.u.) per cell. Cells were incubated in 20 ml ETC10 at 31°C until extensive cytopathic effect (c.p.e.) had developed. Infected cells were harvested into medium by shaking and were pelleted by centrifugation at 1000 r.p.m. in a Sorvall RT6000B centrifuge 15 min. at 4°C. Sterility checks were performed by streaking virus stocks on blood agar plates. Cell associated virus was released by sonicating the cell pellet

in 5ml of ETC10 and the cell debris pelleted by centrifugation at 2000 r.p.m. in a Sorvall RT6000B centifuge 10 min. 4°C. The cells debris were sonicated again, the supernatants pooled and stored in 1ml aliquots at -70°C.

2.2.2.1 TITRATION OF VIRUS STOCKS.

Virus was titrated on subconfluent monolayers of BHK cells in 50mm petri dishes. Serial ten-fold dilutions of virus were made in PBSA containing 5% newborn calf serum. Inocula of 0.2ml were added to BHK monolayers from which grwth medium had been removed, After adsorption of virus for 1h. at 37°C cells were overlaid with ETC10 containing 1% methylcellulose. One set of plates were incubated at 37°C for 2 days and another set at 31°C for 3 days. Monolayers were then overlaid with Giemsa stain for 10 min., the stain wased off with water and virus plates counted under a dissecting microscope.

2.2.3. PREPARATION OF ANTISERA.

2.2.3.1. TUMOUR BEARING SERUM (TBS)

Bn5T cells ($5x10^7$) were injected into the back of eight weeks old anesthetised Hooded Lister rats. Up to three months could be required for a tumour to appear. The rats, anesthetised with chloroform, were bled by cardiac puncture. The serum was stored at -20°C.

2.2.3.2. MOUSE ANTISERA.

To immunise mice three preparations of the antigen were used.

Bn5T cells ($2x10^7$) were injected in the mouse peritoneal cavity. in 0.5 ml of PBS. 2 mice were injected with cells sonicated for 10 seconds, and 2 mice with living cells.

Proteins immunoprecipitated by mouse antisera to Bn5T cells were injected in the mouse peritoneum as immunecomplexes containing mouse antisera, sheep anti-mouse serum, and Pansorbin*. The method used for the preparation of this complex is described in section 2.2.5.

In all cases the mice were injected three times at one month intervals. The mice were bled from the eye to test their immune status. This was kindly performed by Dr Ann Cross of this Institute (The author is not licensed for this procedure). If the titre of the antibody was satisfactory, the animal was selected for a spleen cells fusion experiment and the animal was boosted 5 to 7 days before the fusion by another injection.

2.2.4. IN VIVO RADIOLABELLING.

Bn5T and RE cells were grown to subconfluence, the culture media was removed and EF-2met medium was added. The cells were incubated at 37°C for 4 hours. The medium was removed, and EF-2met medium containing 50 uCi/ml of [^{35}S] L-methionine was added.

The following volumes of EF-2met medium with 50 uCi/ml of [^{35}S] L-methionine were used: 50 mm plates 2ml, 90mmplates 5ml, 140mm plates 12ml and 850 cm2 roller bottles 20ml.

After 17 hours incubation at 37°C the cells were washed three times with PBS and scraped in the buffer used for the i.p. experiment. Cells from 140mm, 90mm and 50mm plates were harvested with 1ml, 0.5ml and 0.2ml of buffer respectively. Cells to be used for protein purification, were harvested in PBS sedimented in 1.5ml tubes and stored dry at -70° C.

Cells were lysed by sonication, left on ice 30 minutes, and and the resulting mixture clarified by centrifugation, 15 minutes at 13,000 r.p.m. in a MSE Microcentaur microfuge. A protease inhibitor mixture containing 300mg benzamidine, 999mg phenanthroline and

340mg phenylmethylsulfonyl fluoride in 10ml of ethanol was added at a ratio of 1% before storing the supernatant at -70° C.

2.2.5. SCINTILLATION COUNTING.

An aliquot of 1ul was spotted on a filter paper disk. The protein was precipitated by two washes of three minutes in 5% trichloroacetic acid, one wash in acetone, and dried under a heat lamp. The filter paper disk was placed into a scintillation vial with four millilitres of scintillation fluid, and counted for one minutes on an Intertechnique scintillation counter.

For a protein profile, $5x10^5$ counts per minute (c.p.m.) were required, the amounts used in i.p. experiment are shown in the following section.

2.2.6. IMMUNOPRECIPITATIONS OF THE POLYPEPTIDES.

For a standard i.p. $4x10^6$ c.p.m were used and to obtain bands intense enough for peptide mapping $4x10^7$ c.p.m. were used. The experiments were set up as follow.

Counts	$4x10^6$	$4x10^7$	$4x10^6$	$4x10^7$	$4x10^6$
TBS	10 ul	50 ul			
Mouse antiserum			10 ul	20 ul	
hybridoma culture supernatant					50 ul
	Incubate 1 h. 4°C				
Sheep anti-mouse			5 ul	10 ul	10 ul
	Incubate 1 h. 4°C				
Pansorbin*	60ul	100ul	60 ul	100 ul	60 ul
	Incubate 1 h. 4°C				

Spin 10 min. at 13,000 r.p.m. in a microfuge.

Wash twice with RIPA buffer.

Wash once with tris saline buffer.

Add 50ul of sample buffer

Heat 10 minutes at 70°C

Load on SDS-PAGE

The immune complexes which were injected into the mice used $2x10^7$ Bn5T cells, which were incubated 1 h. with 20ul of mouse antiserum, 1 hour with 10ul of sheep anti-mouse serum and and 1 hour with 100ul of pansorbin*. The immune complexes were resuspended in 0.5ml PBS and injected intra-peritoneally into the mouse.

2.2.7. ELECTROPHORESIS ANALYSIS OF POLYPEPTIDES.

2.2.7.1. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

The Laemmli system was used (Laemmli, 1970). Gel slabs of the following concentration were used: 7.5%, 9%, 10%, 15% and 16.5%. Acrylamide was cross-linked with N-N'-methylenebisacrylamide, 1 part in 40 (w/w). Gels were polymerized by addition of 0.04% (w/w) of ammonium persulphate and 0.04% (w/v) of TEMED. Gels were overlaid with butanol to obtain a flat surface. Butanol was removed by washing with deionized water, the stacking gel was poured immediately afterwards or the gel was overlaid with 1 X stacking gel buffer and stored at 4°C.

The stacking gel which contained 5% acrylamide in stacking gel buffer, was crosslinked with 1 part in 40 (w/w) N-N'-diallyl-tartar-diamide and polymerized as above. Wells were formed with teflon combs.

Tank buffer was prepared from a 10 X stock.

Samples were disolved in sample buffer, heated 10 minutes at 70°C before loading.

To minimize heating, gels were run at 4°C, except gels for enzymatic digestions. Large gels were run at fixed intensity, the maximal intensity used were according the size of the gels:

160 mm X 190 mm X 1.5 mm : 40 mA.
220 mm X 190 mm X 1.5 mm : 60 mA.
160 mm X 190 mm X 0.75 mm : 30 mA.
Mini-gels were run at fixed voltage : 200 V.

Proteins were fixed by 50% methanol, 7% acetic acid in water (v/v)(1 h.), the gel were then washed with 5% methanol, 7% acetic acid in water (1 h.) and water (1 h.) and dried. Later the gels were simply dried without fixing. Alternatively the gels were fixed (1 h.), soaked in EN^{3}HANCE (1 h.), washed 20 min. maximum in several changes of water and dried at 80°C under vacuum onto a sheet of 3MM filter paper.

Gels were autoradiographed with flashed Kodak X-Omat XS-1 films which were exposed at -70°C

2.2.7.2. COOMASSIE BLUE STAINING.

Gels were stained in a solution of 0.1% Coomassie Blue R-250 in methanol and acetic acid in water (50/7/43, v/v/v), 1 h maximum; destained by several washes in methanol and acetic acid in water (5/7/88, v/v/v), at 37°C to speed up the process.

When proteins were prepared for digestion with Staph. aureus V8 protease and sequencing of the fragments, the gels were stained with a 0.1% solution of Coomassie Blue R-250 but the acetic acid and the methanol concentration was modified to 1% and 10% respectively, and stained for a maximum of 15 min. Gels were destained with the same solution minus dye, until the bands were visible. The 40KD band is then excised and equilibrated with the rehydratation buffer used for peptide mapping for 30 min. before storage at -70°C.

2.2.7.3. PEPTIDE MAPPING.

Enzymatic cleavage was carried out by the method published by Cleveland et al. (1977) and used for Staph.

aureus V8 protease and chymotrypsin digestions. The polypeptides are separated on a first acrylamide gel. The gel may be stained with Coomassie blue R-250, but Enhance* cannot be used (the resolution of the generated peptides is poor). The gel was exposed overnight, and the bands that were visible on the film contain enough radio-activity to produce detectable peptides. Marks with radio-active dye allowed a perfect alignment to cut out the bands. Bands were rehydrated in rehydration buffer pH. 6.8 for an analytical peptide map or pH. 8 to generate larger peptides for N-terminus sequencing, and inserted into the wells of a second gel, taking care to avoid trapping any air bubble. The pH was raised to 8 because at pH.6.8 the enzyme cleaves at aspartate and glutamate residues. At pH 8.1 the specificity may be restricted to the glutamate residues (Aitken et al. 1988). This modification was used in the department for the digestion of the U90. Larger peptides were generated and were successfully sequenced (M. Grassie, personal communication). The stacker of this gel was 5 - 7cm long from the bottom of the well, and the concentration of the resolving gel is 15% or 16.5%. Analytical gels were 1.5mm thick, to facilitate electroblotting, 0.75mm thick gels were used. The gel slice was overlaid with 10ul of rehydration buffer containing 20% glycerol (v/v), and the gel kit was filled with tank buffer. The stock solution of enzyme is prepared by dilution in rehydration buffer containing 10% glycerol, 20ul of the enzyme solution is added to each well.

The electrophoresis was performed as usual, but at room temperature, and the current never exceeding 30 mA. The current was turned off for 1 h. when the dye front was approximatively at 5mm from the bottom of the stacker gel to allow the digestion of the polypeptide by the enzyme.

Chemical cleavage by N-chlorosuccinimide (NCS) was performed as published by Lischwe and Ochs (1982). A solution is made the day of the experiment with 10ml of acetic acid, 10 g of urea and 10ml of water (AUW). The band cut out of the gel was rehydrated for 10min. in AUW at room temperature, the AUW solution was changed once, with

occasional agitation. The gel slice was equilibrated with 3ml of AUW solution containing N-succinimide (2mg/ml), on a rotating wheel, for 20 min. at room temperature. To neutralize acidity the gel slice was equilibrated with 10ml of trisHCl pH. 8, on a rotating wheel. The gel slice was loaded into the well of a 15% gel and overlaid with 20 ul of sample buffer and the gel was run as usual without interruption.

The gels were fixed, enhanced and exposed for three weeks in the first instance.

2.2.8. PROTEIN ASSAY.

The Bio-Rad Protein Assay kit uses the shift of the maximum absorbance of Coomassie Brillant Blue G-250 from 465 nm to 595 nm when protein binding occurs. The usefulness of the method was first pointed out by Bradford (1976). The stock solution is diluted 1 in 5 with deionized water and filtered through Whatman n°1 paper. Five millilitres of the reagent were dispensed in assay tubes. Standards solutions containing 0.14, 0.28, 0.42, 0.56, 0.70 0.98 and 1.4 mg/ml of proteins were prepared. 100ul of standards, sample buffer and unknown are added to 5 ml of reagent. The optical density at 595 nm is read after 5 minutes. A standard curve is drawn and the unknowns are plotted against the standards.

For the micromethod the reagent is used undiluted. The concentration of the protein standards are 1 to 25 ug. 0.8 ml sample is added to 0.2 ml of concentrated reagent.

2.2.9. CELL FRACTIONATION.

Bn5T cells were fractionated, using a method suggested by Dr M. Finbow (Beatson Institute for Cancer Research, Glasgow).

After washing 3 times with PBS, cells were scrapped in 5mM tris-HCl pH 7.4, (One millilitre per 140 mm petri dish).

The cells were broken down in a Dounce homogeniser, until

no more than 90% of the cells were lysed as viewed by phase contrast microscopy and taking care to keep the nuclei intact. The lysate was centrifuged at 1500G (centrifuge Sorval RT6000D 3000 r.p.m.), for 5 min. at 4°C to sediment the membrane fragments and the nuclei: the supernatant is referred to as the cytosolic fraction. The pellet containing the membrane fragments and the nuclei were resuspended in 30% sucrose. The membrane fragments and the nuclei were further separated by centrifugation at 150,000G (centrifuge Sorval OTD50, rotor AH650, 35.000 r.p.m.), for 30min, at 4°C on a 60% sucrose cushion. The membrane fragments floated on top of the 60% sucrose cushion and were collected with a syringe. The pellet was termed the nuclear fraction. Both fraction were washed with 5mM $NaHCO_3$, to remove the sucrose and resuspended in RIPA buffer. The cells are thus fractionated in cytosolic, membrane and nuclear fractions.

2.2.10. PURIFICATION EXPERIMENTS.

2.2.10.1. CELL LYSIS.

Cells were frozen, thawed and lysed with a Dounce homogeniser (40 strokes) in the buffer used by Welsh and Feramisco (1987), to purify the heat shock protein 70 (HSP70): 20mM tris acetate, 20mM NaCl, 0.5 mM EDTA, and 10 mM mercaptoethanol (WF buffer). Cells were sonicated in a sonibath for 5 minutes. The crude extract was centrifuged at 13,000 r.p.m. for 10 min. at 4°C in a MSE Microcentaur microfuge for the pilot experiments or at 10,000 r.p.m., 10 min in a Sorvall SS34 rotor for the preparative experiments. The supernatant was used for the ammonium sulphate fractionation.

2.2.10.2. AMMONIUM SULPHATE FRACTIONATION.

Ammonium sulphate was first ground with a pestle and a mortar. It was added to the cell lysate with continuous stirring at room temperature. After complete solubilisation, the solution was left on ice with stirring for at least half

an hour. Proteins were precipitated by the following percentage of saturation: 30%, 50 %, 70 % for the pilot experiments, 30 %, 60 %, and 80 % for the preparative experiments. After each cut the solutions were cleared by centrifugation at 13,000 r.p.m. for 10 min. at 4°C in a MSE Microcentaur microfuge for the pilot experiments or at 10,000 r.p.m., 10 min in a Sorvall SS34 rotor (10,000G) for the preparative experiments. The proteins soluble in a 70% saturated solution were desalted by dialysis against several washes of 10mM NH_4CO_3. The pellet of the 60-80 % pellet was desalted using a PD 10 column equilibrated with 50mM tris HCl buffer.

2.2.10.3. DESALTING AND CONCENTRATION OF PROTEINS.

The following methods were used to desalt and or concentrate proteins:

2.2.10.3.1. Dialysis.

The protein solution was loaded in dialysis tubing and proteins were dialysed against at least 5 changes of 2.5 litres of 10mM NH_4CO_3. The proteins were concentrated with polyethylene glycol (PEG) 6000 and/or by freeze drying.

2.2.10.3.2. Microdialysis unit.

This system allow desalting of 28 samples which are separated from the 10mM NH_4CO_3 buffer by a dialysis membrane. A peristaltic pump was used to pump the buffer through the microdialysing unit. Samples were dialysed against 2.5 litres of NH_4CO_3 buffer. This method was used to analyse the fractions from the FPLC experiments.

2.2.10.3.3. Gel filtration.

A PD10 column contains 10ml of sephadex G25. The column is first equilibrated with 15ml of the chosen buffer. The volume of the sample was made up to 2.5ml and loaded onto

the column. The polypeptides were eluted free of salt by 3.5ml of the chosen buffer.

2.2.10.3.4. Ultrafiltration.

The centricon 30 tube was used for ultrafiltration. This tube is closed by an ultrafiltration membrane retaining protein having a molecular weight over 30,000 Dalton. The sample (up to 2ml) is put in the top chamber and the small molecules are forced into the lower chamber by centrifugation. The acceleration force used is limited to 7500G, as obtained by a speed of 7500r.p.m. in a Sorval SS34 rotor, a 2ml sample was concentrated with this fixed angle rotor to a volume of 30ul in 20 minutes. The tube was inverted and the concentrated sample was recovered by a 1 minute centrifugation in a cup. This system allowed concentration and desalting at the same time.

2.2.10.3.5. TCA precipitation.

Protein were precipitated by adding trichloroacetic acid to a proportion of 5%. Fifty microlitres of sample buffer made of two millilitres 1M trisbase, 1.5ml glycerol, 0.5ml of 10% SDS and 0.5ml of water was added to each sample followed by 5ul of 1N HCl. The sample were heated as described in section 2.2.6.1 before loading on a gel.

2.2.10.4. ANION EXCHANGE CHROMATOGRAPHY AT pH.8.

Dr A. Darling, of this Institute suggested the following method to separate the 40KD protein using the Mono Q prepacked HR 5/5 column (1ml Mono Q). The polypeptides were resuspended in 0.5ml of buffer A (50mM trisHCl pH.8), and insoluble material pelleted by centrifugation in a MSE Microcentaur microfuge 5 min, 13,000 r.p.m. at room temperature, before loading on the Mono Q column. The void volume of the column was set at three millilitres, the polypeptides were eluted by a 13ml gradient of 50% buffer B (buffer A + 1M NaCl) and the 1ml Mono Q column was washed by

three milliltres of 100% buffer B. In scaled up experiments the void volume was increased to 6ml, but the volume of the gradient remained the same.

As in all chromatography experiments described in this thesis 1ml fraction were collected which were desalted and concentrated with the microdialysis unit or by TCA precipitation before loading onto a SDS-PAGE. The column flow rate used was 1.5ml/min..

2.2.10.5. CATION EXCHANGE CHROMATOGRAPHY.

The Mono S prepacked HR5/5 column was used (1ml Mono S column). The void volume of the anion exchange chromatography at pH.8 step was desalted by dialysis and concentrated by freeze drying. The proteins were resuspended in 500 ul of buffer G (50mM sodium phosphate buffer, Na_2HPO_4 - NaH_2PO_4 pH 7.2). The protein was loaded on the 1ml Mono S column. The void volume was set at 3ml, the proteins were then eluted with a 13ml gradient of buffer H (Buffer G + 1M NaCl). Any remaining proteins were finally eluted by 3ml of buffer H.

2.2.10.6. PREPARATIVE ELECTROFOCUSING.

Electrofocusing is a method of separating proteins according their pI. The machine Rotofor* is sold for preparative electrofocusing. This machine consists of a rotating horizontal column divided by several porous nylon filters with an electrode at each end.

A 5.5ml sample (polypeptides eluting in the void volume of the anion exchange experiment at pH.8), in buffer A, together with 1.5ml of ampholines (Amphoteric molecules used as buffer), 6ml of glycerol and 45ml of H_2O was loaded in the column. When an electric field is applied to the column, a pH. gradient forms, and the proteins migrate toward the part of the column whose pH. equals their pI.. The nylon diaphrams limit the diffusion of the proteins and define chambers which can be harvested separately.

2.2.10.7 ANION EXCHANGE CHROMATOGRAPHY AT pH.9.5.

The buffer containing the polypeptides from the void volume of the anion exchange chromatography at pH.8. was changed to buffer C (20 mM Ethanolamine pH 9.5) using a PD10 column. The polypeptides were loaded on the 1ml Mono Q column in 3,5ml buffer C. The void volume was set at 6ml, the polypeptides were eluted with a 13ml linear gradient of 50% buffer D (buffer C + 1 M NaCl). The column was then washed with 3ml of 100% buffer D.

The gradient was linear for the first experiment. To obtain a better separation of the polypeptides, step gradients of 3ml of 2.5%, 5% and 7.5% of buffer D were introduced between the void volume and the linear gradient of buffer D. After four preliminary experiments the gradients were fixed as follow; 0-5ml void volume, 6-9ml 2.5% buffer B, 10-13ml 5%, 14-17ml 7.5%, 17-32ml linear gradient of 50% buffer D and 32-35ml 100% buffer D.

2.2.10.8. CHROMATOFOCUSING.

The Mono P HR5/20 prepacked column was used (Mono P column). The sample was loaded onto the Mono P column in 7ml of 25 mM diethanolamine pH.9 and eluted by a linear pH. gradient created between pH. 9 and pH. 7 with 34ml of buffer. This buffer consisted of 1ml of Pharmalyte 8-10.5 and 5.2 ml of "polybuffer 96 HCl, pH. 7.0", in 100ml of water, and was titrated to pH.7 with HCl.

2.2.11. PREPARATION FOR SEQUENCING.

2.2.11.1. REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC).

The Aquapore RP-300 column which is a silica based column, and therefore unstable at a pH. above 8. The ethanolamine buffer pH. 9.5, was changed to buffer E (0.1% TFA in water), and each fraction of the void volume were concentrated using a Centricon 30 device. A 50ul loop was

used, and larger volume were loaded in 50ul aliquots. The polypeptides were loaded on the column in buffer E and were eluted by a gradient of buffer F (60% acetonitrile in buffer E). First a linear gradient of 0 to 100% buffer F in 45 minutes was used then to improve the separation the gradient was modified as follow.

t (in minutes)	%B
0	0
3	0
8	45
45	75
48	100
53	100
55	0

2.2.11.2. STAPH. AUREUS V8 PROTEASE DIGESTION OF THE 40KD POLYPEPTIDE TO OBTAIN INTERNAL AA SEQUENCE DATA.

Coomassie blue staining and Staph. aureus V8 protease digestion were described in sections 2.2.6.2. and 2.2.6.3..

2.2.11.3. ELECTROBLOTTING.

Proteins separated by SDS-PAGE were electro-eluted onto Problott* membrane using 10mM CAPS buffer pH 11 in 10 % methanol, according the instruction of the manufacturer Bio-Rad: The membrane was soaked for a few seconds in methanol, and then transfered into a dish containing the CAPS buffer. The transfert was performed at 50 Volts for half an hour with a 0.75mm thick gel or one hour for a 1.5mm thick gel, using a Bio-Rad mini-gel transfert kit. Rainbow markers are used to check the transfer efficiency. For the initial experiments, the gels were also stained with Coomassie blue to make sure that no protein was remaining in the gel. The membrane was then rinsed in distilled water, soaked for a few seconds in methanol, stained 1 min. in 0.1 % Coomassie blue in 40 % methanol and 1% acetic acid in water. The membrane was destained in 50 % methanol in water, the membrane was extensively washed with distilled water before cutting the bands of interest.

✳ Dr Ailsa Campbell from the Department of Biochemistry was also consulted.

2.2.11.4. S-PYRIDYLETHYLATION OF THE PROTEIN.

The method published by Amons, R. (1987) was used. Pyridylethylation was performed in a vial with a constriction. At the bottom of the vial a freshly prepared mixture of 100ul of water, 100ul of pyridine, 20ul of 4-vinylpyridine and 20ul of tributylphosphine was introduced. The sample blotted on Problott, as described in the previous section was suspended over the mixture, taking care that the membrane did not touch the liquid reagent. Oxygen was removed by injecting some helium and the vial was closed. The vial was incubated two hours at 60°C. The membrane was then washed in the following solvents: *n*-heptane, *n*-heptane/ethyl acaetate (2:1, v/v), ethyl acetate and dried.

2.2.12. METHODS USED IN THE ATTEMPT OF RAISING MONOCLONAL ANTIBODIES.

2.2.12.1. FUSION.

Splenocytes from the immunized mice were fused with myeloma cells according a protocol devised by Dr A. CROSS of this Institute*. The mice were immunized and boosted by intra-peritoneal injection of $4x10^7$ cells, five to seven days before the fusion. Mice were anesthetized, exsanguinated and the serum stored.

The spleen was dissected out of the mice and the capsule is teased apart with 18 gauge needles in a few millilitres of medium (The medium referred to in this section is the medium used to grow myeloma cells: Dulbecco's modified Eagle's medium supplemented with 0.11 g Na pyruvate/l, 10% foetal calf serum, 10% horse serum, 100 ug/ml of penicillin, 100ug/ml of streptomycin and 0.1mg/ml gentamycin). The suspension was transfered into a 15ml Falcon tube and left to rest for two to three min. so that clumps and others debris settle. The supernatant was saved and the cells sedimented (500 r.p.m., 10 min). The splenocytes were resuspended in serum free medium and counted. The myeloma

cells were harvested, centrifuged resuspended in serum free medium and counted.

The proportion used for the fusion was 10 spleen cells for 1 myeloma cell. Cells were mixed in a 50ml Falcon tube, and 1 ml of 50 % PEG 1500 in serum free medium was added and mixed gently. After 1 min., 1 ml of serum free medium was added, then at 1 min. intervals, 2, 4, 8, 16ml of serum free medium was added.

The cells were sedimented, and resuspended in medium. Hybridoma were selected by adding 1ml of HAT stock solution per 100ml of medium. They were diluted to a concentration of $3.5x10^6$ cells per ml. One hundred microlitres were dispensed in each well of a 96 well flat bottomed plate.

2.2.12.2. CULTURE OF THE HYBRIDOMA CELLS.

Hybridoma cells were incubated at 37°C with 4% CO2. After 5 to 7 days, the plates were examined for colonies of large cells. After 7 to 10 days 0.1ml of medium was added to each well, after 14 days the medium was changed, aminopterin was removed and medium with HT was provided, after a month hypoxanthine and thymidine was removed from the medium. Growing colonies were first transfered to 24 wells plates and then to small flasks. Supernatant was saved from wells in which the colony occupied 1/3rd of the well for testing.

2.2.12.3. SCREENING OF THE HYBRIDOMA.

2.2.12.3.1. Immunoprecipitation experiments.

I.p. experiments were set up as described in section 2.2.5., $4x10^6$ c.p.m. of Bn5T cell extracts labelled with [^{35}S] L-methionine with 50ul of the hybridoma culture supernatant.

2.2.12.3.2. Dot blots.

The hybridoma culture supernatants were screened using the mouse antibody screening kit RPN 213 (Amersham

International). This kit provides membrane bottomed plates which can be emptied by pulling solutions through membrane under vacuum. The protocol derived from the manufacturer instructions was the following:

Step 1: Add 50ul of cell extract to the wells in PBS + 0.05% SDS. Incubate for 15 min, and pull through membrame under vacuum.

Step 2: Wash membrane once with tris buffered saline with 0.05% Tween-20 (TTBuS).

Step 3: Block membrane with 1% gelatin in TBuS, for 10 minutes. Wash membrane 3 times with TTBuS. Dry off the bottom of the membrane bottomed plate.

Step 4: Incubate with mouse antibody or 50 ul of hybridoma supernatants for 15 minutes. Wash membrane 3 times with TTBuS and dry off the bottom of the membrane bottomed plate.

Step 5: Incubate with horseradish peroxidase (HRP) labelled anti-mouse antibody diluted in TTBuS, Wash membrane three times with TBuS and dry off the bottom of the membrane bottomed plate.

Step 6: Incubate membrane in substrate solution. Wash membrane bottomed plate.

Step 7: Interpret results, a dark purple colour develop in the positive wells.

The peroxidase labelled anti-mouse antibody used was diluted 1/2000 following the instruction of the manufacturer and after testing the validity of this dilution against negative and positive controls.

The substrate solution was prepared by dissolving 60mg of HRP colour reagent (Bio-Rad) in 20ml ice cold (high grade) methanol (solution A). Immediately prior to use, add 60ul of ice cold 30% H_2O_2 to 100ml of room temperature TBuS (solution B). Mix A and B, uses immediately.

The optimal experimental conditions were determined by a checker-board titration carried out using the method recommended by the manufacturer.

The growth of four roller bottles ($8x10^8$ cells) was used to make the antigen solution. The cells were lysed in PBS buffer with 0.05% SDS. This buffer was used because PBS had previously been used before to extract the TBS:40 (see section 3.4.1), it extracts also the U90 albeit poorly. SDS at the concentration of 0.05% was added to improve the extraction of the U90, and this concentration does not prevent binding of the protein to the blotting membrane according the information supplied by the manufacturer (Amersham). The capability of this buffer to extract the U90 and the 40KD was tested by i.p. with both TBS and mouse antiserum.

The concentration of the U90 and the 40KD could not be measured. Instead, the protein concentration of the antigen solution was measured to determine the optimum condition of the experiments and to reproduce them. The Bradford method of protein determination was used, the result was 2.88 mg/ml.

Doubling dilutions of the antigen solution were prepared from 1/2 to 1/64. One hundred microlitres of the neat solution loaded in row A and double dilutions were loaded in rows B to G. Row H was used as the negative antigen control and PBS + 0.05% SDS was loaded in it.

A mouse antiserum was used as antibody, because no positive supernatant was available. Double dilutions of mice antiserum were made in tris buffer saline, from 1/4 to 1/2000, These dilutions were loaded in row 1 to 10. Row 11 was used as negative antibody control and received tris buffer saline.

A 1/2000 dilution of peroxidase labelled anti-mouse antibody was prepared and 100 ul were loaded in each well. After 15 min. incubation, the antibody was removed and the presence of antibodies against Bn5T polypeptides was revealed by adding 100 ul of substrate solution containing 4-chloro-1-naphtol.

The results of the experiments are presented in figure

Figure 2.1a and b. Checker-board titration.

figure 2.1a: plan of the experiment.

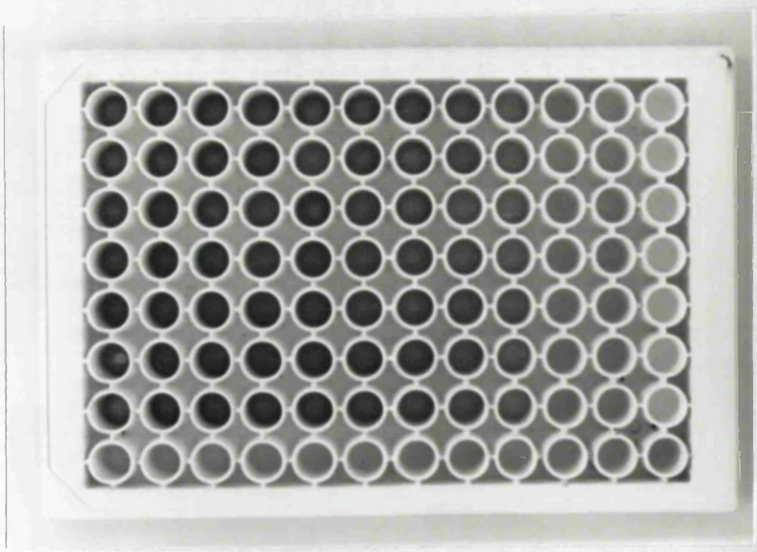
Rows 1-10 : antibody dilutions.
11 : PBS + 0.05% SDS.
Rows A-G : antigen dilutions
H : Tris buffered saline.

figure 2.1b: photograph of the microtitreplate, positive results are visualized by addition of alpha chloro-4-naphthol.

FIGURE 2.1a.

	1 1/4	2 1/8	3 1/16	4 1/32	5 1/64	6 1/128	7 1 256	8 1 512	9 1 1024	10 1 2048	11 C	12
A 1												
B 1/2												
C 1/4												
D 1/8												
E 1/16												
F 1/32												
G 1/64												
H C												

FIGURE2.1b.



2.1.

After processing the PVDF membranes, a positive reaction was detected in most wells. The row H was the negative antigen control, the row 11 was the negative antibody control: Both are negative as expected.

In row 10 (1/2000 dilution of the antibody), the result is negative irrespective of the concentration of antigen.

In row 9 the reaction is weak in well 9 (1/64 dilution of the antigen).

From these results it was decided to use a 1/32 dilution for the cells. As far as the antibody was concerned, the supernatant of the hybridoma would first be used neat and later a dilution would be made to test for positivity.

2.2.13. WESTERN BLOTTING EXPERIMENTS.

Bio-Rad mini gel kits were used for these experiments.

Samples were run on a 9% SDS-polyacrylamide gel. Proteins were then transferred to nitrocellulose using CAPS buffer pH.11, with 10% methanol, the voltage was set at 50 Volts for 1 h. 45 min.

The gel was blocked with 3% gelatin in TBuS for two hours or overnight at 37°C. The gel was washed 3 times with TTBuS.

The mAspAT antibody was diluted 1/1000 in TTBuS with 1% gelatin and 0.01% sodium azide. The blot was incubated 1 hour at 37°C and then washed 10 times with TBuS

The HRP conjugated rabbit anti-goat antibody was diluted in TBuS (1/1000) and incubated for 1 hour at RT, after which the blot was washed 3 times with TBuS (no Tween was added to the washing buffer).

The colour reagent (see section 2.2.11.3.) was then added. Once the colour reagent has developed the blot was washed in distilled water, and the results recorded.

To increase the sensitivity a enhanced chemiluminescence kit marketed by Amersham was used. Horseradish peroxidase catalyzes the oxidation of luminol in the presence of

hydrogen peroxide.

An equal volume of the two detection solutions of the kit are mixed. The excess buffer is washed off the blots and the blots are transferred to petri dishes. The detection reagent is directly added to the blot on the surface carrying the protein.

Incubate exactly 1 min. at RT.

Drain off excess detection reagent and wrap blots in cling film.

Place the blots protein side up, in the film cassette, add an autoradiographic film, and expose for 15 sec..

The autoradiographic film is developed and the exposure time can be modified if necessary according the results.

2.2.14. SLOT BLOT EXPERIMENTS.

The micro-sample filtration manifold (Schleicher & Schuell) was used for these experiments. Protein samples were blotted through slots in a perspex plate onto nitrocellulose by aspiration.

The growth of one roller bottle was harvested, lysed as described in section 2.2.9.1.. The protein concentration was measured by the Bradford method. The protein concentration in the HSV-2 infected and uninfected Bn5T extracts were equalized. Double dilutions of the extracts were loaded in the slots by aspiration. Immunoblotting was carried out as for Western blotting (section 2.2.11)

PART 3
RESULTS.

3.1. BACKGROUND OF THE PROJECT.

The basis from which the proposed tumour specific polypeptides were studied is the original experiment of Macnab, et al. (1985). This experiment showed that the sera of animals bearing a tumour (TBS) to HSV transformed cells (Bn5T) immuno-precipitated (i.p.) a set of polypeptides from these same Bn5T cells but not from rat embryo control cells (RE) . These polypeptides have molecular weights of 200,000, (200KD), 90,000 (a doublet: U90 and L90), 40,000 (40KD), and 32,000 (32KD), the 32KD peptide was later resized as 34KD and was often not i.p. (Hewitt, 1988). Antisera prepared in rats and in mice to HSV-2 infected cells i.p. a similar set of polypeptides (Hewitt et al., 1991).

The monoclonal antibody (Mab) TG7A was raised by D^r N.B. LaThangue against DNA binding proteins from HSV-2 infected BHK clone C13 cells (Macnab et al., 1985; La Thangue and Latchman, 1988) . The Mab TG7A i.p. polypeptides from Bn5T cell lysates which comigrate at the same MW as those i.p. by TBS, and which give very similar peptide maps (Macnab et al., 1985; Hewitt et al., 1991). However, the Mab TG7A i.p. a 35KD polypeptide instead of the 34KD polypeptide i.p. by TBS (Hewitt, 1988). Moreover the Mab TG7A did not immunoprecipitate the polypeptides from RE control cells, but, similar to TBS, only from transformed cells (Macnab et al., 1985; Hewitt et al., 1991). These transformation specific polypeptides could also be i.p. from rat or mouse cells transformed by Rous sarcoma virus or adenovirus 12 as well as from immortalized rat cells, and are therefore clearly cell encoded and not HSV encoded. (Macnab et al., 1985).

These findings suggested that certain cellular polypeptides induced by HSV-2 infection were related to polypeptides expressed at raised levels in cells

transformed by HSV-2 or by other viruses or carcinogens, and thus may play a role in the induction of transformation by HSV-2 (Macnab et al., 1985).

3.2. PRELIMINARY EXPERIMENTS.

3.2.1. IMMUNOPRECIPITATION EXPERIMENTS.

To verify the original findings of Macnab et al., 1985, i.p. and peptide mapping studies were carried out. Bn5T cell lysates in RIPA buffer were i.p. by TBS (figure 3.1). Two 90KD polypeptides (U90 and L90) were consistently i.p., but TBS did not always i.p. the 40KD polypeptide which is the subject of this thesis. At the beginning of the study, sixty six percent (16/24) of the TBS tested by the author were found to be positive. The 40KD polypeptide i.p. by TBS from Bn5T cells extracted in RIPA buffer will be referred to as TBS:40 in this thesis.

This ability to precipitate the 40KD polypeptide was tested in a standard i.p. experiment. Bn5T cells in vivo labelled with [^{35}S] L-methionine were lysed by sonication in a sonibath, in RIPA buffer. The protease inhibitor mixture (300mg benzamidine, 999mg phenanthroline and 340mg phenylmethylsulfonyl fluoride in 10ml ethanol) was added at a concentration of 1% to all preparations.

The radio-activity of a one microlitre aliquot was counted. A volume of lysate containing $4x10^6$ counts per minute (c.p.m.). was incubated for 1 hour with 10 ul of TBS at 4°C then 60 ul of a suspension of Staph. aureus protein A containing cells (Pansorbin*) was added for another hour at 4°C.

The samples were analysed on SDS-PAGE. Only SDS-PAGE and the buffer system described by Laemmli (1970) were used in this thesis.

An example of the ability of TBS to i.p. the 40KD polypeptide is given in figure 3.1. In this figure three different TBS were used to immunoprecipitate Bn5T cell extracts. All three TBS precipitate a 200 KD polypeptide and the 90 KD polypeptide doublet (U90 and L90) but the 40

Figure 3.1 : Immunoprecipitation experiments with TBS.

Autoradiograph of a 7.5% SDS polyacrylamide gel which shows i.p. experiments performed on [^{35}S] L-methionine labelled polypeptides extracted in RIPA buffer from RE control and Bn5T tumour cells with the antiserum TBS. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

These experiments detect a set of tumour specific polypeptides and show that the affinity of TBS for the 40KD protein is variable.

Track 1 shows the MW markers.

Track 2 shows the Bn5T cell polypeptides profile (labelled Bn5T).

Tracks 3-5 show the RE control cells polypeptides i.p. by 3 different TBS (labelled RE ip).

Tracks 6-8 show the Bn5T cell polypeptides i.p. by the same three different TBS (labelled Bn5T ip).

Track 9 shows the RE cell polypeptides profile (labelled RE).

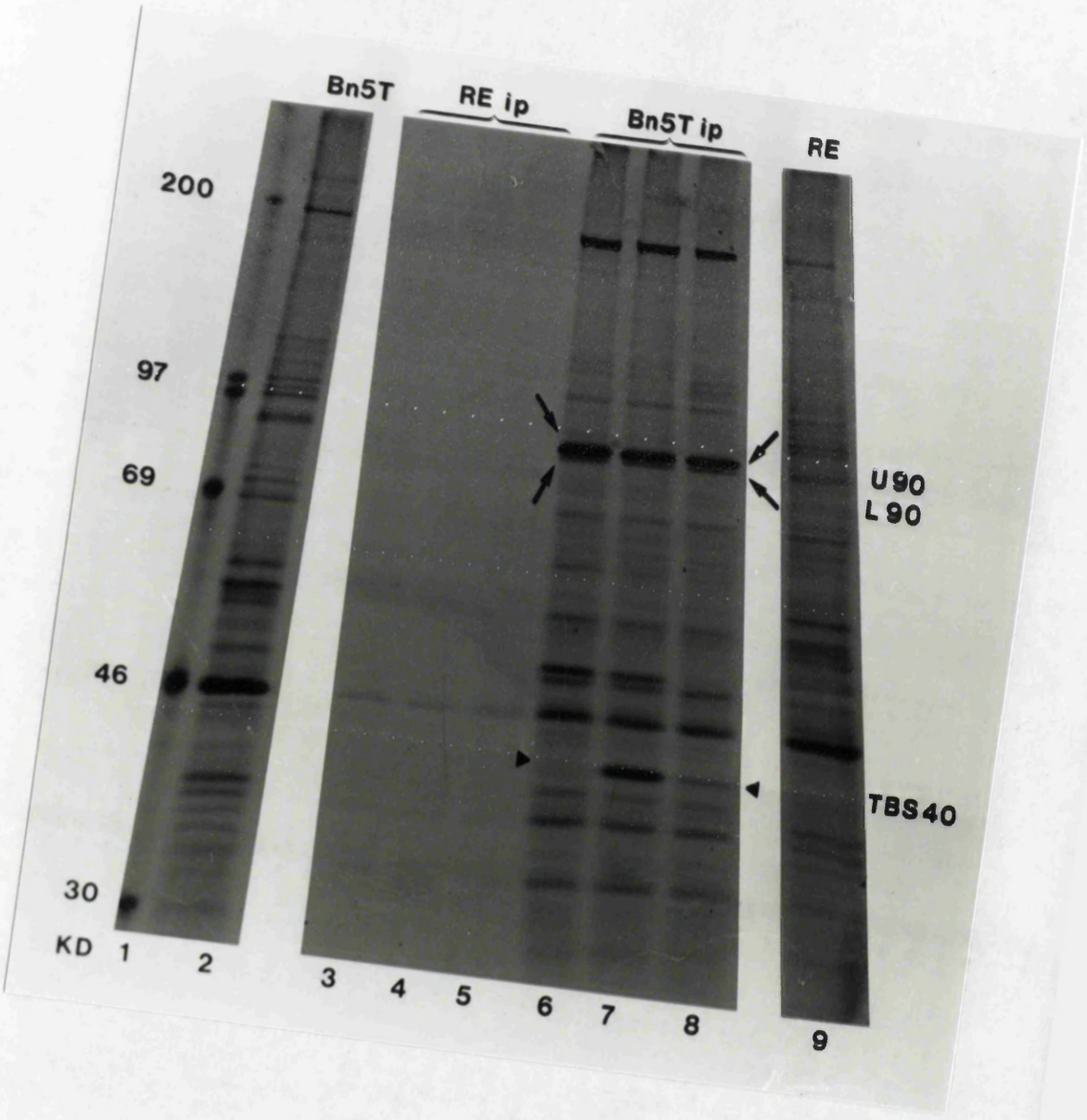
The U90 and L90 are indicated by arrows.

The position of the TBS:40 is indicated by triangles.

The MW of the markers is at the left of the gel

The U90 and L90 are i.p. by the three TBS in the Bn5T extract, but the TBS:40 is i.p. strongly in track 7 faintly in track 8 and not at all in track 6.

Two different TBS i.p. a 56 KD protein. This proteins was not further studied due to lack of resources.


Bn5T
RE ip
Bn5T ip
RE
200
97
69
46
30
KD
1
2
3
4
5
6
7
8
9
U90
L90
TBS40

KD polypeptide was precipitated very well by only one antiserum, faintly by a second and not at all by a third.

3.2.2. STAPH. AUREUS V8 PROTEASE DIGESTION.

The immunoprecipitated polypeptides were further characterized by enzymatic digestion. The enzyme used was Staph. aureus V8 protease because the results obtained could be compared to previously published work (Macnab et al., 1985; Hewitt, 1988; Hewitt et al.,1991) and was a well characterized proteolytic enzyme for peptide analysis in the laboratory where this work was carried out.

The ability to detect peptide bands after overnignt exposure was essential. So the i.p. experiment was expanded ($4x10^7$ c.p.m.) to obtain a polypeptide band on an unenhanced gel. The bands were excised from the gel and digested by the method of Cleveland et al., (1977) previously described by Macnab et al., (1985) and Hewitt et al.,(1991). The amount of Staph. aureus V8 protease used was always 5 ug unless otherwise stated, as it was previously shown that this gives reproducible identifiable results after digestion (Macnab et al., 1985; Hewitt et al.,1991; J.C.M. Macnab, personal communication). The products of the digestion were analyzed on a 15% SDS-PAGE. The gel was enhanced, dried and exposed for three weeks in first instance, and then for longer periods of time.

The figure 3.2 shows the result of digestion of the TBS:40 by Staph. aureus V8 protease compared with the digests of the U90 and L90. The peptide map of the TBS:40 was quite dissimilar to that of the 90KD polypeptides showing that they were most likely different proteins although they might share an epitope in common. This epitope similarity could account for their i.p. by the Mab TG7A.

The TBS:40 peptide map is characterized by two groups of peptides one group greater that 14KD and composed of 4 bands and a second group of bands of about 4-8 KD. Between those groups a faint band can be distinguished. This experiment used as a control was repeated 37 times. The relative intensity of

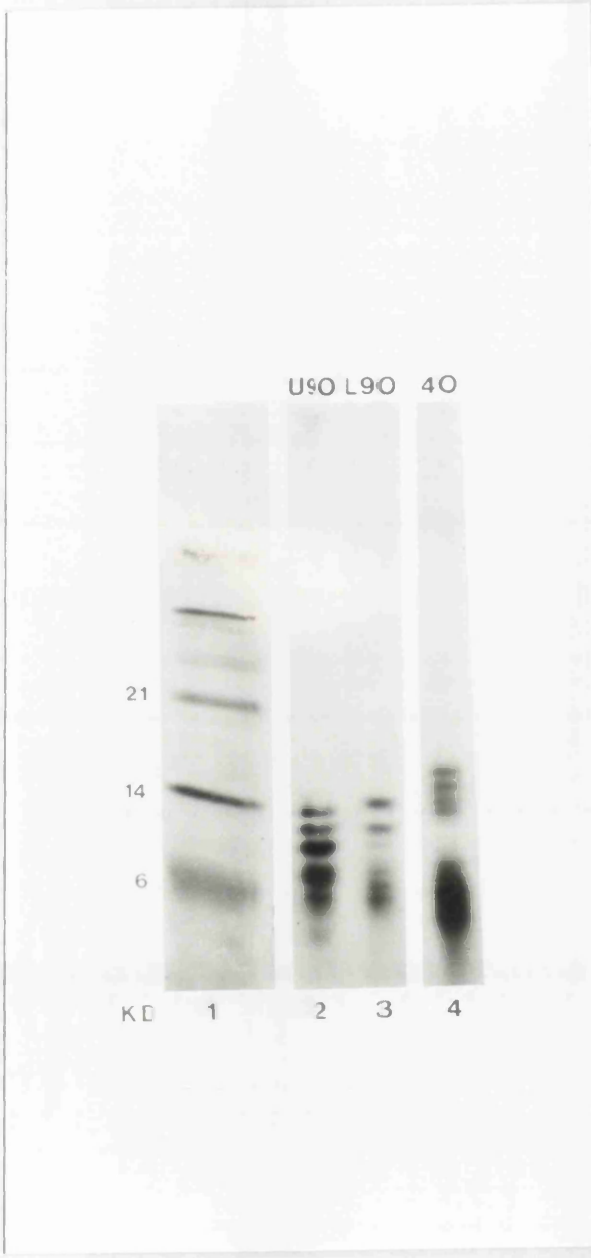
Figure 3.2. Staph. aureus V8 digests of the U90, the L90 and the 40KD proteins.

The U90, L90 and 40KD [^{35}S] L-methionine labelled polypeptides i.p. by TBS (figure 3.1) were digested by 5ug of Staph. aureus V8 protease. The result was analysed on a 15% polyacrylamide gel and visualized by autoradiography. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

Track 1 shows the MW markers.
Track 2 shows the digest of the U90
Track 3 shows the digest of the L90.
Track 4 shows the digest of the 40.

The MW of the markers is at the left of the gel

This experiment shows that the three proteins all have different peptide maps.


U90 L90 40
21
14
6
KD
1
2
3
4

radio-label of the two groups of bands can vary considerably and the faint band described here can be much more apparent, but the general pattern always remains the same.

Only TBS was used in this thesis because the original ascitic fluid from Mab TG7A used for the i.ps. was exhausted by the time I started this project. Dr J.C.M. Macnab obtained some more material and the original hybridoma from Dr N.B. LaThangue. However, unfortunately Dr J.C.M. Macnab was unable to detect antibodies to any of the tumour specific polypeptides set in the hybridoma fluid, nor was it possible to detect the polypeptides in ascitic fluid or after purification of IgG from either the hybridoma or the ascitic fluid. These results were confirmed by me (data not presented) and we were forced to conclude that the hybridoma TG7A had ceased to produce antibodies. No further source of hybridoma was located.

3.2.3. METHODS USED FOR IDENTIFICATION OF THE 40 KD POLYPEPTIDE.

The subject of the study presented in this thesis was the purification and sequencing of the TBS:40. The polypeptide of interest during the purification will be identified in all the experiments described by a combination of the following:

1/ Comparison of the apparent molecular weight of the protein in question with the 40KD polypeptide as i.p. by TBS from Bn5T cells extracted in RIPA buffer and referred to as TBS:40.

2/Immunoprecipitation i.e. recognition of the polypeptide in question by TBS.

3/Comparison of the enzymatic digest of the protein in question with the digest of the TBS:40.

For these experiments cells were radioactively labelled in vivo with [^{35}S] L-methionine. All the experiments described in this thesis used radioactively labelled cells except the samples prepared for the determination of the primary structure of the proteins (amino-acid sequence) and the Bn5T cell extract prepared for the dot blot experiments.

3.2.4. METHODS USED TO PURIFY THE TBS:40.

The first method was to try to raise monoclonal antibodies against the tumour specific polypeptides. For this, pure antigen was not required. Living cells have been used to raise monoclonal antibodies. (Harlow and Lane, 1987). If a monoclonal antibody is produced, an affinity column could be constructed for the purification of the polypeptide.

Affinity columns can successfully be made with polyclonal antibodies, but have severe drawbacks. Elution conditions are usually harsh. The affinity of the antibodies for the protein is variable therefore the protein is eluted with different strength of eluent, which result in a considerable tailing (Reviewed by Goding, 1986). An affinity column was made with TBS. It was possible to bind the protein to it, but no method was devised which succeeded in eluting the tumour specific polypeptide from the column made with TBS (J.C.M. Macnab, personal communication).

The second method was to purify the 40KD polypeptide using biochemical techniques. Techniques available in the Institute included ion exchange chromatography available on a computer controlled system allowing fast and reproducible chromatographic separations, the Fast Protein Liquid Chromatography (FPLC) system (Pharmacia). Ion exchange chromatography had also the advantage of preserving the native conformation of the proteins and therefore was the method of choice. Several prepacked columns are available covering anionic and cationic exchange chromatography as

well as electrofocusing.

Although infection with both HSV-1 and HSV-2 increases the amount of radiolabel in the 40KD protein recognized by TBS this result could reflect an increase in stability rather than a large increase in absolute quantity of protein (J.C.M. Macnab, personal communication). This was not known at the commencement of this work. Therefore the extra work required in generating virus stocks and in infecting Bn5T cells was not considered justified by the author for the work presented in this thesis.

3.3. MONOCLONAL ANTIBODIES.

3.3.1. IMMUNIZATION OF THE MICE.

Female Balb C mice were used. Before immunization a test bleed was taken from the eye by Dr A.Cross (the author is not licensed for this technique) and lack of antibodies to the 40KD and the 90KD doublet was confirmed in i.p. experiments (Results not presented). Three methods were used to immunize the mice.

3.3.1.1. IMMUNIZATION WITH LIVING CELLS.

Initially no purified 40KD protein was available. Mice were immunized by injecting living cells. The cells were resuspended in the same buffer used to immunize rats: PBS.

3.3.1.2. IMMUNIZATION WITH SONICATED CELLS.

Fractionation studies had shown that the TBS:40 was a cytoplasmic protein, (see section 3.4.1). It was thought that limited lysis of the cells might improve the immunogenicity of the TBS:40. PBS was suitable for extracting the TBS:40 and has been used extensively in cell fractionation experiments (See section 3.4.1.). I.p. of the TBS:40 from the cytoplasmic fraction of cells extracted in PBS is shown in figure 3.9 ,(Section 3.4.1).

3.3.1.3.IMMUNIZATION BY IMMUNE COMPLEXES.

The mice were immunized with proteins i.p. by the mouse antisera raised against Bn5T cells. Although the 40KD protein precipitated by immune mouse antiserum appeared different from the TBS:40, there was a strong possibility that the TBS:40 composed of more than one precipitated polypeptide (See sections 3.4-3.5). Therefore i.ps. might have increased the possibility or producing an antibody to a component of the TBS:40 and would definitely enhance the

possibility of producing an immune response to the U90 (Harlow and Lane, 1987).

Using data from the antibody screening experiment it was estimated that the extract from $2x10^7$ Bn5T cells could be i.p. by 20ul of mouse antisera followed by a further incubation with 10 ul of sheep anti-mouse serum; the immune complexes were trapped with 100ul of Pansorbin*. Four mice were immunized with these immune complexes and four other mice with sonicated cells. The mouse antisera were tested as previously described.

3.3.1.4. THE IMMUNIZATION PROTOCOL

A total of four mice were immunized; two with $2x10^7$ Bn5T cells injected as live cells and two with $2x10^7$ Bn5T cells which had been sonicated for 30 seconds in a sonibath. The cells, sonicated or live, were injected into the peritoneum four times at one month intervals. A week after the last injection the mice were bled to test the antibody response.

In a separate experiment, the interval between the injections was also reduced to two weeks but the antibody response was less, and two further injections were necessary before a titre similar to the first method was reached.

3.3.2. TESTING THE ANTIBODY RESPONSE.

3.3.2.1. IMMUNOPRECIPITATION EXPERIMENTS.

The antibody response of the mouse was tested in i.p. experiments by reaction with the Bn5T cell lysate, followed by incubation with 5ul of sheep anti-mouse serum. Most mouse immunoglobulin classes bind poorly to protein A, and a second antibody is included which binds to the mouse IgG and binds strongly to protein A.

Figure 3.3 shows the results of these i.p. experiments. Mice injected with live cells developed antibodies against

Figure 3.3. Test of an antiserum from a mouse immunized by Bn5T.

Autoradiograph of a 9% SDS polyacrylamide gel which shows i.p. experiments performed on [^{35}S] L-methionine labelled polypeptides extracted in RIPA buffer from RE control and Bn5T tumour cells with TBS and one mouse antiserum. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

Track 1 shows the MW markers.

Track 2 shows the Bn5T cell polypeptides profile (labelled B).

Track 3 shows Bn5T cells polypeptides i.p. with TBS (labelled B.TBS).

Track 4 shows the RE cell polypeptides profile (labelled RE).

Track 5 shows RE control cells i.p. with TBS (labelled RE.TBS).

Track 6 shows Bn5T cells polypeptides i.p. with mouse antiserum (labelled B.m).

Track 7 shows RE control cells i.p. with mouse antiserum (labelled RE.m).

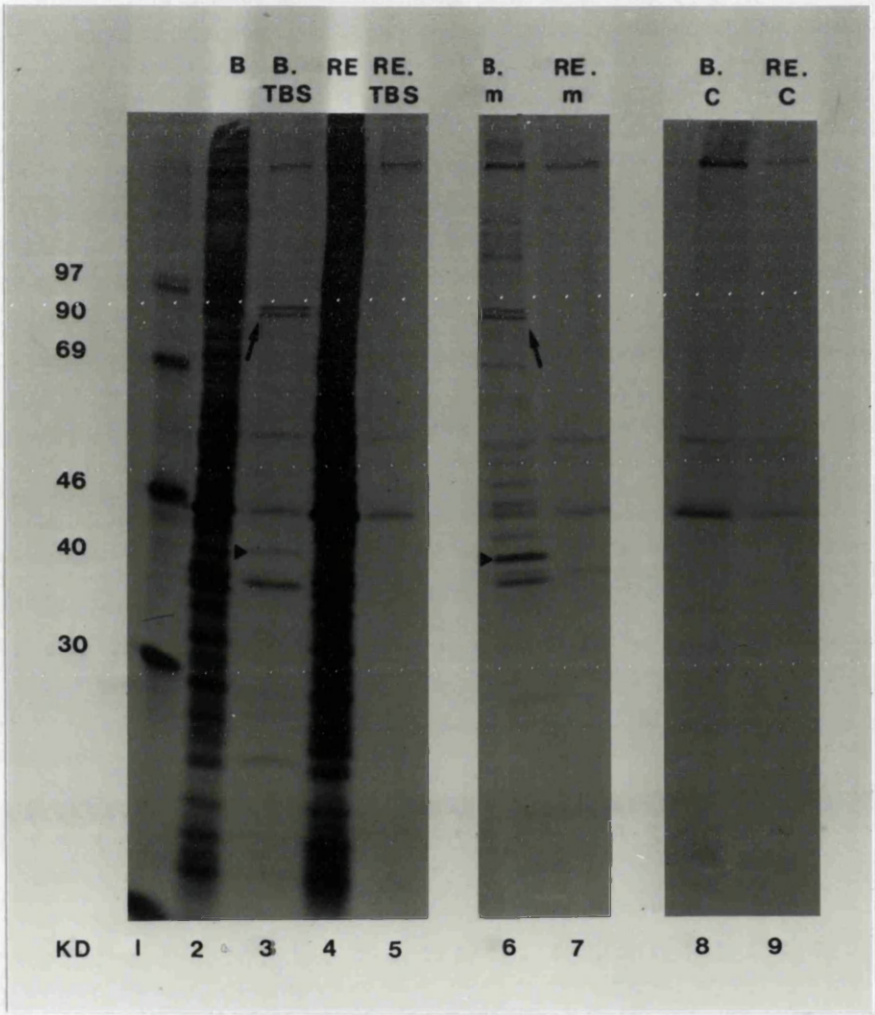
Track 8 shows Bn5T cells i.p. with sheep anti-mouse antiserum (labelled B.C).

Track 9 shows RE control cells i.p. with sheep anti-mouse antiserum (labelled RE.C).

The positions of the 90KD doublet is showed by arrows and the 40KD proteins are indicated by triangles.

The MW of the markers is at the left of the gel

A mouse can be immunized against Bn5T cells and its antiserum can i.p. a 90KD doublet and a 40KD protein from Bn5T cells extract and not from RE cells extract similar to those i.p. by TBS.


B
B. TBS
RE
RE. TBS
B. m
RE. m
B. C
RE. C
97
90
69
46
40
30
KD
1
2
3
4
5
6
7
8
9

a few polypeptides expressed in Bn5T cells and not in RE cells.

A 90KD doublet (U90 and L90) and a 40KD polypeptide are prominent, these bands co-migrate with the 90KD doublet and the 40KD bands observed in the TBS i.p. track. In addition mouse antisera immunoprecipitate three proteins of 120-150 KD. These bands are fuzzy suggesting they are glycoproteins. Four other bands one of MW between 56KD and 46KD and one each of 42KD, 38KD and 36KD are seen in figure 3.3.

Only the actin band is i.p. in the RE cell track. One control track shows that sheep anti-mouse serum i.p. actin and a 56KD band (figure 3.3).

3.3.2.2. STAPH. AUREUS V8 PROTEASE DIGESTION.

The experiment was repeated using 200ul of cell extract containing $4x10^7$ c.p.m. and i.p. to use the 40KD and the U90 digest with Staph. aureus V8 protease.

The results are shown in figure 3.4.

The proteolytic digests of the U90 polypeptides precipitated by either TBS or mice antisera are indistinguishable.

The digests of the 40KD polypeptides precipitated by mice antisera (Tracks 3 and 4) and TBS (track 2) are different. However, in track 2, fainter bands of higher molecular weight shown by arrows, comigrate with bands in the TBS:40 digest (track 2); this suggests that mice may recognize two proteins, but these proteins have different immunogenicity which when run on a gel produce the result presented in figure 3.4.

3.3.2.3.TITRATION OF THE MOUSE ANTISERA.

Before starting to fuse the spleen cells with myeloma cells the antisera were titrated. Immunoprecipitation was used as the means of assay, because the difference between Bn5T cells and RE control cells was demonstrated by i.p. and because i.p. is a means of testing the suitability of an

Figures 3.4a and b. Comparison of Staph. aureus V8 digests of the U90 and the 40KD proteins i.p. by TBS and mice antisera.

The U90 and 40KD [^{35}S] L-methionine labelled polypeptides extracted from Bn5T cells and i.p. by TBS and mice antisera were digested by 5ug of Staph. aureus V8 protease. The results were analysed on a 15% polyacrylamide gel and visualized by autoradiography. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

Figure 3.4a.

Track 1 shows the MW markers.

Track 2 shows the digests of the U90 i.p. by a mouse antiserum raised against Bn5T cells injected as live cells.

Track 3 shows the control digest of the U90 i.p. by TBS.

Figure 3.4b.

Track 1 shows the MW markers.

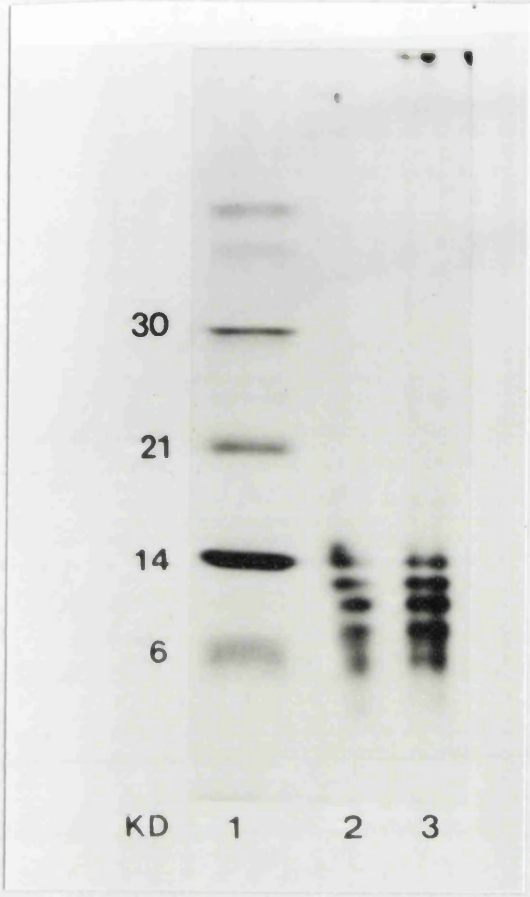
Track 2 shows the control digest of a TBS:40.

Track 3 shows the 40KD i.p. by a mouse antiserum raised against an immune complex made from Bn5T cells polypeptides i.p. by a mouse antiserum.

Track 4 shows the digests of the 40KD i.p. by a mouse antiserum against Bn5T cells injected as live cells. Arrows show the faint bands co-migrating with the high MW bands of the TBS:40 control track (track 2).

The MW of the markers is at the left of the gels

A



B

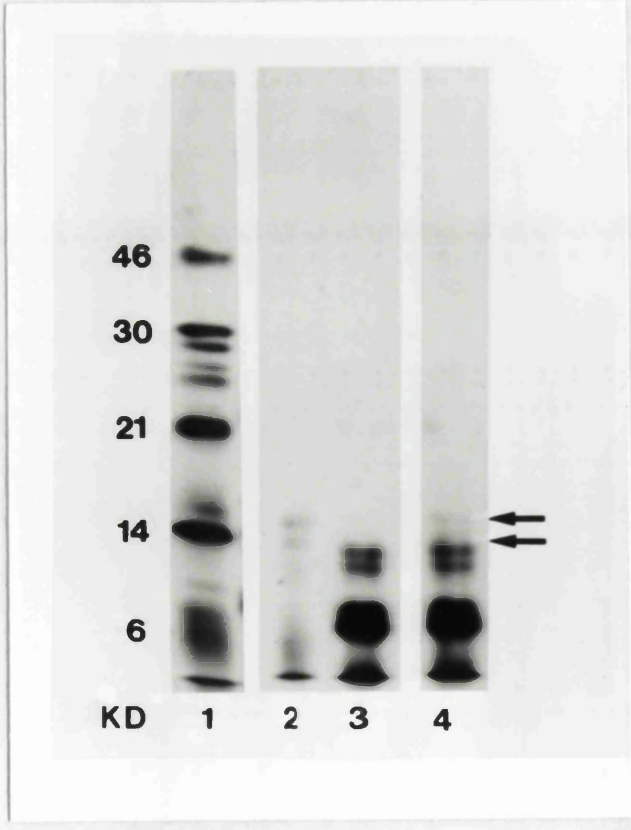


TABLE 3.1. SEQUENTIAL I.P.

TITRATION OF THE MOUSE ANTISERUM
FOLLOWED BY I.P. WITH TBS.

Bn5T cells extract 4.10^6 c.p. m. in each tubes
incubate 1 h with the following volumes of mouse antiserum

20 ul	2 ul	0.2 ul	0.02 ul
add 60 ul of pansorbin* incubate 1 h	add 60 ul of pansorbin* incubate 1 h	add 60 ul of pansorbin* incubate 1h	add 60 ul of pansorbin* incubate 1 h
spin, wash, load on gel. (figure 3.5)	spin, wash, load on gel (figure 3.5)	spin, wash, load on gel (figure 3.5)	spin, wash, load on gel (figure 3.5)
save supernatant			save supernatant
add 20 ul of TBS			add 20 ul of TBS
incubate 1 h			incubate 1 h
add 60 ul of pansorbin*			add 60 ul of pansorbin*
incubate 1h			incubate 1 h
spin, wash and load on gel (figure 3.6)			spin, wash and load on gel (figure 3.6)

In the first tube only the antigens not immunoprecipitated by mice antisera will appear in the 2^{nd} gel (figure 3.6).

antibody for affinity chromatography. Tenfold dilutions of antisera were used, with a fixed amount of cell extract ($4x10^6$ c.p.m.).

An experiment used to show the relatedness of the proteins recognized by TBS and mouse antisera was carried out. It consisted of preadsorbing the protein of interest. In this experiment the proteins were first i.p. with a mouse antiserum and then the resulting supernatant i.p. with TBS. The protocol is described as a flow diagram in table 3.1.

The results of the titration experiments are shown in figure 3.5.

A mouse antiserum i.p. a 40KD band in tracks 3, 4 and 5, and a U90 band in tracks 3 and 4 . The dilution is 1/100 in track 5, and 1/10 in track 4. The titres were sufficient to undertake fusion experiments. Titres of 1/5 or 1/10 are satisfactory according to the antisera used by Harlow and Lane (1987). A band can be seen under the U90 band in tracks 3 and 4 but it is also present in the control track 7, therefore it is unlikely to be the L90.

The result of the second i.p. experiment is shown in figure 3.6.

Twenty microlitres of a mouse antiserum immunoprecipitated all the U90 polypeptide. The U90 band was absent in the analysis of the proteins subsequently precipitated from the supernatant by TBS. A 1/1000 dilution (0.02 ul of a mouse antiserum) was insufficient to precipitate the U90 completely and in this case TBS i.p. the remainder from the supernatant.

The L90 polypeptide and the 40KD polypeptide bands have the same intensity in both i.p. experiments, therefore the intensity of the bands is not altered by the dilution of the mouse antiserum used in the titration experiment. Twenty microlitres of mouse antiserum may not have been sufficient to bind to all the 40KD band which can be i.p. by TBS

The proteolytic digestion data showed that the Staph. aureus V8 protease digests of the 40KD polypeptide i.p. by mouse antisera (mice:40) was different from TBS:40. TBS

Figure 3.5. Titration of an immune mouse antiserum.

Autoradiograph of a 9% SDS polyacrylamide gel which shows i.p. experiments performed on [^{35}S] L-methionine labelled polypeptides extracted in RIPA buffer from Bn5T tumour cells with ten fold dilutions of a mouse antiserum. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

Track 1 shows the MW markers.

Track 2 shows Bn5T cells polypeptides i.p. with TBS .

Tracks 3-6 show Bn5T cells polypeptides i.p. with 20, 2, 0.2 and 0.02ul of a mouse antiserum to Bn5T cells.

Track 7 is a control track for the second antibody. It shows Bn5T cells i.p. with 10ul of the sheep antiserum used as second antiserum.

The position of the U90 and L90 are indicated at the left of track 2 by two arrows and the TBS:40 is indicated by a triangle at the left of track 2. The 90KD polypeptide i.p. by the mouse antiserum is indicated by an arrow at the left of track 3. The 40KD polypeptide i.p. by the mouse antiserum is situated between the two empty triangles.

The MW of the markers is at the left of the gel

A 1/10 dilution of the mouse antiserum i.p. an U90 and a 1/100 dilution i.p. a 40KD protein

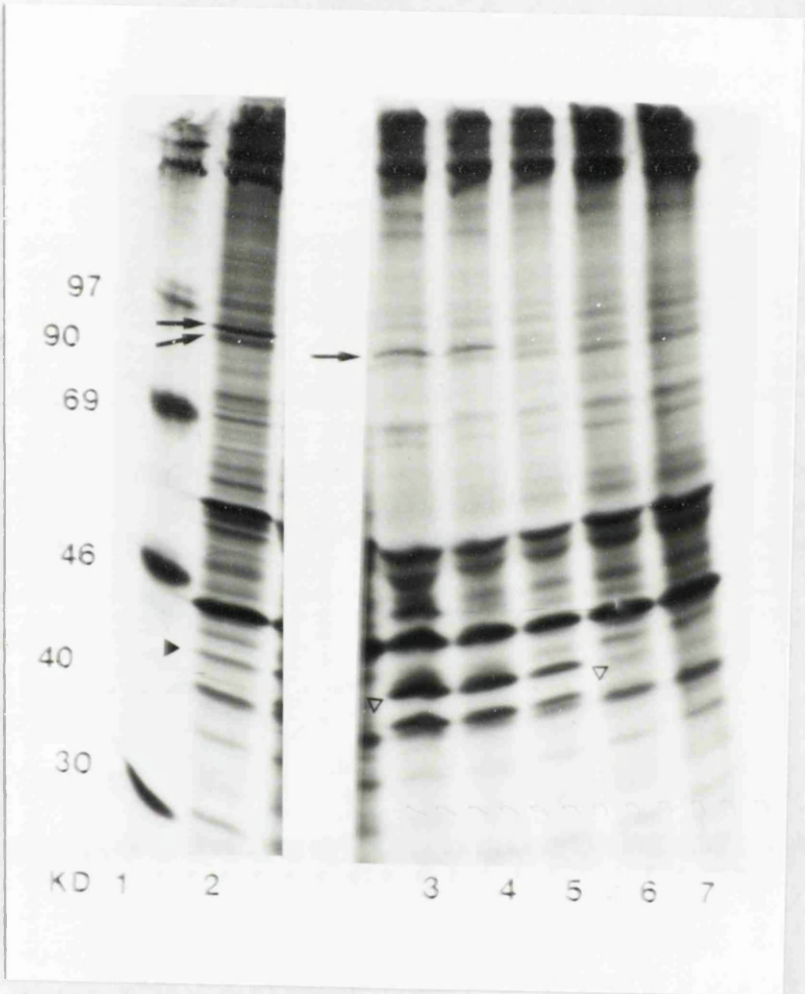

97
90
69
46
40
30
KD
1
2
3
4
5
6
7

Figure 3.6. Identification of the polypeptides i.p. by mice antisera.

Autoradiograph of a 9% SDS polyacrylamide gel which shows i.p. experiments with TBS performed on supernatant of i.p. experiments with mice antisera (figure 3.5). The polypeptides extracted in RIPA buffer from Bn5T tumour cells were labelled with[^{35}S] L-methionine. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

Track 1 shows the MW markers.

Track 2 shows a control Bn5T cells i.p. with TBS (labelled c).

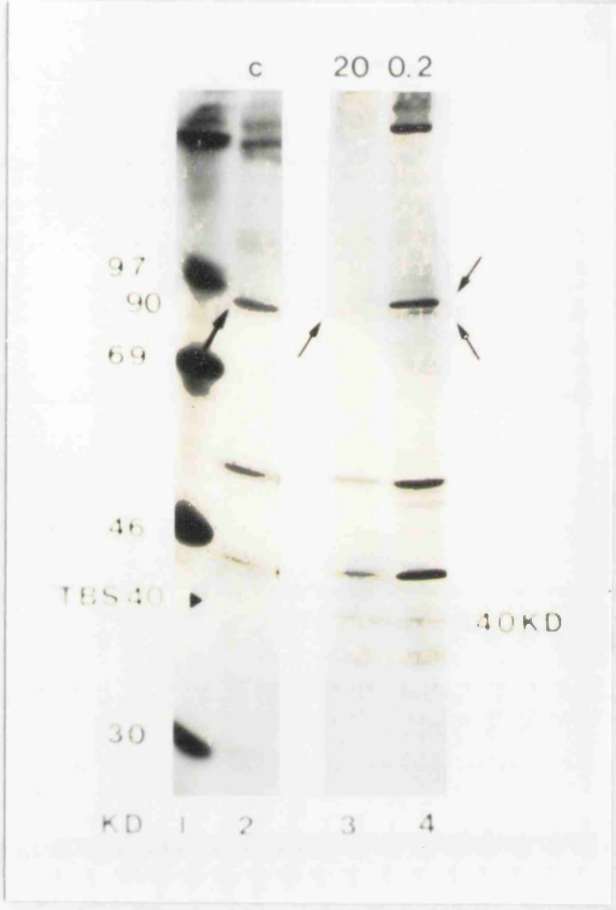
Track 3 shows Bn5T cells polypeptides i.p. with TBS from an extract preadsorbed with 20ul of mouse antiserum (labelled 20).

Track 4 shows Bn5T cells polypeptides i.p. with TBS from an extract preadsorbed with 0.02ul of mouse antiserum (labelled 0.2).

The MW of the markers is at the left of the gel

The U90 and L90 are shown by arrows, the TBS:40 by a filled triangle, and the 40KD polypeptides by empty triangles.

This figure shows that twenty microlitres but not 0.02ul of mouse antiserum i.p. the U90. The dilution of mouse antiserum has no effect on the i.p. of the 40KD by TBS.


c
20
0.2
97
90
69
46
TBS 40
40KD
30
KD
1
2
3
4

and mouse antisera, therefore, precipitate two different 40KD polypeptides. The U90 polypeptides digests are, however, indistinguishable.

It would be impossible to produce a monoclonal antibody to the TBS:40 using spleen cells from these mice. Monoclonal antibodies could be raised, however, against the U90 polypeptide and a 40KD polypeptide. For the reason now to be explained, it was nevertheless decided to proceed with the fusions.

The Mab TG7A used in previous studies i.p. both the U90 and a 40.000 MW protein showing they are either related or share an epitope. Thus it was possible that Mabs prepared to the 90KD protein could also recognize the 40KD protein. The work presented in the sections 3.4-3.6 relates to the purification of the 40KD protein which TBS i.ps. from Bn5T lysate. This work was carried out at the same time and it became clear that the TBS:40 was a polypeptide band which consisted of several different proteins precipitated as a complex (see section 3.4.5.3. figure 3.21). Moreover the proteolytic digestion pattern of one of the 40KD protein (referred as the "VOID VOLUME 40", see section 3.4.5.3.) was similar to the pattern of digestion of the 40KD i.p. by mice antisera (figure 3.21 tracks 5, 6 and 7). This result suggested that the TBS:40 was a composite protein produced by several polypeptides one of which was of major immunogenic dominance to the mouse. Furthermore similarities between U90 and the TBS:40 where investigated by experiments to determine the mechanisms of induction of both proteins. Originally these proteins were recognized by Mab TG7A (generated by N. LaThangue). Mab TG7A was raised against the DNA binding proteins of HSV-2 infected BHK C13 cells and not Bn5T implying that both proteins could bind to DNA. Experiments to determine if alterations in the DNA topology could alter the expression of the 40KD and the 90KD led to the use of ciprofloxacin (Ciproxin*), an antibiotic with activity against DNA topoisomerase type II (information obtained from the manufacturer: Bayer). Addition of this drug to the culture medium abolished the

ability of all the transformation specific polypeptides U90, L90 and 40KD to be i.p. by TBS fom Bn5T cells.

3.3.3. EFFECT OF CIPROXIN*.

Bn5T cells were passaged and an equal number of cells were seeded in medium with or without Ciproxin*, (1ul/ml). After 24h incubation the medium was removed and the cells labelled with [^{35}S] L-methionine for 17h as described in materials and methods, using medium without Ciproxin*. The cellular incorporation of radiolabel into cell coded proteins was similar as shown in this 3 separate experiments (c.p.m./ul) (Table 3.2).

TABLE 3.2. INCORPORATION OF [^{35}S] L-METHIONINE IN CELLS GROWN IN MEDIUM WITH OR WITHOUT CIPROXIN*.

Experiment	Cells grown in medium with Ciproxin (c.p.m./ul)	without Ciproxin (c.p.m./ul)
1	3 x10^5	2.5x10^5
2	0.6x10^5	1 x10^5
3	2 x10^5	1.7x10^5

An aliquot containing 4x10^7 c.p.m. was i.p. by 50ul of TBS and the immunecomplexes trapped with 100ul of Pansorbin*. The results are shown in figure 3.7. Addition of Ciproxin* to the culture medium resulted in a considerable reduction in the intensity in the bands i.p. by TBS, i.e. TBS:40 and 90KD doublet i.p. by TBS. This result shows that inhibition of the DNA topoisomerase type II prevents either directly or indirectly the expression of the U90 and the TBS:40 as detected by i.p..

3.3.4. FUSION OF THE SPLEEN CELLS.

The method developed by D^r A. Cross in this Institute

Figure 3.7. Effect of Ciproxin*.

Autoradiograph of a 9% SDS polyacrylamide gel which shows i.p. experiments performed on [^{35}S] L-methionine labelled polypeptides extracted in RIPA buffer. Bn5T cells were grown without or with Ciproxin* 1ug/ml. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

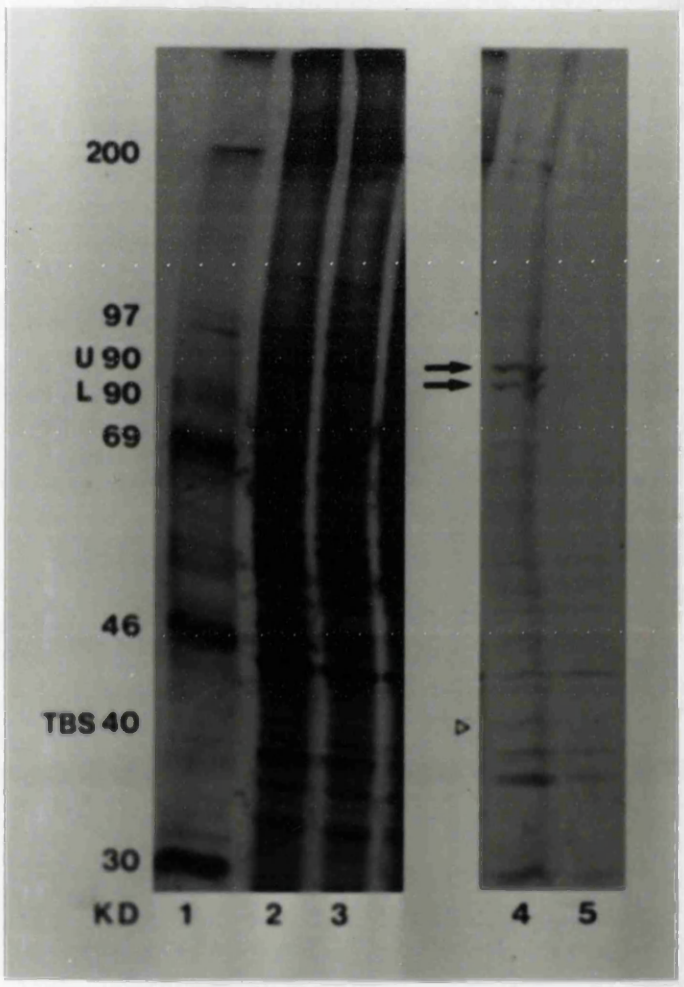
Track 1 shows the MW markers.

Tracks 2 and 3 show the polypeptide profile of Bn5T cells grown without and with Ciproxin*.

Track 4 and 5 show the polypeptides i.p. with TBS from Bn5T cells grown without or with ciproxin.

The U90 and the L90 are shown by two arrows, and the TBS:40 by an empty triangle.

The MW of the markers is at the left of the gel


200
97
U 90
L 90
69
46
TBS 40
30
KD
1
2
3
4
5

was used. The four mice immunized with Bn5T cells developed antibodies against the U90, the L90 and the 40KD polypeptide, they were all used for fusion experiments and were boosted 5 to 7 days before the fusion by an intraperitoneal injection.

The myeloma cells line used was the P3-X67 Ag8, which secretes IgG (Köhler and Milstein, 1975).

On the day of the fusion, a mouse was killed and the spleen dissected out of the animal. Myeloma cells and spleen cells were counted and mixed in the ratio of 10 spleen cells to one myeloma cell. The cells were fused using 1ml of 50% polyethylene glycol (PEG) 1500. The PEG was progressively diluted and then removed by centrifugation (5 min at 500 r.p.m.). The cells were then resuspended at $3.5x10^6$ cells/ml in selective HAT medium (Stock HAT contains 20ml 10^{-2}M hypoxanthine, 3.2ml 10^{-2} thymidine and 1.6ml 10^{-3}M aminopterin. 1ml of this stock solution was added to every 100ml of complete medium). Cells were plated out 0.1ml/well in 96 well plates.

After the experiments, cultures were observed each week for growing fused cells, but only 15 wells showed growth. The supernatants were tested by immunoprecipitation, but none was positive. I decided to repeat the experiment, and immunized four mice with sonicated cells and four mice with the Bn5T cell polypeptides i.p. by mice antisera as described in section 3.1.3.

3.3.5. TESTING THE SUPERNATANTS OF THE HYBRIDOMA FUSIONS.

3.3.5.1. IMMUNOPRECIPITATION EXPERIMENTS.

Three mice were used for fusion experiments according to the protocol described in the previous section. Mice N°1 and 2 were immunized with sonicated cells, N°3 was immunized with immune complexes. The results are shown in table 3.3

TABLE 3.3. RESULTS OF THE I.P. OF BN5T POLYPEPTIDES BY THE CULTURE MEDIUM OF HYBRIDOMA.

Mouse N°	Spleen cells 10^8	Wells filled	Wells screened	Positive wells
1	2	280	37	2
2	2.2	540	19	0
3	2.2	480	236	0

The supernatant of the two positive wells i.p. a 40KD protein as shown in figure 3.8. The clones were subcultured when well grown into 24 well plates as is the routine method of clonal expansion used in this Institute. Unfortunately both positive clone ceased to grow at this stage.

3.3.5.2. DOT BLOTS.

As an alternative to immunoprecipitation, supernatants were tested by dot blot analysis. A system marketed by Amersham was used. This system consists of a 96 well microtitre plate sealed at the bottom by a polyvinyldifluoride (PVDF) membrane. The microtitre plate fits a manifold which is linked to a vacuum pump so that the contents of all the wells can be sucked out at the same time. With this system 96 samples can be tested simultaneously.

One hundred microlitres of the antigens were added to the wells and incubated for 15 min. at RT. The membrane was blocked with 1% gelatin in tris buffer saline (TbuS) for 15 min. and washed three times with TBuS containing 0.05% Tween 20. Fifty microlitres of hybridoma supernatants were added and incubated 15 min. at RT. The membrane was washed three times with TTBuS and incubated with 100 ul of a 1/200 dilution of horse radish peroxidase labelled anti-mouse

Figure 3.8. Immunoprecipitation with the culture medium of the hybridoma to test for hybridoma clones positive for antibodies to the U90, the L90 or the 40KD.

Polypeptides labelled with [^{35}S] L-methionine, extracted in RIPA buffer from Bn5T tumour cells were i.p. with the culture medium of hybridomas to test for clones positive for antibodies to the U90, the L90 or the 40KD. The polypeptides were analysed on a 9% SDS-PAGE and visualized by autoradiography. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

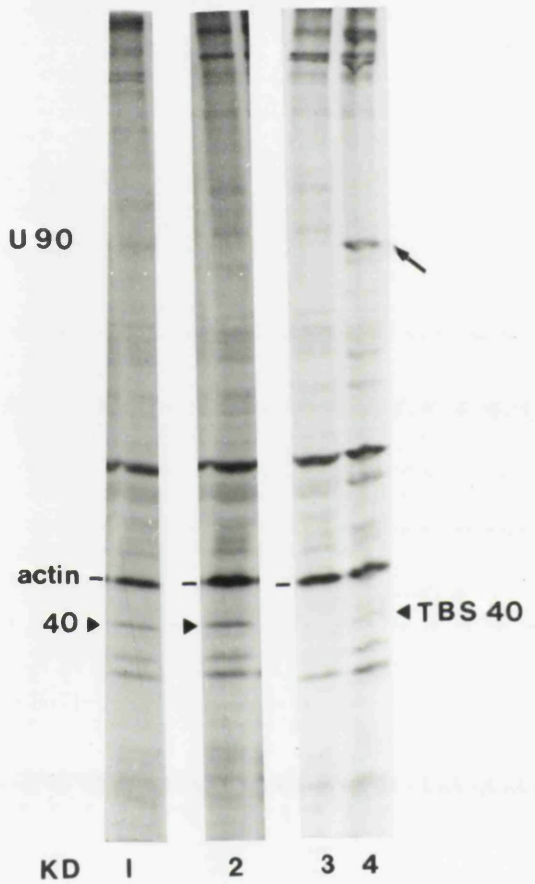
Track 1-3 show Bn5T cells i.p. with the culture medium of three different hybridoma clones.

Track 4 shows a control Bn5T cells i.p. with TBS.

The position of the U90 is indicated by an arrow and the TBS:40 by a triangle at the right of track 4.

A 40 KD polypeptide is i.p. by the culture media tested in track 1 and 2. Their position is indicated by triangles at the left of the tracks.

The MW of the markers is at the left of the gel.


U 90
actin
40
TBS 40
KD
1
2
3
4

antibody. After washing three times with TBuS, the colour developer containing alpha chloro naphthol was prepared and 50 ul were added to each well. A purple colour developed in the positive wells.

A total of 196 supernatants were screened by this method, but no positives were found.

3.3.6. THE ATTEMPT TO RAISE MONOCLONAL ANTIBODIES: CONCLUSIONS.

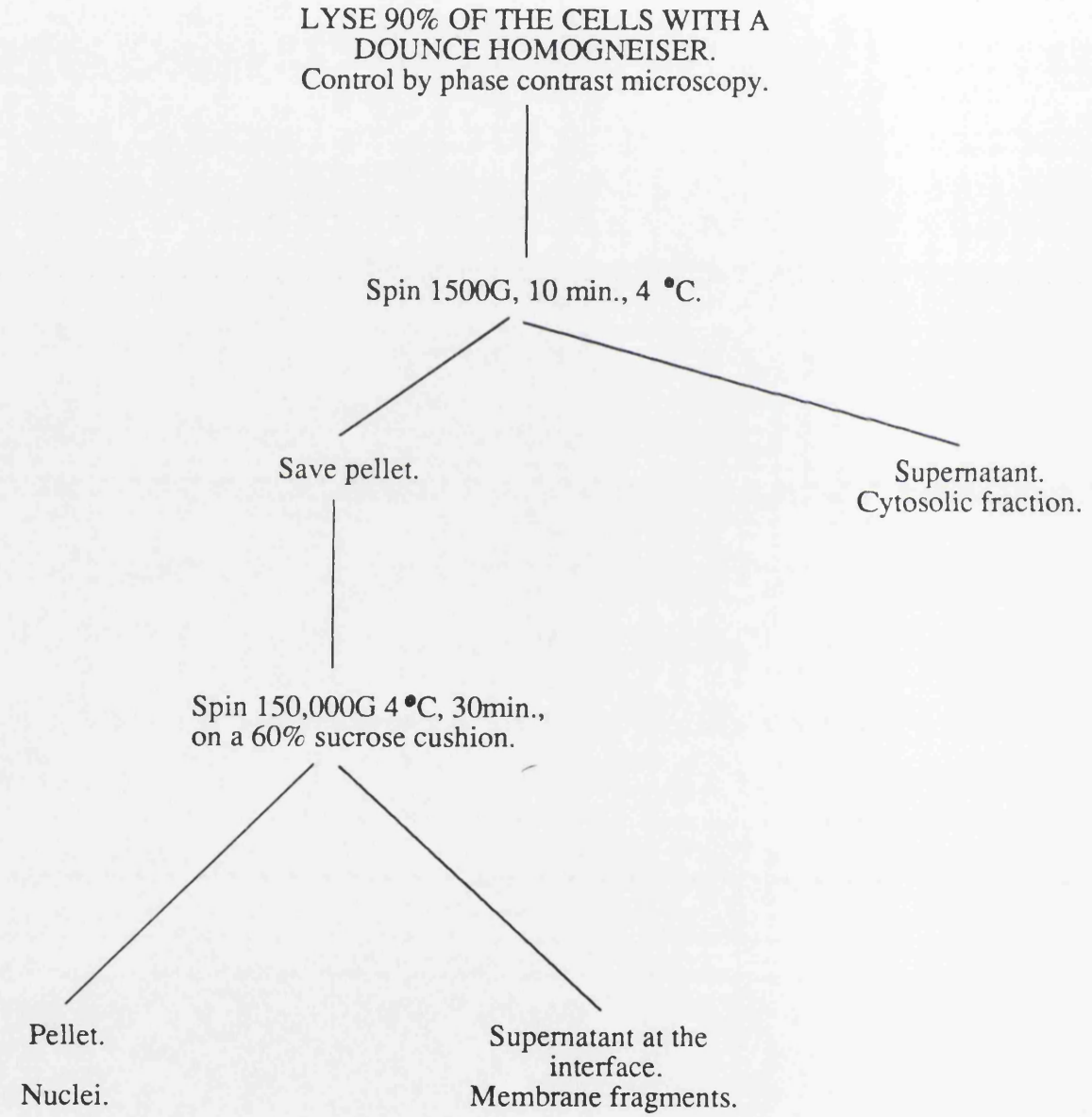
Figure 3.4b showed that the Staph. aureus V8 digest of the TBS:40 differs from the digest of the mice:40. Figure 3.7 showed that the i.p. of a 40KD polypeptide by a mouse antiserum does not affect the i.p. of the TBS:40.

Therefore the antibody response of rats to the tumour and mice to the injection of Bn5T cells is different. The mice were expected to develop antibody to all the proteins antigenic for the rats since they are xeno-antigens for the mice. Instead the mice failed to react to a 40KD protein of identical peptide map to the TBS:40. This could be partly explained by the results of the purification experiments to be presented in section 3.4 onwards.

The purification experiments described in the following chapters appeared to show that the TBS:40 consists of a complex of at least three proteins. However not all three could have been immunogenic in the mouse, a fact which may explains the difference between the peptide map of the TBS:40 and the mouse:40.

TABLE 3.4.

CELL FRACTIONATION.



*The experiment used $4x10^7$ cells labelled with EF2-met and 50uCi/ml of [^{35}S] L-methionine.

3.4. PURIFICATION PROCEDURES.

3.4.1. THE 40 000 MW POLYPEPTIDE IS A CYTOPLASMIC POLYPEPTIDE.

The TBS:40 was i.p. from a whole cell extract, however the exact intracellular location was not known. Knowledge of the intracellular location of a polypeptide could assist in devising a purification procedure.

Bn5T cells were fractionated, using a method suggested by Dr M. Finbow (Beatson Institute for Cancer Research, Glasgow). which is described in table 3.4. The cells were broken down in a Dounce homogeniser , in a hypotonic buffer until no more than 90% of the cells were lysed as viewed by phase contrast microscopy, taking care to keep the nuclei intact. The method then involves centrifugation at 1500G (centifuge Sorval RT6000D 3000 r.p.m), for 5 min. at 4°C to sediment the membrane fragments and the nuclei: the supernatant is referred to as the cytosolic fraction. The membrane fragments and the nuclei are further separated by centrifugation at 150,000G (centrifuge Sorval OTD50, rotor AH650, 35.000 r.p.m.), for 30min, at 4°C on a 60% sucrose cushion. The membrane fragments float on top of the cushion and the nuclei sediment. The cells are thus fractionated into cytosolic, membrane and nuclear fractions.

* The volume of the cytosolic fraction was 12 ml. The membrane fraction was first washed in 1 ml of 5mM NH_4CO_3, resuspended in RIPA buffer to extract insoluble proteins, sonicated, and centrifuged for 10 min. at 13,000 r.p.m. (4°C) in an MSE Microcentaur microfuge. The nuclear fraction dissolved instantaneously in 1 ml of RIPA buffer. All fractions were centrifuged 10 min. at 13,000 r.p.m. (4°C) in an MSE Microcentaur microfuge to pellet insoluble material before the i.p. experiment. The radio-activity of a one microlitre aliquot of each fraction was measured to calculate the total radio-activity of each fraction. The total radio-active count of each fraction was as follow: cytosolic, $9x10^8$; membrane, $1.4x10^7$ and nuclear, $3.3x10^7$.

An aliquot containing $4x10^6$ counts of each fraction was

i.p. with 10ul of TBS and the immune-complexes trapped with 60ul of Pansorbin.

Figure 3.9 shows the results of these experiments as analysed on a 7.5% SDS polyacrylamide gel.

By the method of cell fractionation used in this study, there is hardly any 40KD i.p. in the membrane fraction. There is a strong 40KD band in the cytoplasm and in the nuclear tracks. The result shows that the 40KD protein can be purified from the cytoplasmic fraction. The total radio-active count of the cytoplasmic fraction was 30 times the total radio-active count of the nuclear fraction. Although the intensity of the band reflects the amount of radio activity incorporated and not the total amount, it was considered that a large part of the 40KD protein was soluble.

Figure 3.9. Cell fractionation.

Bn5T cells were fractionated into nuclear, cytoplasmic and membrane fractions by differential centrifugation. Each fraction was i.p. by TBS, The results were analysed on a 7.5% polyacrylamide gel and visualized by autoradiography. The polypeptides were labelled with[^{35}S] L-methionine. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

Track 1 shows the nuclear fraction i.p. with TBS (labelled p).

Track 2 shows the cytoplasmic fraction i.p. with TBS (labelled c).

Track 3 shows the membrane fraction i.p. with TBS (labelled m).

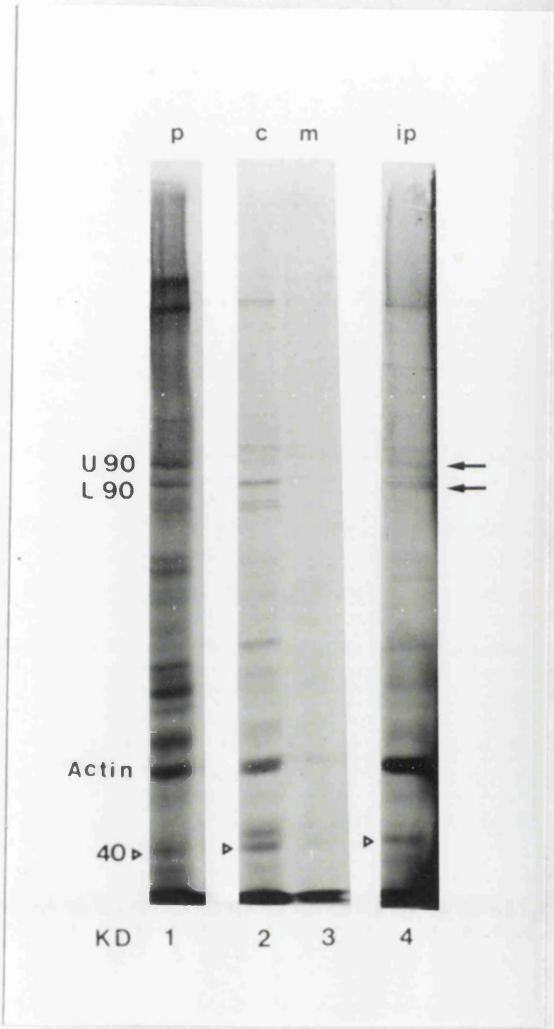
Track 4 shows a control Bn5T cells i.p. with TBS (labelled ip).

The position of the 40KD polypeptides is shown by empty triangles on the left of the tracks. The position of the U90 and L90 is shown by two arrows on the right of track 4.

The U90 is i.p. in the nuclear fraction.

A protein with a MW slightly over 40KD is i.p. in the cytoplasmic fraction.

The 44KD band labelled "actin" is i.p. in the nuclear and cytoplasmic fraction.


p
c
m
ip
U 90
L 90
Actin
40
KD
1
2
3
4

3.4.2.TRIAL OF A BUFFER WITHOUT DETERGENTS.

The previous experiment located the 40KD polypeptide to the cytoplasmic compartment. It was reasonable to deduce that the protein was probably soluble and that the detergents present in RIPA buffer were not required for its solubilisation. RIPA buffer contains SDS, sodium deoxycholate and triton X 100. SDS precludes the use of an ion exchange chromatography column.

To use anion exchange chromatography, another buffer without SDS had to be found. The 90KD polypeptide recognized by the Mab TG7A was reported to be related to HSP 90 (La Thangue and Latchman, 1988). It was assumed that the TBS:40 might conceivably be a similar type of protein as it was immunologically related to the 90KD polypeptide. In addition other HSPs are frequently coprecipitated with oncogenes and anti-oncogenes such as ras and p53 (Pinhasi-Kimhi, et al., 1986).

Therefore buffers used in the extraction of the HSPs were tested and the buffer used to successfully purify the HSP70 by ion exchange chromatography (Welsh and Feramisco, 1985) was chosen (20mM tris acetate pH.7,4; 20mM NaCl; 0.1mM EDTA; 15mM beta-mercaptoethanol). It will be referred hereafter as the WF buffer. Bn5T cells were extracted with this WF buffer. This extract was i.p. by TBS and the result compared with a standard i.p in which Bn5T cells were lysed in RIPA buffer.

Figure 3.10 compares the results of the i.p. of Bn5T cells extracted in WF buffer and in RIPA buffer.

Cells from two 50mm petri dishes seeded with equal numbers were radio-labelled with [^{35}S] L-methionine using the same conditions. One plate was harvested with WF buffer and the other with RIPA buffer. Cells extracted in WF buffer do not contain as high levels of radio-activity as similar cells extracted in RIPA buffer (total count per 50mm dish : WF buffer, 1.6×10^6 c.p.m. against 4×10^6 c.p.m. for RIPA buffer). Total protein from both samples were used for an i.p. experiment with TBS. In this experiment only a

Figure 3.10. Extraction of Bn5T polypeptides in a buffer without detergent.

Autoradiograph of a 9% SDS polyacrylamide gel which shows i.p. experiments performed on [^{35}S] L-methionine labelled polypeptides extracted in RIPA buffer (control i.p.) and in 20mM TrisHCl pH.7.4 (WF) buffer from Bn5T tumour cells. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

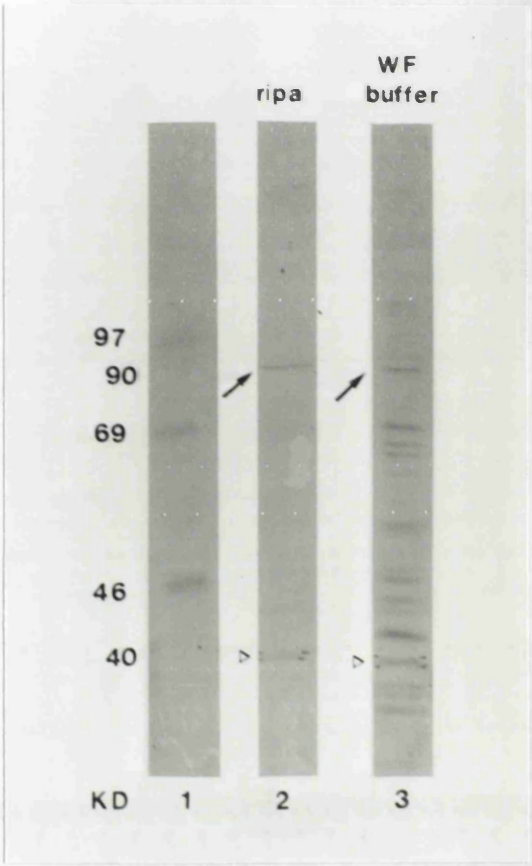
Track 1 shows the MW markers.

Track 2 shows a control Bn5T cells i.p. with TBS.

Track 3 shows Bn5T cells extracted in WF buffer and i.p. with TBS.

The MW of the markers is at the left of the gel

The position of the U90 is indicated by arrows and the 40KD polypeptide by open triangles at the left of the tracks. (The TBS used in this experiment does not i.p. the L90).


ripa
WF
buffer
97
90
69
46
40
KD
1
2
3

90KD and a 40KD polypeptide is i.p. by TBS from Bn5T cells extracted in RIPA buffer. A 90KD and a 40KD can also be i.p. from cells extracted in WF buffer; in addition twelve polypeptides were i.p., nine of MW between 90KD and 40KD and three of MW inferior to 40KD (fig 3.10). In some preparation U90 is well extracted by WF buffer, but generally good extraction of the U90 requires the addition of detergent. The reason why WF buffer can occasionally extract the U90 is not understood.

The WF buffer, which contains no detergent, extracts a 40KD polypeptide which was i.p. by TBS. To further identify the 40KD, the polypeptide band was cut out of the gel and digested with Staph. aureus V8 protease together with a TBS:40 band extracted by RIPA.

Figure 3.11 shows that by proteolysis the two peptides maps were similar therefore the 40KD polypeptide extracted in and i.p. from WF buffer was very similar to the TBS:40. Bn5T cells were therefore lysed in this WF buffer at the start of all further purification experiments.

Figure 3.11. Comparison of the Staph. aureus V8 protease digest of the 40KD polypeptides extracted by WF buffer and RIPA buffer.

The 40KD [^{35}S] L-methionine labelled polypeptides extracted in RIPA and WF buffer and i.p. by TBS (figure 3.10) were digested with 5ug of Staph. aureus V8 protease. The results were analysed on a 15% polyacrylamide gel and visualized by autoradiography.

Track 1 shows the MW markers.

Track 2 shows the digest of the control TBS:40.

Track 3 shows the digest of the 40KD polypeptide extracted by WF buffer and i.p. by TBS.

The MW of the markers is at the left of the gel

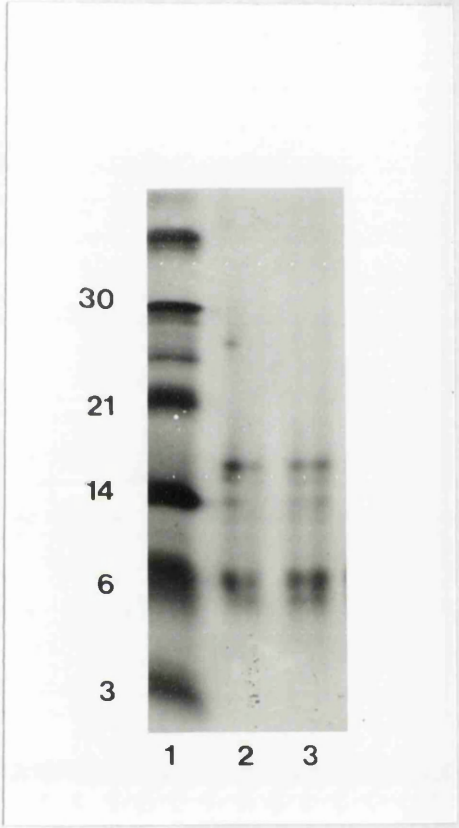
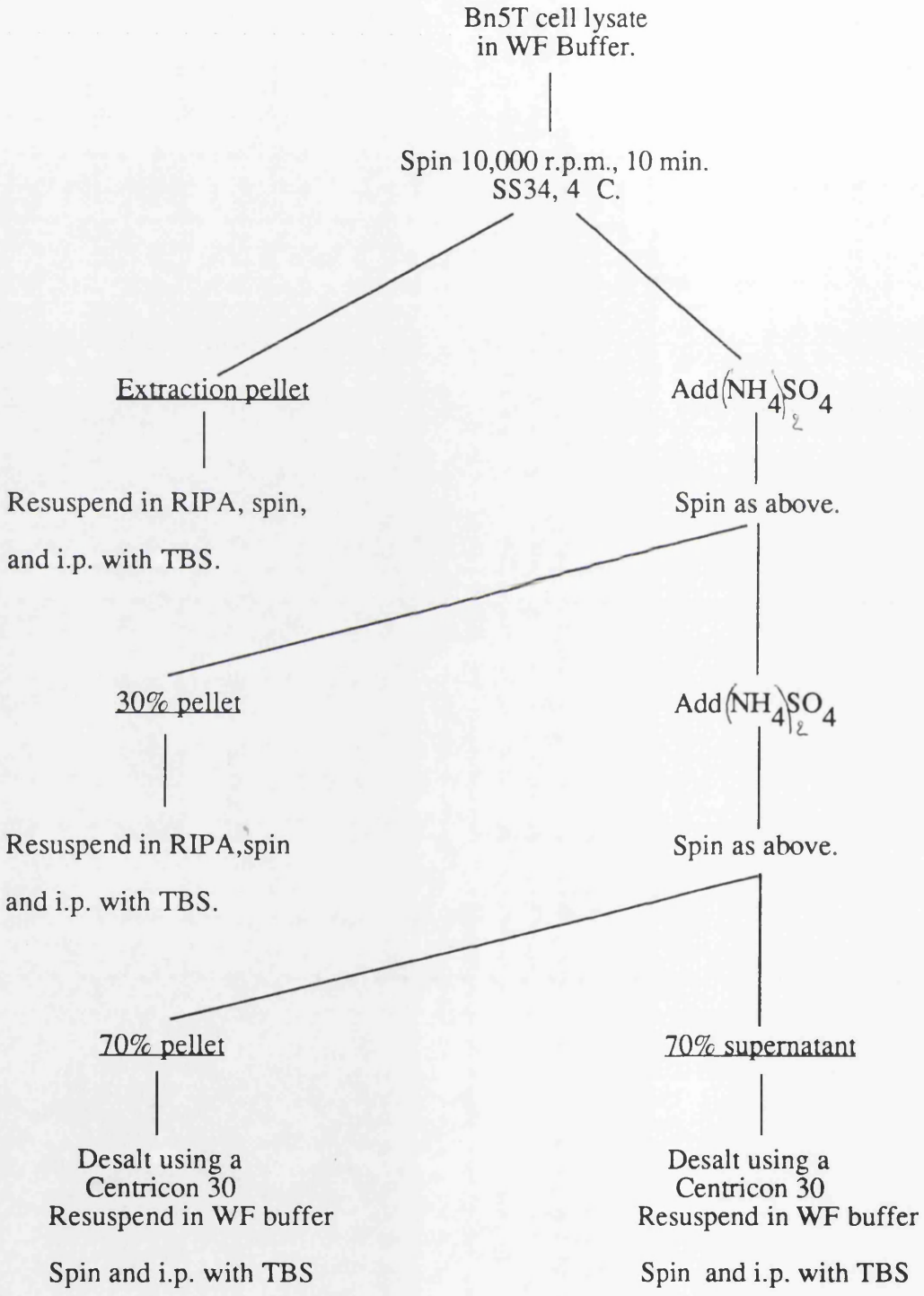

30
21
14
6
3
1
2
3

TABLE 3.5.

IMMUNOPRECIPITATION OF THE AMMONIUM SULFATE FRACTIONS.



3.4.3. 1ST PURIFICATION STEP.
AMMONIUM SULPHATE PRECIPITATION.

Precipitation of proteins by a high concentration of salt is a well established procedure. This technique is therefore applied as a preliminary procedure in protein purification. It can be used on a large amount of material, the precipitated proteins are extremely stable in $(NH_4)_2SO_4$ at 4°C and can be redissolved in a small volume to concentrate the protein and this is advantageous for stability. Its main drawback is that recovery is limited by differential solubility of the protein. The peak of recovery of proteins has a leading and a trailing fraction.

In the pilot experiments $2x10^9$ Bn5T cells (8 roller bottles and four 140mm plates labelled with [^{35}S] L-methionine) were lysed in a total volume of 12ml of WF buffer. Cells were first disrupted with a Dounce homogeniser (40 strokes), and then sonicated in a sonibath for at least 2 min. at 0°C.

The lysate was aliquoted in 1.5ml tubes and was cleared by centrifugation in an MSE Microcentaur microfuge by spinning at 13,000 r.p.m. for 10 min. (4°C). The pellet hereafter called the extraction pellet, and the supernatant were kept.

For the preliminary experiment, proteins were sequentially precipitated from the supernatant by adding NH_4SO_4 to make a 30%, 50% and 70% saturated solution. In the third experiment which is presented in figure 3.12, proteins were precipitated by a 30% saturated solution and then $(NH_4)_2SO_4$ was added to make a 70% saturated $(NH_4)_2SO_4$ solution. Precipitated proteins were pelleted by centrifugation as above, these were the 30% and 70% pellet and the supernatant was saved and called the 70% supernatant hereafter. The flow chart of the experiment is shown in table 3.5.

The extraction pellet was resuspended in RIPA buffer and sonicated for two minutes to solubilize any membrane proteins present.

An aliquot of the 30% pellet was resuspended in 1ml of RIPA buffer and desalted in a Centricon 30 tube. This tube is closed by an ultrafiltration membrane retaining protein having a molecular weight of over 30,000 Daltons. The sample is put in the top chamber and the molecules below 30KD are forced into the lower chamber by centrifugation. The volume of the sample can readily be reduced to 30ul. The proteins were desalted twice by adding 1ml of RIPA each time, and the final volume was made up to 400 ul. RIPA buffer was used because the 30% pellet could contain aggregated proteins as well as particulate material which could be redisolved by the detergents contained in RIPA buffer.

The 70% pellet was desalted by the same procedure but WF buffer was used instead of RIPA buffer.

The 70% supernatant was desalted by dialysis against 5mM NH_4CO_3 and then concentrated by freeze drying. An aliquot was resuspended in 200 ul of WF buffer.

All samples (resuspended extraction pellet, 30% pellet, 70% pellet and 70% supernatant) were centrifuged (13,000 r.p.m., 5 min., 4°C in an MSE microcentaur microfuge) to remove insoluble proteins before i.p.. An aliquot of each sample was saved to run as a control on the gel, and the remainder was used in the i.p. experiments with 50ul of TBS and 100ul of Pansorbin*.

The result of the experiments is shown in figure 3.12.

TBS precipitates a 40KD polypeptide from each fraction. To identify which polypeptide was the TBS:40, the bands were cut and digested with Staph. aureus V8 protease, and a TBS:40 band from an i.p. experiment of Bn5T cell lysate was used as a positive control.

A 90KD polypeptide was also present in the extraction pellet and in the 30 % pellet when re-extracted with RIPA. Digestion with Staph. aureus. V8 protease showed that these two polypeptides were similar to the U90 (Data not shown), a finding used to aid its purification.

Figure 3.12. 1st purification step, Ammonium sulphate precipitation.

Bn5T cells were lysed in WF buffer, insoluble material was pelletted (extraction pellet). The polypeptides of the supernatant were precipitated by a 30% and a 70% saturated solution of ammonium sulfate, resuspended in RIPA buffer or WF buffer, desalted and i.p. with TBS. The results were analysed on a 9% polyacrylamide gel and visualized by autoradiography. The polypeptides were labelled with [^{35}S] L-methionine.

Track 1 shows the MW markers.

Track 2 shows the Bn5T cell polypeptides profile.

Track 3 shows the profile of the extraction pellet which contains polypeptides insoluble in WF buffer but extracted by the detergents contained in RIPA buffer.

Track 4 shows the polypeptides of the extraction pellet i.p with TBS.

Track 5 shows the profile of the polypeptides insoluble in a 30% saturated solution of $(NH_4)_2SO_4$ (30% pellet).

Track 6 shows the polypeptides of the 30% pellet i.p. with TBS.

Track 7 shows the profile of the polypeptides soluble in a 30% but insoluble in a 70% saturated solution of $(NH_4)_2SO_4$ (70% pellet).

Track 8 shows the polypeptides of the 70% pellet i.p. with TBS.

Track 9 shows the profile of the polypeptides soluble in a 70% saturated solution of $(NH_4)_2SO_4$ (70% supernatant).

Track 10 shows the polypeptides of the 70% supernatant i.p with TBS.

The MW of the markers is at the left of the gel

The position of the U90 is indicated by an arrow and the 40KD polypeptides is indicated by a triangle.

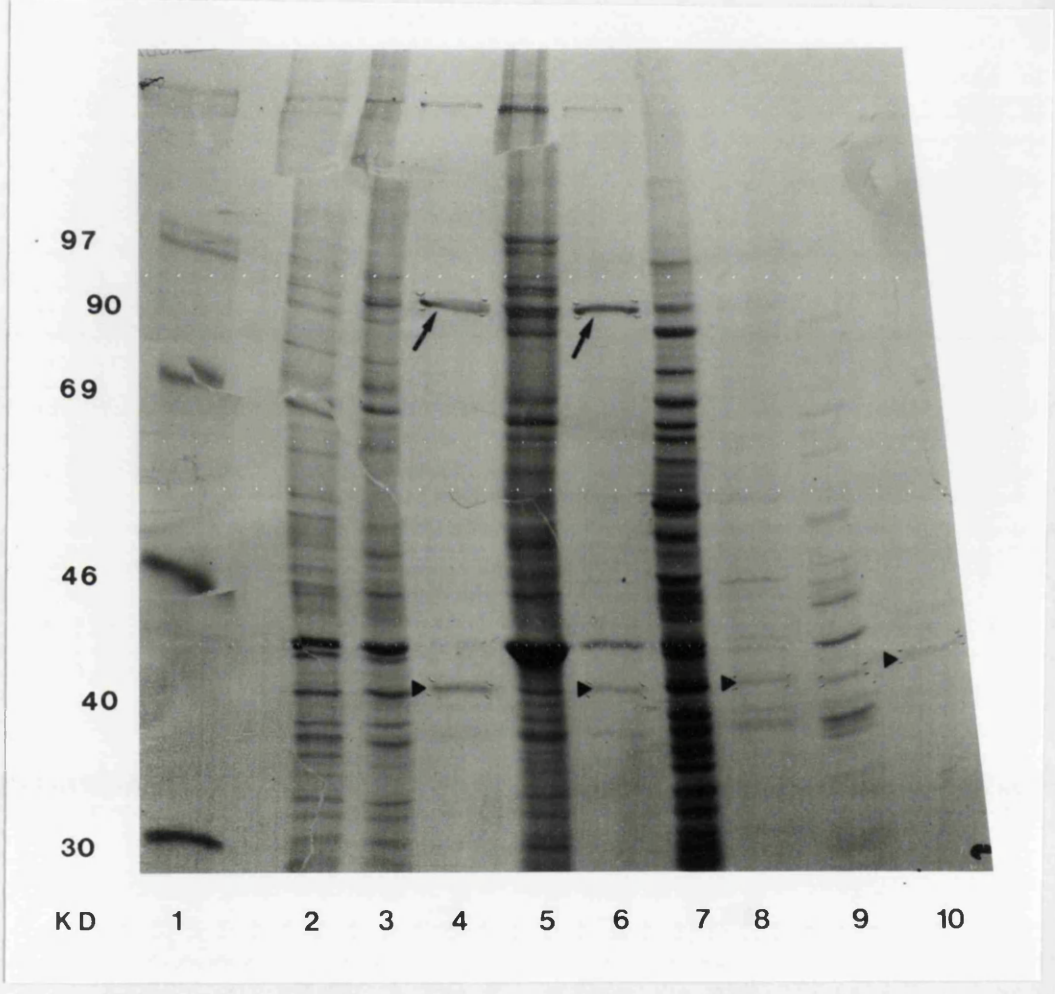

97
90
69
46
40
30
KD
1
2
3
4
5
6
7
8
9
10

Figure 3.13 shows that the peptide maps of the 40KD polypeptides i.p. by TBS in the extraction pellet, 30% pellet and 70% pellet are significantly different from the TBS:40 peptide map. However, there are fainter bands in the 30% and 70% pellet polypeptides, co-migrating with the largest peptides of the TBS:40 and the 70% supernatant 40KD.

The 40KD polypeptide precipitated by TBS in the 70% supernatant and the TBS:40 have similar peptide map patterns. The fainter bands observed in digests of the 30% and 70% pellets suggest that some of the polypeptide of interest may precipitate at a lower ammonium sulphate concentration.

These experiments showed that the 70% supernatant contains a soluble polypeptide similar to the TBS:40. This fraction could then be used for further purification. It was realized that the protein i.p. from the pellets could be a component of the TBS:40 and that the two proteins could interact with each other.

The procedure had to be modified for scaling up the purification procedure. When 60 roller bottles were used ($1.2x10^{10}$ cells) the volume of lysate used was 120ml. Fifty millilitre polycarbonate tubes were used for centrifugation in a Sorval SS34 rotor at 10,000 r.p.m. for 10 min at 4°C.

The method of desalting had to be changed. In the pilot experiments, the samples were desalted by dialysis, which resulted in considerable losses in recovery; on one occasion only 10% the radio-activity was recovered. In addition a lower ammonium sulphate concentration might improve the yield of the purified 40KD polypeptide. To increase recovery of the 40KD protein, the fractionation procedure was modified to 30%, 60% and 80% fractions. The polypeptide was then recovered in the 80% pellet, from which it was desalted on a PD10 column and could be immunoprecipitated by TBS. The protein could be preserved for three to five days at 4°C in the 80% pellet. In the experiments which follow, the 70% supernatant were first

Figure 3.13. 1st purification step, Staph. aureus V8 protease digests

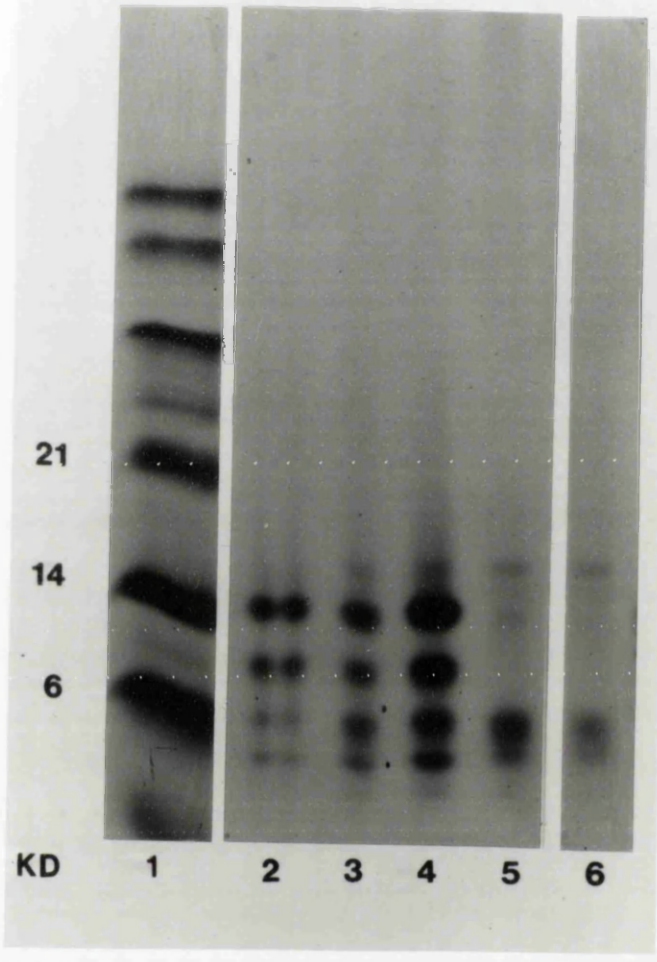
The 40KD polypeptides from the NH_4SO_4 fraction i.p. by TBS were digested by 5ug of Staph. aureus V8 protease. The results were analysed on a 15% polyacrylamide gel and visualized by autoradiography. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

Track 1 shows the MW markers.

Track 2 to 5 show the digests of the 40KD polypeptide i.p. by TBS from the extraction pellet, the 30% pellet, the 70% pellet and the 70% supernatant (bands cut from the gel shown in figure 3.11, tracks 4, 6, 8 and 10).

Track 6 shows the digest of the control TBS:40.

The MW of the markers is at the left of the gel


21
14
6
KD
1
2
3
4
5
6

investigated (figures 3.14 and 3.15). In all further experiments the 40KD protein was purified from the 60%-80% pellet.

It was verified that the supernatant of the 80% pellet contained no 40KD protein. This modification did not result in any loss of 40KD protein (Data not shown).

3.4.4. 2nd PURIFICATION STEP.

3.4.4.1. ION EXCHANGE CHROMATOGRAPHY: Anion exchange chromatography at pH.8.

Anion exchange chromatography was chosen for the second purification step. A prepacked column from the FPLC system was used.

The Mono Q prepacked HR 5/5 column was used, hereafter referred to as the Mono Q column. This column contains 1ml of the Mono Q resin, it is an anion exchange column. The capacity of this column is 25mg, and it was used for all the experiments. When the sample exceeded the capacity of the column, the sample was divided, and two to four runs were made.

The charged groups on the matrix of Mono Q are all quaternary amines. The ionic charge capacity is totally independent of the pH. over the range 2 to 12. The Mono Q column separates sample components at a pH. value of 1 pH. unit above their isoelectric point (pI.). Negatively charged sample components interact with the quaternary amines until a rising salt concentration causes the component to elute from the column.

Cells from 12 roller bottles and four 140 mm plates labelled with [^{35}S] L-methionine were used for the initial experiment ($2x10^9$ cells). The 70% supernatant was desalted by dyalisis against several washes of 10mM NH_4CO_3 and concentrated by freeze drying.

Dr A. Darling, a protein chemist working in our Institute, supplied the following method of separating proteins using the Mono Q column. The polypeptides were resuspended in 0.5ml of buffer A (50mM trisHCl pH.8), and insoluble material pelleted by centrifugation in an MSE Microcentaur centrifuge for 5 min (RT), at 13,000 r.p.m., before loading onto the Mono Q column. The void volume of the Mono Q column was set for three millilitres, the polypeptides were then eluted by a 13ml gradient of 50% buffer B (buffer A + 1M NaCl) and the Mono Q column was

finally washed by three millilitres of 100% buffer B.

To avoid overloading the Mono Q column and to set the parameters for the detector, the amount of protein applied was measured by the Bradford method. The value found was 1.2 mg, and therefore well within the capacity (25 mg) of the column. The amplification was set at 0.1.

For the initial experiments, the fractions obtained were desalted using a microdyalisis unit (Bethesda Research Laboratory), against two changes of 10 mM NH_4CO_3, concentrated by freeze drying before loading on an SDS-PAGE. Protein was lost by this method and microdyalisis was subsequently replaced by precipitation with 5% Trichloroacetic acid (TCA) using a method suggested by Dr H.Lankinen of this Institute. In these studies TCA precipitation was not found more efficient at protein recovery but was certainly faster.

In the subsequent experiments the 80% pellet was desalted by gel filtration using a PD10 column. The PD10 column was first equilibrated with buffer A. The 80% pellet was resuspended in 2.5 ml of buffer A and loaded onto the column. The proteins were eluted from the column free of $(NH_4)_2SO_4$, by 3.5 ml of buffer A.

The void volume of the Mono Q column was increased to 6ml to take into account the larger volume (3.5ml) of the sample loaded onto the Mono Q column, but the volume of the gradient remained unchanged.

The amount of protein contained in the 80% pellet was 39 mg. To avoid overloading the column the sample was divided into 4 aliquots which were each loaded separately on the column.

Figures 3.14a and b show the result of the first experiment. The polypeptides were loaded in 0.5ml buffer A (50mM trisHCl pH.8). The void volume was three millilitres and the polypeptides were eluted by a 13ml linear gradient of 50% buffer B (buffer A + 1 M NaCl). The column was washed with three millilitres of buffer B.

One millilitre fractions were collected from the 1ml Mono Q column and the polypeptides analyzed on two separate

Figures 3.14a and b. 2nd purification step, anion exchange chromatography.(opposite page and next page).

The polypeptides from the 70% supernatant were desalted concentrated and loaded in 0.5ml of buffer A (50mM trisHCl pH.8) onto the 1ml Mono Q column. The void volume was 3ml, and the proteins were eluted with a 13ml gradient of 50% buffer B (buffer A + 1M NaCl). The column was then washed with 3 ml buffer B. Each 1ml sample was desalted, concentrated and analysed on two 9% polyacrylamide gels. The polypeptides which were labelled with [^{35}S] L-methionine, were visualized by autoradiography. The track number is indicated at the bottom of the gel and the fraction number at the top of the gel.

In figure 3.14a

Track 1 shows the MW markers.

Track 2 shows a control Bn5T cells i.p. with TBS, (labelled ip).

Track 13 shows a Bn5T polypeptides profile (labelled B).

Tracks 3-12 show the polypeptides of the 1ml fractions eluted from the 1ml Mono Q column numbered 1-10 above the gels. They are eluted by:

Fractions 1-3 : Void volume.

Fractions 4-16 : Linear gradient of 50% buffer B.

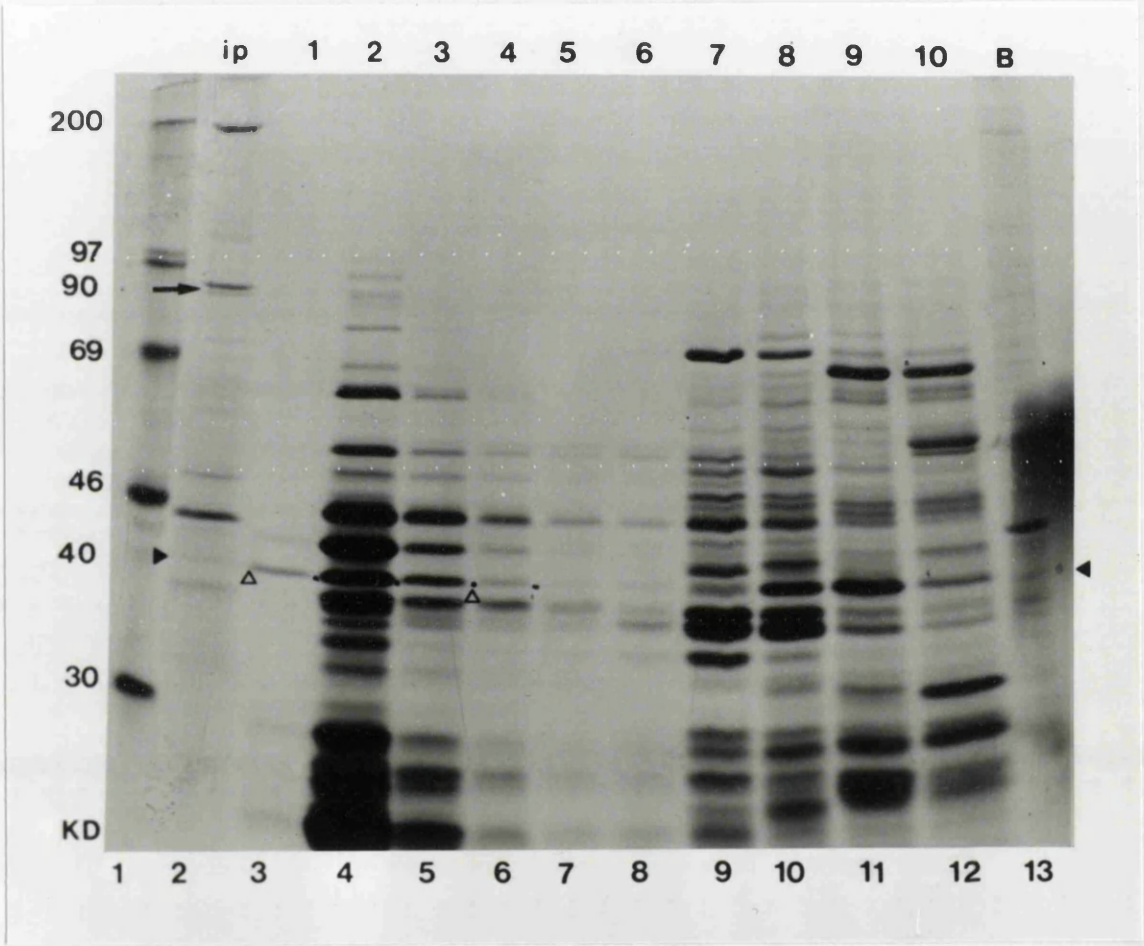
Fractions 17-19 : 100% buffer B.

The MW of the markers is at the left of the gel

The filled triangle left of track 2 shows the position of the TBS:40. Another filled triangle on the right of fraction 13 shows the position of a 40KD band . The position of the 40KD protein eluting in the void volume is indicated by two empty triangles. The position of the 90 doublet is indicated by arrows.

A

Fractions numbers



Tracks numbers

In figure 3.14b

Track 14 shows the MW markers.

Track 25 shows a control Bn5T cells i.p. with TBS, (labelled ip).

Track 15 shows a Bn5T polypeptides profile (labelled B).

Tracks 16-24 shows the polypeptides of the 1ml fractions eluted from the 1ml Mono Q column numbered 11-19 above the gel. They are eluted by:

Fractions 1-3 : Void volume.

Fractions 4-16 : Linear gradient of 50% buffer B.

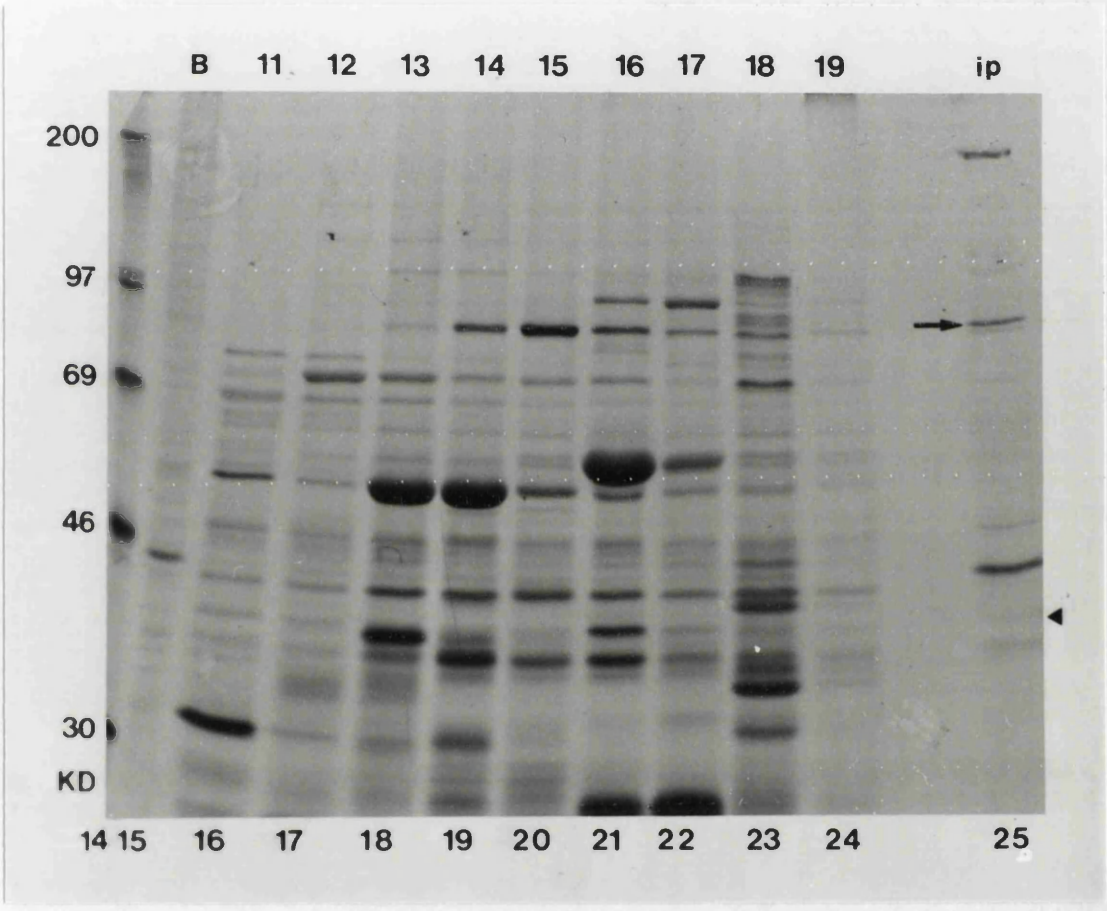
Fractions 17-19 : 100% buffer B.

The MW of the markers is at the left of the gel

The filled triangle right of track 25 shows the position of the TBS:40 band. The position of the 90 doublet is indicated by arrows.

B

Fractions numbers



Tracks numbers

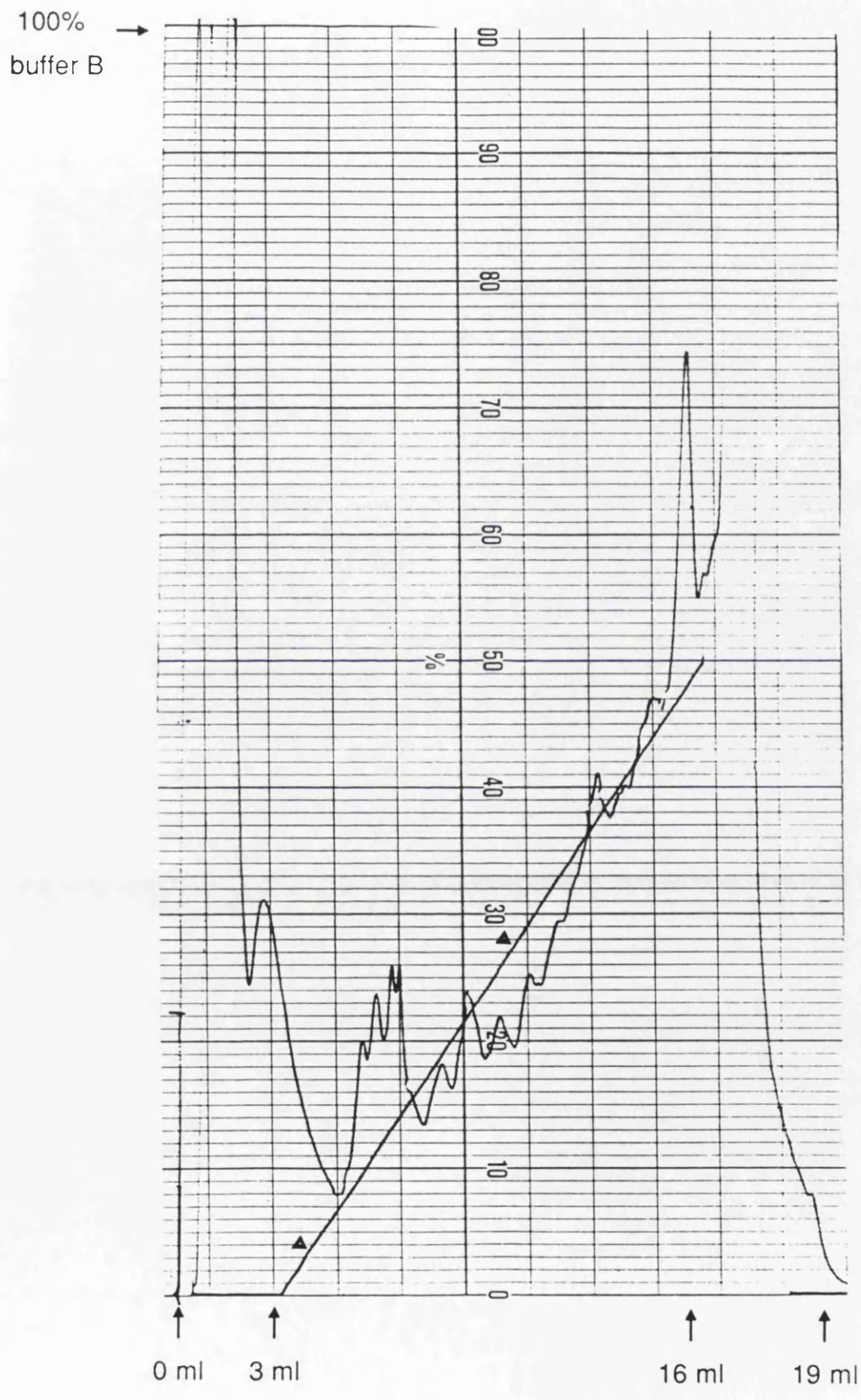
Figure 3.14c, Graph 1.

Graph showing the elution polypeptides from the 1ml Mono Q column (anion exchange chromatography at pH.8. Polypeptides were loaded in buffer A (50mM trisHCl, pH.8) and eluted by a gradient of buffer B (buffer A + 1M NaCl) as follow.

Fractions 1-3 : Void volume (0-3ml).
Fractions 3-16 : Linear gradient of 50% buffer B (3-16ml).
Fractions 17-19 : 100% buffer B (16-19ml).

The line representing the % of buffer B is indicated by triangles.

1ml fractions.
Flow rate : 1 ml/mn.
Wave length : 260nm
AUFS : 0.1


100%
buffer B
100
90
80
70
60
%
50
40
30
20
10
0
0 ml
3 ml
16 ml
19 ml

SDS-PAGE run in parallel, using the same conditions.

There was one polypeptide of 40KD in each of the 19 fractions collected. Digestion of these 40KD proteins with Staph. aureus V8 protease was used to identify the polypeptides similar to the TBS:40.

The elution profile is presented in figure 3.14c.

3.4.4.2. DIGESTION WITH STAPH. AUREUS V8 PROTEASE.

Figure 3.15 shows the results of the Staph. aureus V8 protease digest for all the 40KD bands. A 40KD band from each fraction was digested with the exception of fractions 5 6 and 19 which contained too little radio-activity to be able to be analyzed. Polypeptides could be grouped into three different patterns of peptide maps according to the following fractions: 1-4, 7, 8-18. The slight differences between the peptides map may be the results of the dimerization of proteins (H. Marsden, personal communication).

Unfortunately the positive control TBS:40 did not digest correctly. The analysis had therefore to be based on controls digested in other gels, which was less accurate but essentially feasible.

The peptide map patterns from the fractions 1 to 4, i.e. void volume are very similar to those obtained from TBS:40. This similarity was verified later. However, the polypeptides of these bands migrate with a slightly lower molecular weight than the control TBS:40.

The other digests are clearly different from the positive control TBS:40 digest.

These experiments showed that the polypeptide eluting in the void volume was probably similar to the TBS:40. This similarity had to be confirmed by other experiments.

3.4.4.3. CONFIRMATION OF THE SIMILARITY OF TBS:40 WITH THE 40KD ELUTING IN THE VOID VOLUME OF THE ANION EXCHANGE COLUMN AT pH.8.

The similarity of the 40KD eluting in the void volume and the TBS:40 was further tested by the following

Figure 3.15. 2nd purification step, Staph. aureus V8 protease digests.

The 40KD polypeptides in each fraction eluted from the anion exchange chromatography at pH. 8, (shown in figure 3.14) except fractions 5, 6 and 19 were digested by 5ug of Staph. aureus V8 protease. The results were analysed on a 15% polyacrylamide gel. The polypeptides were labelled with [^{35}S] L-methionine and visualized by autoradiography. The track number is indicated at the bottom of the gel and the fraction number at the top of the gel.

Tracks 1 and 11 show the MW markers.

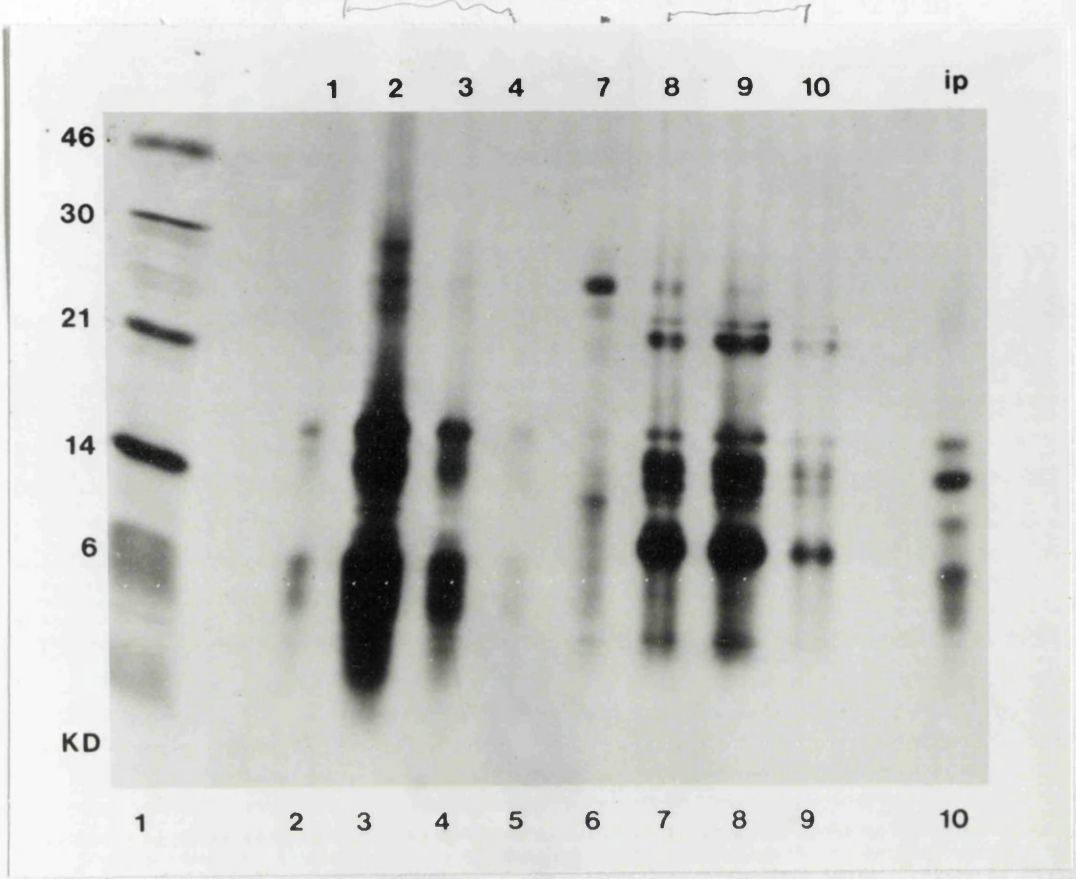
Tracks 2-9 and 12-19 shows the digest of each 40KD polypeptide.

The fraction in which the polypeptide eluted is indicated above the gel. Fractions 1-3 are the void volume.

Tracks 10 and 21 show the digest of the control TBS:40 (labelled ip).

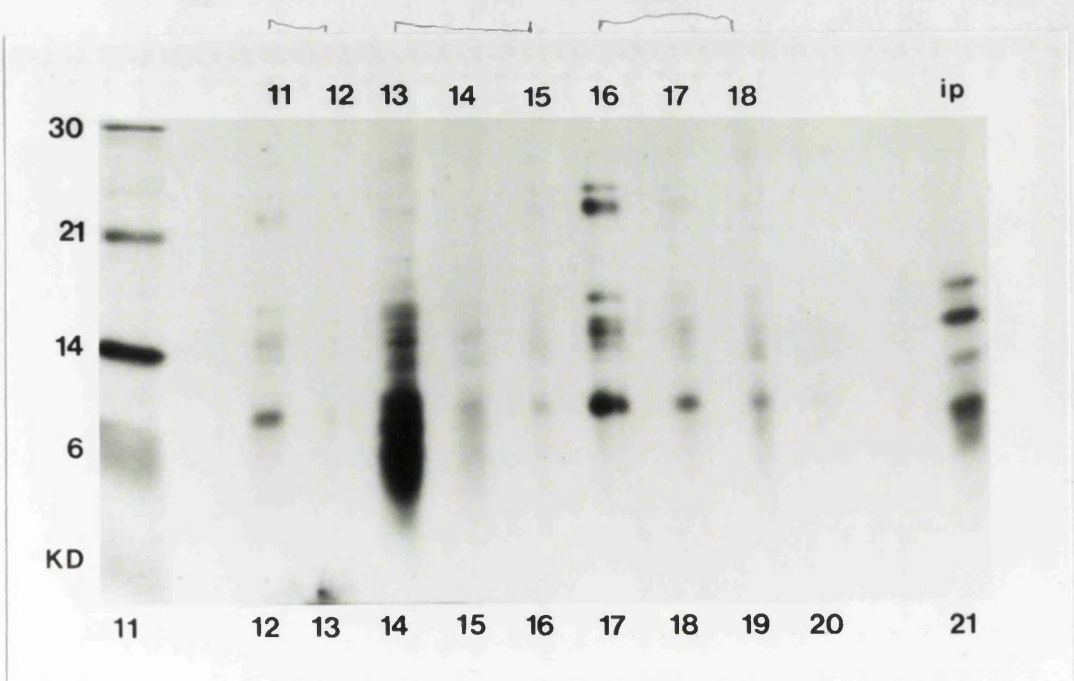
The MW of the markers is at the left of the gel

Fractions numbers



Tracks numbers

Fractions numbers



Tracks numbers

experiments :

1/ Digestion of the 40KD polypeptides with a variable amount of Staph. aureus V8 protease.

2/ Enzymatic digestion with chymotrypsin.

3/ Chemical cleavage with N-chlorosuccinimide.

4/ Immunoprecipitation of the 40KD polypeptides by TBS.

3.4.4.4. DIGESTION BY AN INCREASING AMOUNT OF STAPH. AUREUS V8 PROTEASE.

In the experiments routinely carried out and presented, in the thesis, the polypeptides were always digested with 5ug of Staph. aureus V8 protease. Digestion with lower amounts of enzyme will generate less complete digests and larger peptides, and this may highlight differences between the polypeptides.

Figure 3.16 shows the result of the digestion of four TBS:40 and four 40KD polypeptides eluting in the void volume of the Mono Q column, using an increasing amount of Staph. aureus V8 protease, namely 0.25, 0.5, 1 and 5ug.

When the polypeptide bands digested by the same amount of enzyme are compared, all the main bands are the same, but the polypeptides immunoprecipitated by TBS produce peptides of MW 39,000, 35,000 and 30,000 when digested with 0.25ug of V8, which have no counter-parts in the 40KD purified by FPLC.

All the other bands, used as controls, were digested by 5ug of Staph. aureus V8 protease.

1/ A 40KD eluting in fraction 8 has a digest clearly different from the TBS:40 digest, and therefore this polypeptide can be disregarded.

2/ A 40KD band from the 60%-80% pellet was digested for comparison with the 40KD purified by FPLC (fig 3.16.). This digest is very similar to the digest of the TBS:40, however considerable variation can be observed in the Staph. aureus digests of the 40KD protein from the flow through. (see figure 3.21a tracks 4 and 12).

Figure 3.16. 2nd purification step, Enzymatic digestion with an increasing amount of Staph. aureus V8 protease to identify the 40KD protein similar to TBS:40.

The 40KD polypeptides eluting in the void volume of the anion exchange chromatography experiment at pH.8, and TBS:40 were digested by 0.25, 0.5, 1 and 5 ug of Staph. aureus V8 protease. The 40KD polypeptides eluting in fraction 8 of the anion exchange chromatography experiments and 40KD polypeptides from a different 70% supernatant was digested with 5ug of Staph. aureus V8 protease. The results were analysed on a 15% polyacrylamide gel. The polypeptides were labelled with [^{35}S] L-methionine and visualized by autoradiography.

Track 1 shows the MW markers.

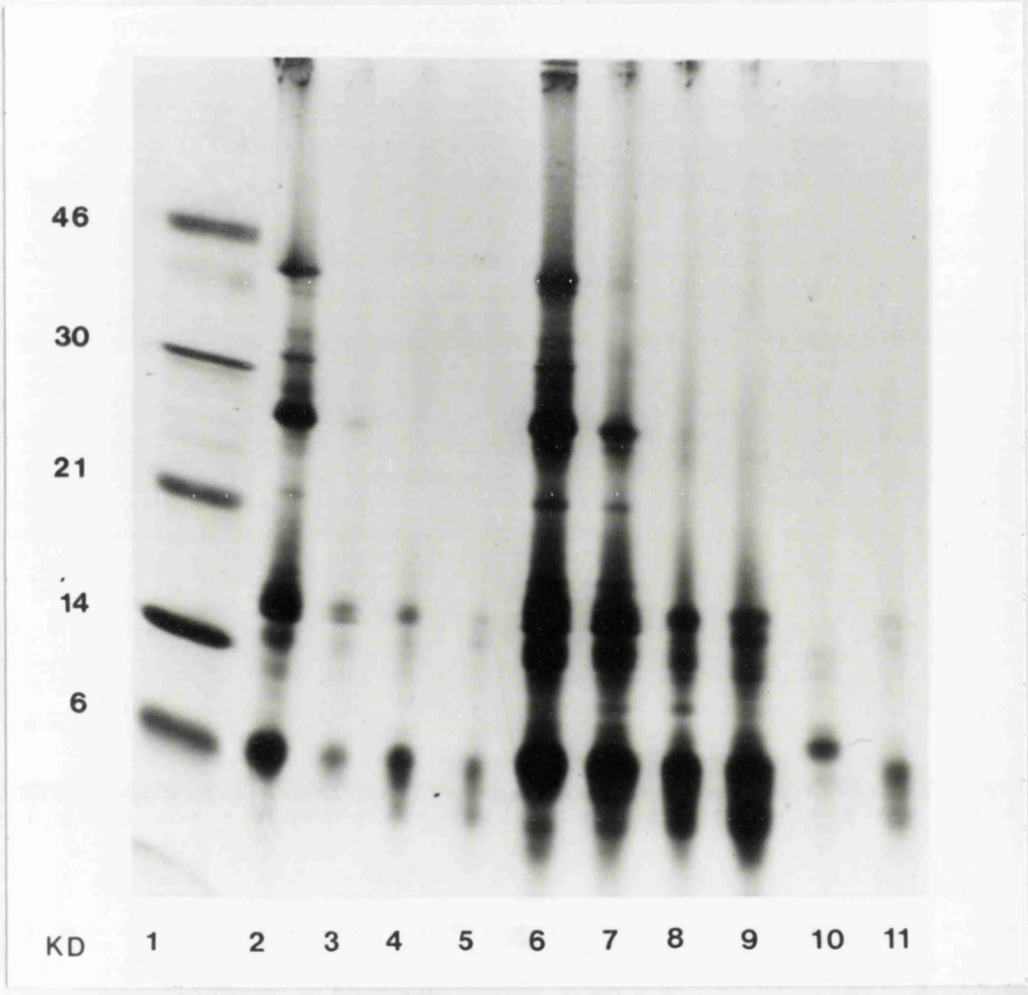
Tracks 2-5 show the digests of the 40KD polypeptides eluting in the void volume of the 1ml Mono Q by 0.25, 0.5, 1 and 5ug of Staph. aureus V8 protease.

Tracks 6-9 show the digests of the TBS:40 by 0.25, 0.5, 1 and 5ug of Staph. aureus V8 protease.

Track 10 shows the digest of the 40KD polypeptides eluting in fraction 8 of the anion exchange chromatography experiments.

Track 11 shows the digest of 40KD polypeptides from a70% supernatant.

The MW of the markers is at the left of the gel


46
30
21
14
6
KD
1
2
3
4
5
6
7
8
9
10
11

✳ Aromatic: tryptophane, tyrosine and phenylalanine.

3.4.4.5. DIGESTION BY CHYMOTRYPSIN.

The 40KD polypeptide bands were digested by chymotrypsin. Chymotrypsin cleaves the following peptide bonds: X-CO—NH_2-R. X is any amino-acid, R is an aromatic amino-acid. This enzyme was used with the same method and the same buffer as used for Staph. aureus V8 protease digestion. Five microgrammes of enzyme were used in each track.

Figure 3.17 shows the results of the experiment.

All the bands found in the FPLC track were also in the track of the TBS:40 but the FPLC track had bands with a slightly higher molecular weight. The TBS:40 track gives more bands than the FPLC:40 almost certainly because it was more highly radio-labelled. The lowest band in the i.p. track migrates with a lower apparent MW than in the FPLC track.By contrast with the i.p. track, no bands can be seen in the FPLC track between a 14KD band and the lowest band. The two peptide maps are nevertheless similar.

Figure 3.17. 2nd purification step, digestion with 5ug of chymotrypsin.

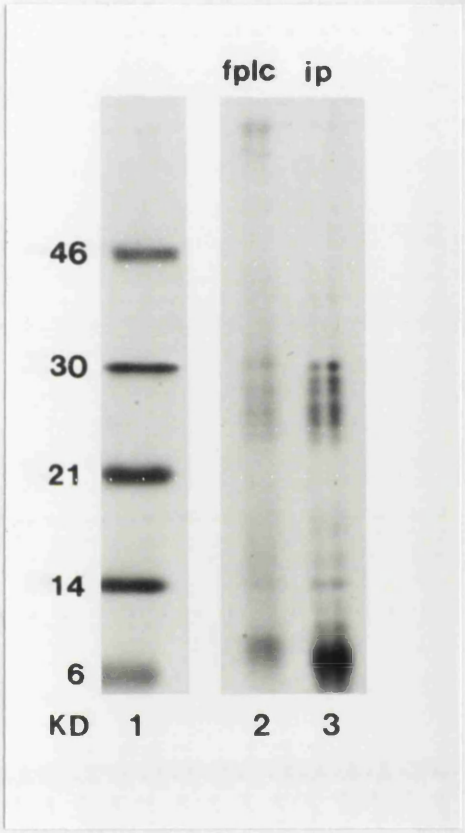
The 40KD polypeptides labelled with [^{35}S] L-methionine, eluting in the void volume of the anion exchange chromatography experiment at pH.8, and a TBS:40 were digested by 5ug of chymotrypsin. The result was analyzed on a 15% SDS-PAGE and visualized by autoradiograph. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

Track 1 shows the MW markers.

Track 2 shows the digest of the 40KD polypeptides eluting in the void volume of the anion exchange chromatography experiment at pH.8 (labelled fplc).

Track 3 shows the control digest of a TBS:40 (labelled ip).

The MW of the markers is at the left of the gel


fplc
ip
46
30
21
14
6
KD
1
2
3

3.4.4.6. CLEAVAGE BY N-CHLOROSUCCINIMIDE.

The method used was developed by Lischwe and Ochs (1982).N-chlorosuccinimide cleaves peptide bonds Trp-COO-NH2-X, X being any amino-acid. Because tryptophan is a rare amino-acid the result enhances similarities.

Figure 3.18 shows the result of digestions with N-chlorosuccinimide.

The experiment confirmed the similarity of the TBS:40 and the 40KD eluting in the void volume of the Mono Q column at pH 8. There are only two main bands of 30 and 8 KD in addition to the 40KD band. The 8KD band is slightly higher in the TBS:40 track and may consist of one band as opposed to two seen in the FPLC track. Fainter bands of 25, 16 and 14KD are visible in both tracks. It is of interest that the U90 polypeptide did not resemble the FPLC:40 by this digestion protocol which shows similarities in protein rather than differences (data not shown).

Figure 3.18. 2nd purification step, cleavage with N-chlorosuccinimide.

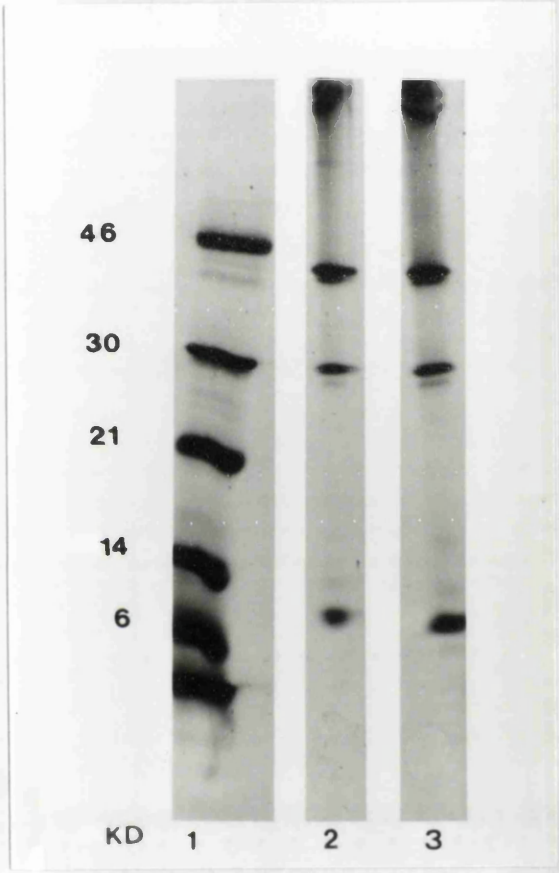
The 40KD polypeptides eluting in the void volume of the anion exchange chromatography experiment at pH.8, and a TBS:40 were cleaved with N-chlorosuccinimide. The results were analysed on a 15% polyacrylamide gel. The polypeptides were labelled with [^{35}S] L-methionine and visualized by autoradiography. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

Track 1 shows the MW markers.

Track 2 shows the cleavage of the 40KD polypeptides eluting in the void volume of the anion exchange chromatography experiment at pH.8

Track 3 shows the cleavage of the TBS:40.

The MW of the markers is at the left of the gel


46
30
21
14
6
KD
1
2
3

3.4.4.7. IMMUNOPRECIPITATION BY TBS.

Polypeptides from the anion exchange chromatography fractions 2 (void volume), 8 and 14 were i.p. by TBS. Before freeze drying the three fractions were each divided into two tubes, 600 ul were used for i.p. and 400ul were analysed on a separate gel, run in parallel, together with all the other fractions of the anion exchange chromatography experiment at pH.8 (not shown).

Fraction 8 was chosen as a control to further confirm that the 40KD band in this fraction was not related to the TBS:40, and also because a 34KD band was noticed which might be related to the 34KD polypeptide often seen in i.p. by TBS from Bn5T cells (Hewitt, 1988).

Fraction 14 was chosen as a second negative control.

Figure 3.19 shows the result of the i.p. experiments.

TBS immunoprecipitated a 40KD polypeptide only in fraction 2, which is a fraction of the void volume.

TBS immunoprecipitated a 34KD band in fraction 8, no 40KD band was immunoprecipitated in either fractions 8 or fraction 14.

The flow through was therefore considered to contain the 40KD protein i.p. by TBS.

Figure 3.19. 2nd purification step, i.p. by TBS of the fractions from the anion exchange chromatography at pH.8.

The fractions 2, 8 and 14 from the anion exchange chromatography at pH.8 were concentrated, desalted and i.p. by TBS. Bn5T tumour cells and RE cell extracts were i.p. by TBS as positive and negative controls. The results were analysed on a 9% polyacrylamide gel. The polypeptides were labelled with [^{35}S] L-methionine and visualized by autoradiography. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

Track 1 shows the MW markers.

Track 2 shows the Bn5T cell polypeptides profile (labelled Bn5T).

Track 3 shows Bn5T cells i.p. with TBS (labelled Bn5T ip).

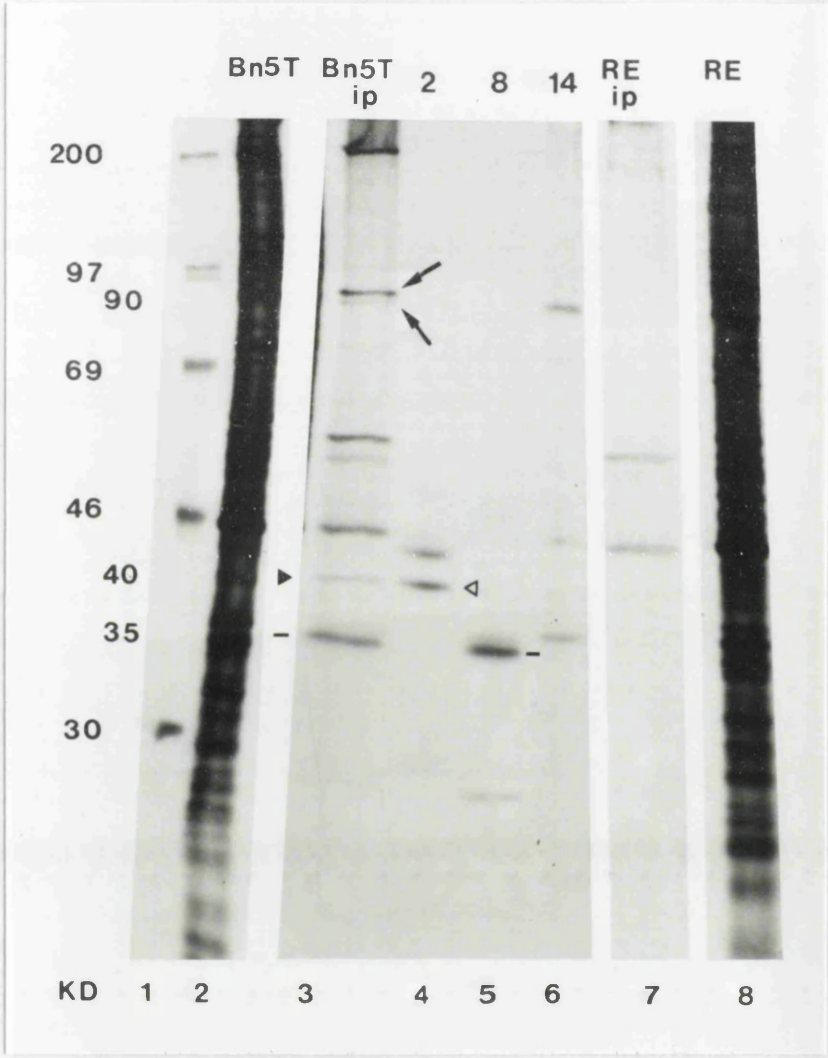
Tracks 4-6 show the i.p. of fractions 2, 8 and 14 respectively i.p. by TBS.

Track 7 shows RE control cells i.p. with TBS (labelled RE ip).

Track 8 shows the RE control cell polypeptides profile (labelled RE).

The MW of the markers is at the left of the gel.

The position of the U90 and L90 is shown by arrows. A filled triangle shows the position of the TBS:40 and an empty triangle the position of the 40KD polypeptide i.p. from fraction 2. Two horizontal strokes - indicates the position of 34KD polypeptides. (Such proteins have been previously i.ped. by TBS from Bn5T cells)


Bn5T
Bn5T ip
2
8
14
RE ip
RE
200
97
90
69
46
40
35
30
KD
1
2
3
4
5
6
7
8

3.4.4.8. THE 2ND PURIFICATION STEP: CONCLUSIONS

The previously described experiments demonstrated a great similarity between the TBS:40 and the 40KD band eluting in the void volume (fraction 2) of the Mono Q column at pH.8. This band will be called FPLC:40. The variable intensity of the peptides generated by Staph. aureus V8 protease digestion of the TBS:40 suggested that the TBS:40 band was made up of several polypeptides. The variation in the intensity of the peptide bands could be the result of differences in the relative amounts of these 40KD polypeptide i.p. by the different TBS samples. To confirm this hypothesis the components of the FPLC:40 which appears to contain the same polypeptides as the TBS:40 were further purified.

3.4.5. THE 3rd PURIFICATION STEP.

Four methods were tried to further purify the FPLC:40. They are described in the following section.

3.4.5.1. 3rd PURIFICATION STEP: CATION EXCHANGE CHROMATOGRAPHY.

The Mono S prepacked HR5/5 (Pharmacia FPLC system) hereafter referred as the Mono S column contains 1ml of Mono S beads. Mono S beads share the same matrix as Mono Q beads but the reactive groups are sulphonyl groups instead of quaternary amine groups. The sulphonyl groups interact with proteins having a net positive charge. The Mono S is effective in separating sample components with pH. values greater than 1 pH. unit below their pI. Attempts were made to use the Mono S column to further purify the TBS:40.

The void volume of the anion exchange chromatography purification step was desalted by dialysis and concentrated by freeze drying. The proteins were resuspended in 500 ul of buffer G (50mM sodium phosphate buffer, Na_2HPO_4 - NaH_2PO_4 pH 7.2). The protein was loaded onto the 1ml Mono S column. The total void volume was 3ml; the proteins were then eluted with a 13ml gradient of 50% buffer H (Buffer G + 1M NaCl). Any remaining proteins were finally eluted by 3ml of buffer H.

Most of the polypeptides did not bind to the column including the FPLC:40, and no separation was achieved. The experiment was repeated twice with the same poor results, and finally abandoned.

Although theoretically it should have been possible to lower the pH. of the buffer so that the 40KD would bind to the column, this was not done. This decision was made on the advice Dr Joe Connor, protein chemist working in this Institute who had obtained good separations using the Mono Q columnn at higher pH. values. This system will be described later (section 3.4.5.3.1).

3.4.5.2. 3rd PURIFICATION STEP: ELECTROFOCUSING.

Electrofocusing separates proteins according to their isoelectric point (pI.). The aim of this experiment was to separate the proteins and obtain an approximation of their pI.. The machine Rotofor* (Bio Rad) is sold for preparative electrofocusing. This machine consists of a rotating horizontal column divided by several porous nylon filters.

The sample (Bn5T polypeptides eluting in the void volume of the anion exchange experiment at pH.8), 5.5ml in buffer A, 1.5ml of ampholines (Amphoteric molecules used as buffer), 6ml of glycerol and 45ml of H_2O were loaded in the column. When an electric field is applied to the column, a pH. gradient forms, and the proteins migrate in the column toward the part of the column whose pH. equals their pI.. The nylon diaphragm limits the diffusion of the proteins and defines chambers which can be harvested separately.

There is no set running time for the experiment, the experiment is run at constant power (12 watts) and is stopped when the voltage reaches a plateau. In this experiment the voltage reached a plateau of 1200 Volts.

The fractions were harvested, the pH. of each fraction was measured, a pH. gradient between pH.2 and pH.10 had formed. The fractions were desalted by precipitation with 10 volumes of methanol and loaded onto an SDS-PAGE, with a standard TBS i.p. as control. The autoradiograph of the gel showed that all the fractions contained the same bands including the 40KD band. Therefore the result was merely a dilution of the protein.

The experiment was not repeated although the experimental conditions could have been adjusted according to the manufacturer's suggestions. This was not carried out because separation at high pH. on a Mono Q column was being tested and its result looked promising (see next section).

3.4.5.3. 3rd PURIFICATION STEP: ANION EXCHANGE CHROMATOGRAPHY AT pH. 9.5.

3.4.5.3.1. Description and results of the anion exchange chromatography experiment at pH 9.5.

Anion exchange chromatography was used again, but the pH. of the buffer was increased to 9.5 and the ionic strength of the buffer was decreased to 20mM to try to retain the polypeptides on the column.

The buffer A in which the polypeptides of the void volume were disolved was changed to buffer C (20 mM Ethanolamine pH 9.5) using a PD10 column. The polypeptides were loaded on the 1ml Mono Q column in 3,5ml buffer C with a total void volume of 6ml, and eluted with a 13ml linear gradient of 50% buffer D (buffer C + 1 M NaCl). The column was then washed with 100% buffer D.

The gradient was linear for the first experiment. (Data not shown). Examination of the autoradiograph showed that a 40KD polypeptide eluted in the void volume and also ahead of all the other polypeptides when the salt gradient was applied. When the percentage of buffer D reached 15 %, a stronger 40KD band, of slightly higher molecular weight, was seen which trailed through the remaining fractions. Examination of the graph showed that most of the polypeptides were eluted by 20% buffer D.

Using these data the gradient was modified to obtain a better resolution of the polypeptides. Step gradients using 3ml of 2.5%, 5% and 7.5% buffer D were introduced between the void volume and the linear gradient of buffer D. After four preliminary experiments the gradients were fixed as follow; 0-5ml void volume, 6-9ml 2.5% buffer D, 10-13ml 5%, 14-17ml 7.5%, 17-32ml linear gradient of 50% buffer D and 32-35ml 100% buffer D.

In the experiment presented in figure 3.20a (3rd experiment), the void volume is 9ml and the gradient is only 6ml with 25% buffer B. The elution profile is presented in figure 3.20b.

A 40KD polypeptide eluted in fraction 2 (void volume) and from fraction 17 onwards. 40KD bands from this gel were cut out and digested with Staph. aureus V8 protease.

Figure 3.20a. 3rd purification step, anion exchange chromatography at pH. 9.5.

The polypeptides eluting in the void volume of the anion exchange chromatography at pH. 8 were concentrated and the buffer was changed to buffer C (20mM Ethanolamine pH 9.5). The polypeptides were loaded onto a 1ml Mono Q column in 3.5ml buffer C (20 mM Ethanolamine pH. 9.5) and eluted by a linear gradient of buffer D (Buffer C + 1M NaCl) interupted by 3ml steps as follow : Fractions 1-9, void volume; fractions 12-14, step of 2.5% buffer D fractions 16-18, step of 5% buffer D; fractions 20-22, step of 7.5% buffer D then fractions 23-28 linear gradient of 25% buffer D . The column was washed by 4 ml of buffer D. The results were analysed on a 9% polyacrylamide gel. The polypeptides were labelled with [^{35}S] L-methionine and visualized by autoradiography.

Track 1 shows the MW markers.

Track 2 shows the fraction 2 (void volume).

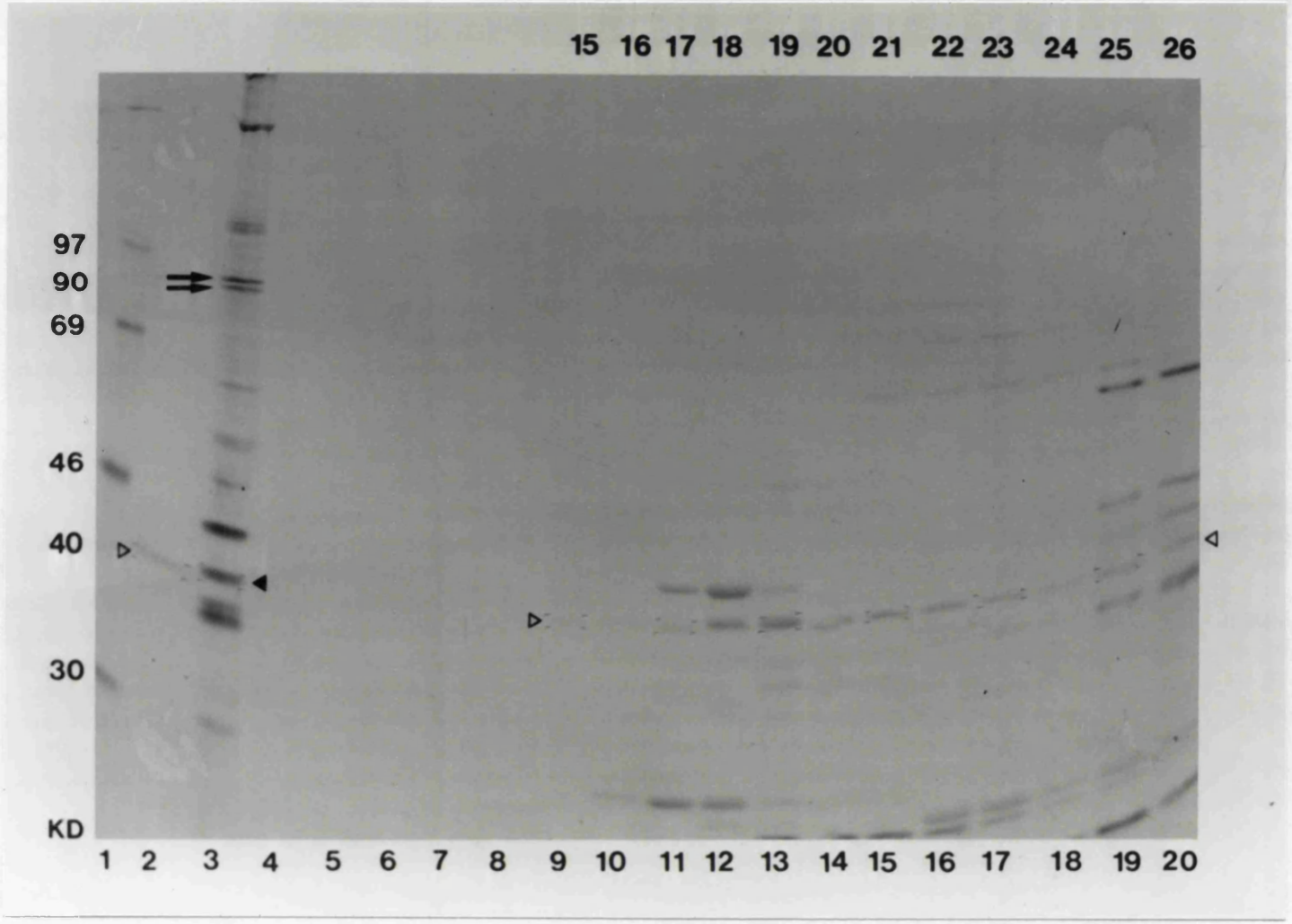
Track 3 shows Bn5T cells i.p. with TBS.

Tracks 4-20 show the polypeptides eluted by the gradient of buffer D, from fraction 10 onwards. Polypeptides of 40KD MW are detected from fraction 15 onwards. The fraction number is indicated at the top of the gel.

The MW of the markers is at the left of the gel

The position of the 40KD polypeptides is indicated by empty triangles and the position of the TBS:40 by a filled triangle. Two arrows show the U90 and the L90.

Fractions numbers



Tracks numbers

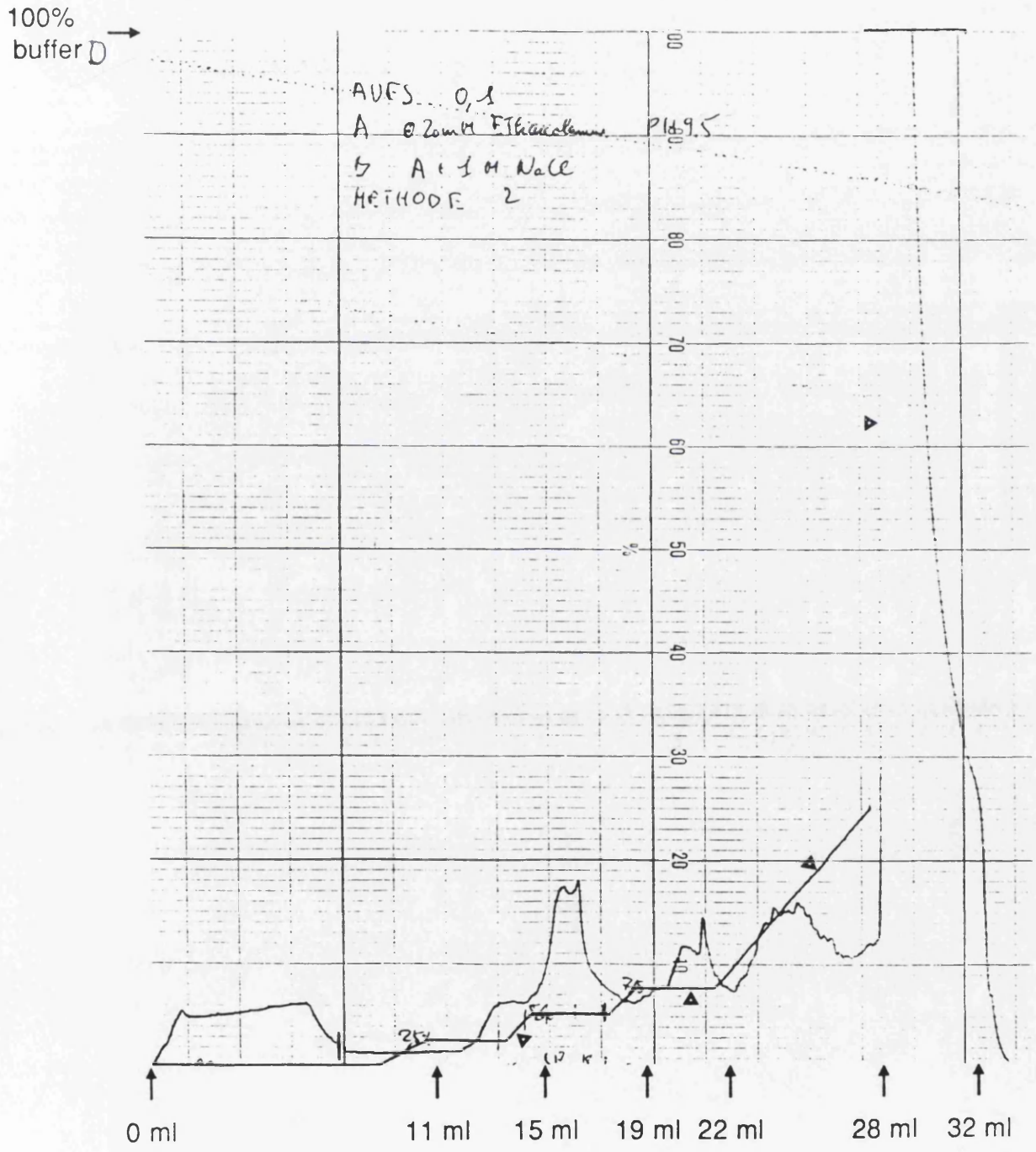
Figure 3.20b. Graph 2.

Graph showing the elution profile of polypeptides from the 1ml Mono Q column (anion exchange chromatography at pH.9.5). Polypeptides were loaded in buffer C (20mM ethanolamine pH.9.5) and eluted by a gradient of buffer D (buffer C + 1M NaCl) as follow.

Fractions 1-9 : Void volume (0-9ml).
Fractions 10-11 :linear gradient of 0% to 2.5% buffer D.
Fractions 12-14 : 2.5% buffer D (11-14ml).
Fraction 15: linear gradient of 2.5% to 5% buffer D.
Fractions 16-18 : 5% buffer D (15-18ml).
Fraction 19: linear gradient of 5% to 7.5% buffer D.
Fractions 20-22 : 7.5% buffer D (19-22ml).
Fractions 23-28 : linear gradient of 25% buffer D (22-28ml).
Fractions 28-32 : 100% buffer D (28-32ml).

The line representing the % of buffer D is indicated by triangles.

1ml fractions.
Flow rate : 1.5 ml/mn.
Wave length : 260nm
AUFS : 0.1


100%
buffer D
AUFS 0,1
A 0,2 mM Ethanolamin pH 9,5
B A + 1 M NaCl
METHODE 2
0 ml
11 ml
15 ml
19 ml
22 ml
28 ml
32 ml

3.4.5.3.2. Enzymatic digestion with Staph. aureus V8 protease.

The results of the anion exchange chromatography experiment at pH.9.5 indicated the possible existence of two 40KD polypeptides. One polypeptide eluted in the void volume at a low salt concentration, and a second polypeptide eluted with a salt concentration of 0.15M onward. To confirm the existence of two different polypeptides the bands were cut out, digested with Staph. aureus V8 protease and compared with digests of a TBS:40

Figure 3.21a shows that two polypeptides both eluting in the flow through of the anion exchange experiment at pH.8 are separated by anion exchange chromatography at pH 9.5.. They are characterized by different peptide maps.

One elutes in the void volume(fraction 2, track 5) and is eluted by 5% buffer D (fractions 15 and 17, tracks 6 and 7). The digest is composed of 3 main bands of molecular weight 10, 7 and 5KD.

Another protein elutes from fractions 19 to 23 (tracks 8, 10 and 11). The peptide map is composed of a group of 3 bands of molecular weight 14KD and over, and a band of about 6KD.

The 40KD protein eluting in the void volume and at low salt concentration will be referred to hereafter as the "VOID VOLUME 40" whereas the 40KD protein eluting from the column will be referred as the "COLUMN 40".

The peptide maps of the "VOID VOLUME 40" and of the "COLUMN 40" are both different from the peptide map of the TBS:40. Four TBS:40 digests are shown in figure 3.21, they illustrate the different intensity of the bands discussed in section 3.2.1. and allow the comparison with the digests of the "VOID VOLUME 40" and "COLUMN 40" digests. This comparison suggests that each band of both peptide map could have a homologue in the peptide map of the TBS:40. The peptide maps of the "VOID VOLUME 40" and the "COLUMN 40" shown in figure 3.21b were superposed and photographed but the result was not clear enough to show the homology with the peptide map of a TBS:40. The TBS:40 peptide map

Figures 3.21a and b. 3rd purification step, Staph. aureus V8 protease digests.(opposite page and next page)

The 40KD polypeptides in fractions eluted from the anion exchange chromatography experiment at pH. 9.5 were digested by 5ug of Staph. aureus V8 protease. The results were analysed on 15% polyacrylamide gels. The polypeptides were labelled with [^{35}S] L-methionine and visualized by autoradiography.

Figure 3.21a

Track 1 shows the MW markers.

Tracks 2, 3, 9 and 13 show the digests of control TBS:40 (labelled ip).

Tracks 4 and 12 show the digests of polypeptides eluted from the void volume of the anion exchange experiment at pH. 8 (FPLC:40, labelled ft).

Tracks 5, 6, 7, 8, 10 and 11 show the digests of the 40KD polypeptides eluted from the anion exchange chromatography experiments at pH 9.5 in fractions 2, 15, 17, 19, 21 and 23 of the experiment presented in the figures 3.20 a and b, as labelled on top of the gel.

Fraction 2 is one of the fractions of the void volume.

Fraction 15 contains polypeptides eluted by 2.5 to 5% buffer D.

Fraction 17 contains polypeptides eluted by 5% buffer D.

Fraction 19 contains polypeptides eluted by 5 to 7.5% buffer D.

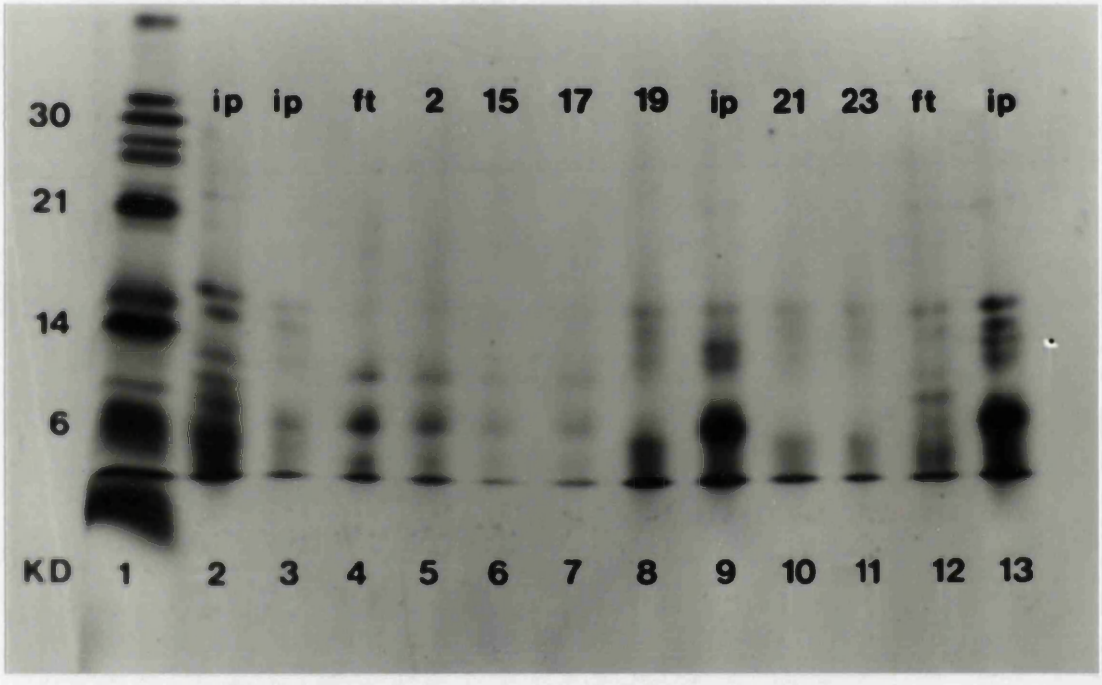
Fraction 21 contains polypeptides eluted by 7.5% buffer D.

Fraction 23 contains polypeptides eluted by over 7.5% buffer D.

The MW of the markers is at the left of the gel.

A

Fractions numbers



Tracks numbers

Figure 3.21b

In another experiment the 40KD proteins eluting in the void volume and in fraction 20 of an anion exchange chromatography experiment at pH. 9.5 were digested by 5ug of Staph. aureus V8 protease and compared to the digest of a TBS:40. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

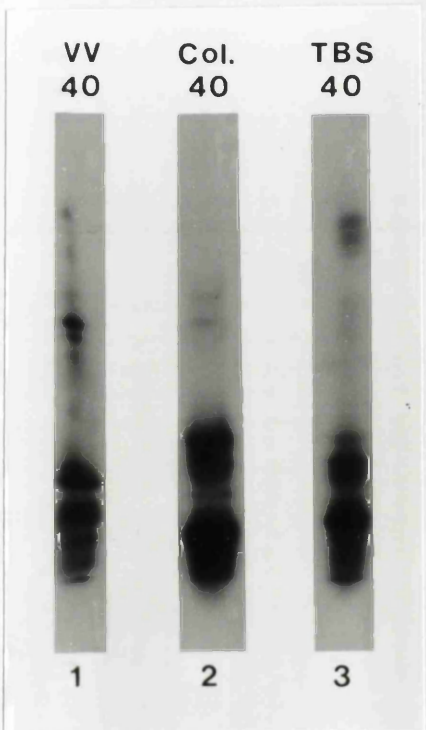
Track 1 shows the digest of the 40KD protein eluting in the void volume (labelled VV 40).

Track 2 shows the digest of the 40KD protein eluting in fraction 20 (labelled Col. 40).

Track 3 shows the digest of a TBS:40 (labelled TBS:40).

This figure shows that the peptide map pattern of the TBS:40 could be the result of the combination of the patterns of the "VOID VOLUME 40" and the "COLUMN 40".

B



could nevertheless result from the digestion of a combination of the two 40KD proteins which i.p. separately or together as a complex with TBS. I.p experiments were set up to determine which protein was i.p. by TBS.

3.5.4.3.3. Immunoprecipitation by TBS of THE "COLUMN 40" and the "VOID VOLUME 40".

After the experimental conditions of the anion exchange chromatography experiment at pH. 9.5 were definitly set up, examination of the Coomassie blue stained gels showed that the "VOID VOLUME 40" always eluted in the void volume,(but trails in the following fractions). The "COLUMN 40" started to elute in fraction 14 (7.5% b uffer D) with a maximum in fraction 20.

The early experiments were unfortunately failures, the proteins were present in very small amounts and were lost on changing the buffer in Centricon tubes to RIPA. I decided to i.p. the proteins without changing the buffer (Buffer C) or concentrating the sample.

Finally it was verified, in a separate experiment, that the 40KD polypeptide was immunoprecipitated by TBS from Bn5T cell lysates extracted in buffer C (20 mM Ethanolamine pH.9.5). To do this the cells from two 140mm plates were labelled with 40ul of [^{35}S] L-methionine. One of the plates was harvested in 1ml of RIPA buffer and the other in 1 ml of Buffer C. The radio-activity of a 1ul aliquot of each extract was counted. The results were: RIPA buffer, $6x10^5$ c.p.m. and buffer C, $4x10^5$. I.p. experiments were set up with 7.5ul of RIPA extact and 11ul of buffer C extract and 5ul of TBS, The immune complexes were captured with 60ul of Pansorbin* and analysed as presented in figure 3.22. TBS i.p. a 40KD protein in this buffer, however one of the proteins may not be i.p. in this buffer.

The i.p. experiment was set up using two fractions of the anion exchange chromatography at pH. 9.5:

Fraction 2 (void volume) to test the "VOID VOLUME 40".

Figure 3.22. Immunoprecipitations of Bn5T tumour cells polypeptides extracted in RIPA buffer and buffer C (20mM ethanolamine pH. 9.5) with TBS.

Autoradiograph of a 9% polyacrylamide gel which shows i.p. experiments performed on [^{35}S] L-methionine labelled polypeptides extracted in RIPA buffer and in buffer C from Bn5T tumour cells with TBS. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

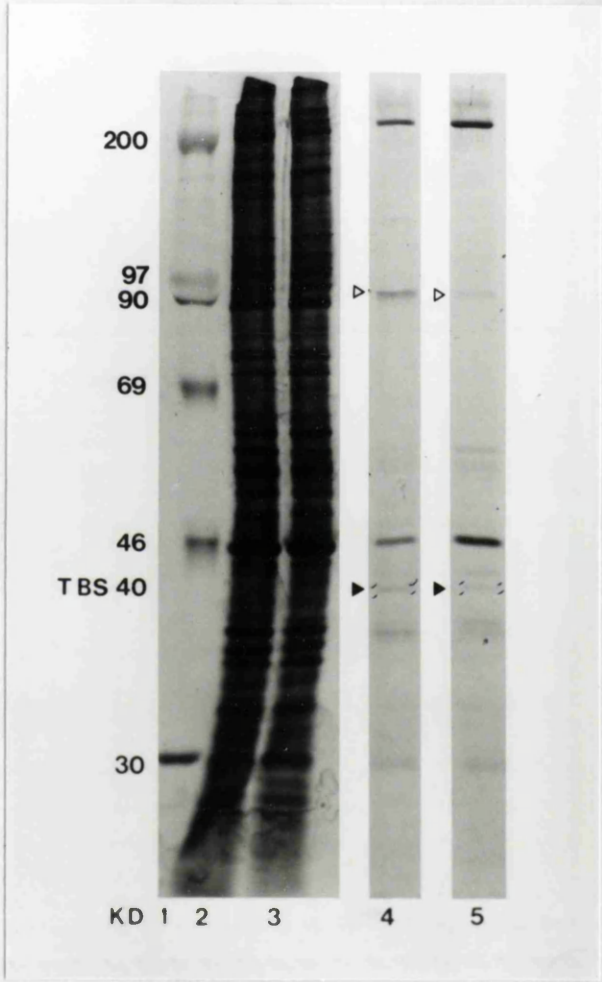
Track 1 shows the MW markers.

Tracks 2 and 3 show the Bn5T cell polypeptides profile extracted in RIPA buffer and buffer C respectively.

Tracks 4 and 5 show the Bn5T cell polypeptides extracted in RIPA buffer and buffer C respectively, i.p. by the same TBS.

The position of the 90KD doublet is shown by two empty triangles and the position of the TBS:40 by two filled triangles.

The MW of the markers is at the left of the gel


200
97
90
69
46
TBS 40
30
KD 1 2 3 4 5

Fraction 20 to test the "COLUMN 40".

Two positive controls were included:

1/ A Bn5T extract in RIPA for a standard i.p. experiment.

2/ A 1ml fraction of the void volume of the anion exchange experiment at pH.8., which is the substrate of the anion exchange experiment at pH. 9.5.

Fifty microlitres of TBS was added to improve the precipitation of the polypeptides because it was estimated that the low radio-activity did not reflect the total quantity of protein present.

Figure 3.23 shows the result of the experiment.

A 40KD is present in both fractions 2 (track 5) and 20 (track 7), however, the 40KD protein in fraction 2 has a slightly lower MW than the protein i.p. from the control track.

The i.p. tracks showed:

TBS i.p. a 40KD protein from the two positive controls; a Bn5T cell extract (track 2) and a fraction of the void volume of the anion exchange experiment at pH. 8 (track 4).

A 40KD protein is i.p. by TBS in fraction 2 but only faintly, and at a lower MW than in the protein profile of fraction 2.

The "COLUMN 40" in fraction 20 (track 8), is strongly i.p. by TBS.

The "VOID VOLUME 40" was identified by AA sequencing (see sections 3.5.4. and 3.5.5.) as the mitochondrial aspartate aminotransferase and two proteins were identified from the "COLUMN 40" 1-6 diphosphate aldolase A and phosphoglycerate kinase (see section 3.5.8.1.). The preparations used for sequencing the "COLUMN 40" were not

Figure 3.23. 3rd purification step, i.p. by TBS of the fractions from the anion exchange chromatography at pH.9.5.

The fractions from the anion exchange chromatography experiment at pH. 9.5 2 containing the "VOID VOLUME 40" and 20 containing the "COLUMN 40" are i.p. by TBS. A fraction from the void volume of the anion exchange chromatography experiment at pH. 8 is i.p. by TBS and a standard i.p. are used as controls. The results were analysed on a 9% polyacrylamide gel. The polypeptides were labelled with [^{35}S] L-methionine and visualized by autoradiography. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

Track 1 shows the Bn5T cell polypeptides profile (labelled Bn5T).

Track 2 shows Bn5T cells i.p. with TBS (labelled Bn5T i.p.).

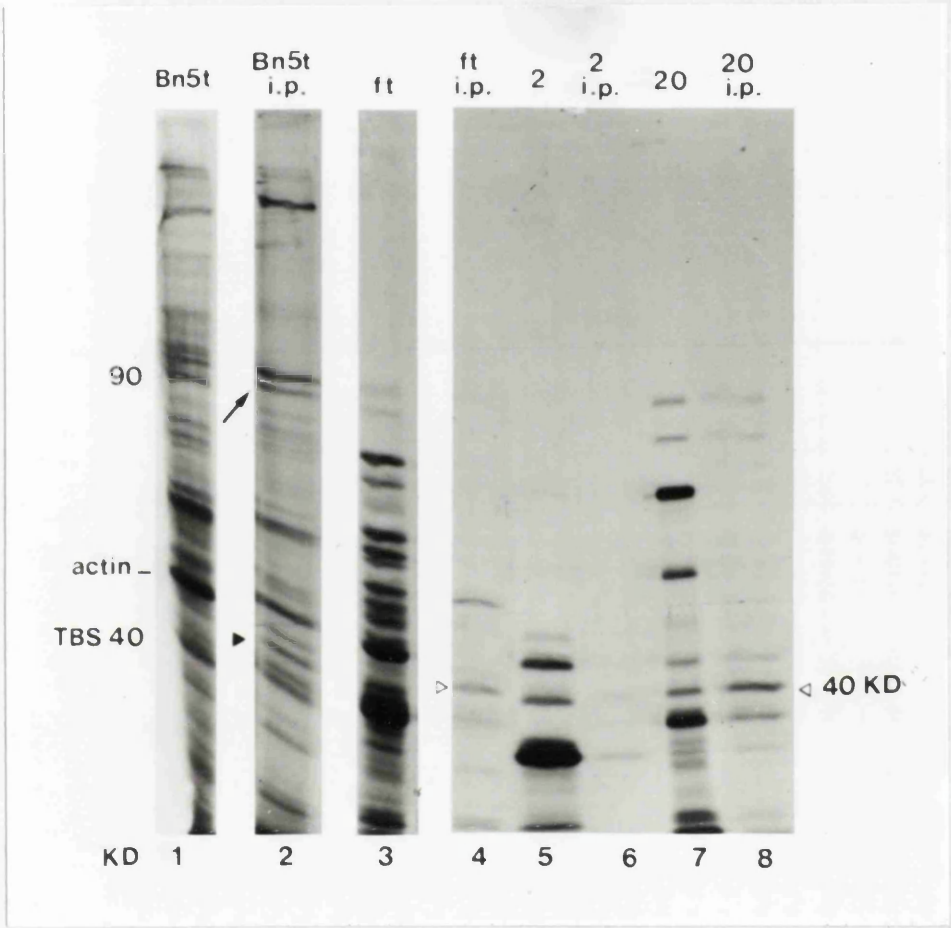
Track 3 shows the polypeptides profile of the fraction from the void volume of the anion exchange chromatography experiment at pH. 8 (labelled ft). The polypeptides i.p. by TBS from this fraction are shown in track 4 (labelled ft i.p.)

Track 5 shows the polypeptides profile of the fraction 2, i.e. void volume of the anion exchange experiment at pH. 9.5 (labelled 2). The polypeptides i.p. by TBS are shown in track 6 (labelled 2 i.p.).

Track 7 shows the polypeptides eluting in fraction 20 of the anion exchange experiment at pH. 9.5 (labelled 20). The polypeptides i.p. by TBS from fraction 20 are shown in track 8 (labelled 20 i.p.).

The MW of the markers is at the left of the gel

The position of the TBS:40 is indicated by a filled triangle. The position of the 40KD i.p. from the FPLC fractions are indicated at the left of track 4 and at the right of track 8 by empty triangles. The position of the 90 KD doublet is indicated by an arrow.


Bn5t
Bn5t i.p.
ft
ft i.p.
2
2 i.p.
20
20 i.p.
90
actin
TBS 40
40 KD
KD
1
2
3
4
5
6
7
8

radiolabelled. It was not possible to say if these proteins could be i.p. by TBS.

3.4.5.4. 3rd PURIFICATION STEP: CHROMATOFOCUSING.

Chromatofocusing is an analytical or preparative technique for the separation of biomolecules according their pI.. The Mono P HR5/20 prepacked column, hereafter referred as the Mono P column was used, it is part of the FPLC system.

The Mono P matrix is made of the same monobeads as the Mono Q and Mono S but they are substituted with quaternary amines and tertiary amines. The quaternary amines are always charged ensuring sample binding. The tertiary amines provide buffering capacity, they are charged or neutral depending on the pH..

A molecule has a negative charge above its pI. Following sample application, the polybuffer is used to titrate the Mono P and the sample components. Titration of the Mono P results in the generation of a linear descending pH. gradient and titration of the sample molecules results in focused zones of molecules with different pI. A polypeptide is eluted from the Mono P column when the pH. of the buffer is equal to or lower than its pI.

The polypeptides which elute in the void volume of the 1ml Mono Q at pH.8 would be expected to have a pI above 7. A pH. gradient from 9 to 7 was therefore chosen to separate them using the Mono P column.

The sample was loaded onto the Mono P column in 7ml of 25 mM diethanolamine pH.9 and eluted by a 34ml linear pH. gradient created between pH. 9 and pH. 7. The buffer used consists of 1ml of "Pharmalyte 8-10.5*" and 5.2 ml of "polybuffer 96 HCl*" in 100ml of water, and the pH. is adjusted to 7.0 with hydrochloric acid,. Polybuffer consists of amphoteric buffering substances dissociating at different pHs.

Figure 3.24 shows the presence of a 40KD polypeptide in fraction 4 (track 3); one of the seven fractions which constitute the void volume, the other fractions were saved

Figures 3.24a and b. 3rd purification step, chromatofocusing.

The polypeptides eluted in the void volume of the anion exchange chromatography at pH 8 were loaded onto the Mono P column in 25mM diethanolamine pH.9, and eluted by 34ml of a buffer consisting of 1ml pharmalyte 8-10.5 and 5.2ml polybuffer 96 pH. 7 in 100ml of water, generating a pH. gradient from 9 to 7. The results were analysed on two 9% polyacrylamide gels. The polypeptides were labelled with [^{35}S] L-methionine and visualized by autoradiography.

Figure 3.24a

Track 1 shows the MW markers.

Track 2 shows a Bn5T polypeptides profile.

Track 3 shows the 40KD polypeptide eluting in 25mM diethanolamine pH.9. (Only one of the 7 fractions is shown).

Tracks 4-20 show the polypeptides eluted by the pH. gradient. 40KD polypeptides are detected from fraction 17 onwards (indicated on top of the gel).

Figure 3.24b (next page)

Track 21 shows the MW markers.

Track 22 shows a Bn5T polypeptides profile.

Tracks 23-35 show the polypeptides eluted by the pH. gradient.

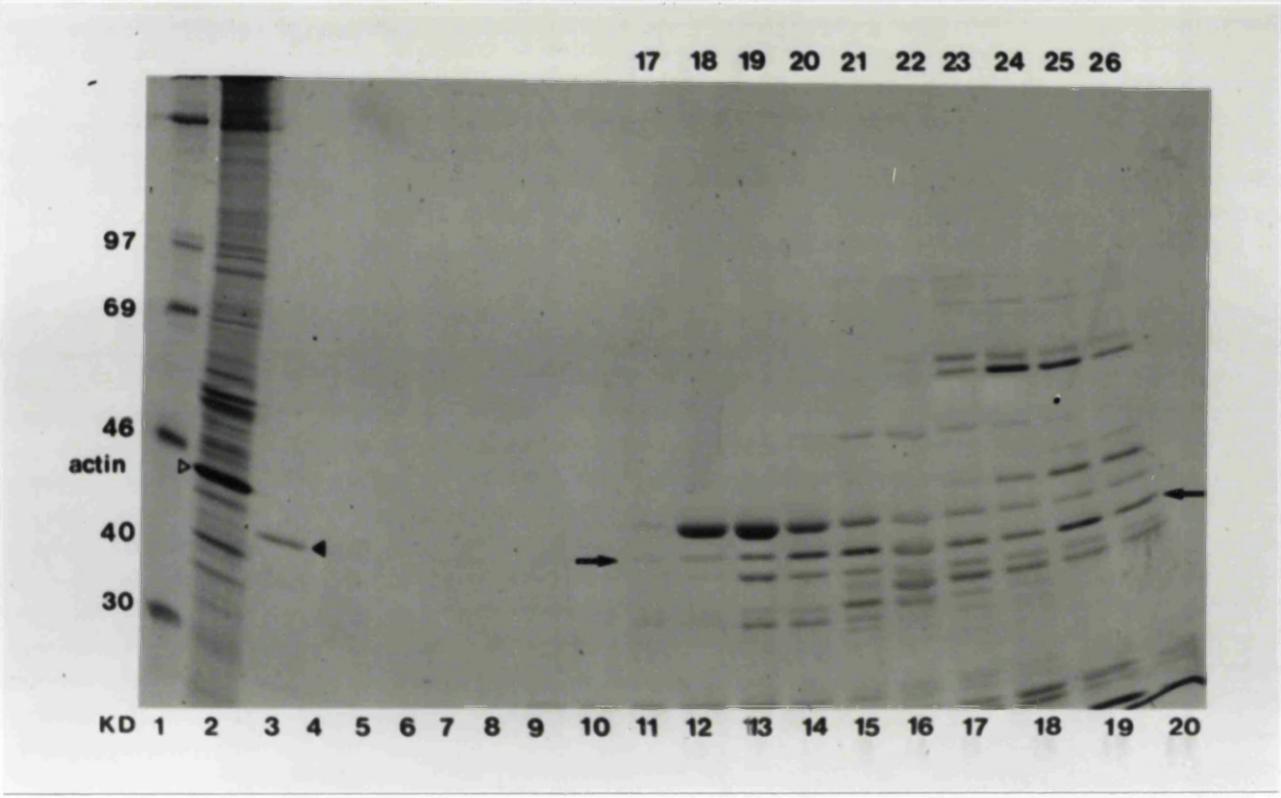
Track 36 shows a Bn5T:i.p., the TBS:40 is indicated by an empty triangle.

The MW of the markers is at the left of the gel

The position of the 40KD polypeptide eluting in 25 mM diethanolamine is indicated by a filled triangle, the 40KD polypeptides eluted by the pH. gradient are indicated by arrows. The position of actin is indicated by an empty triangle.

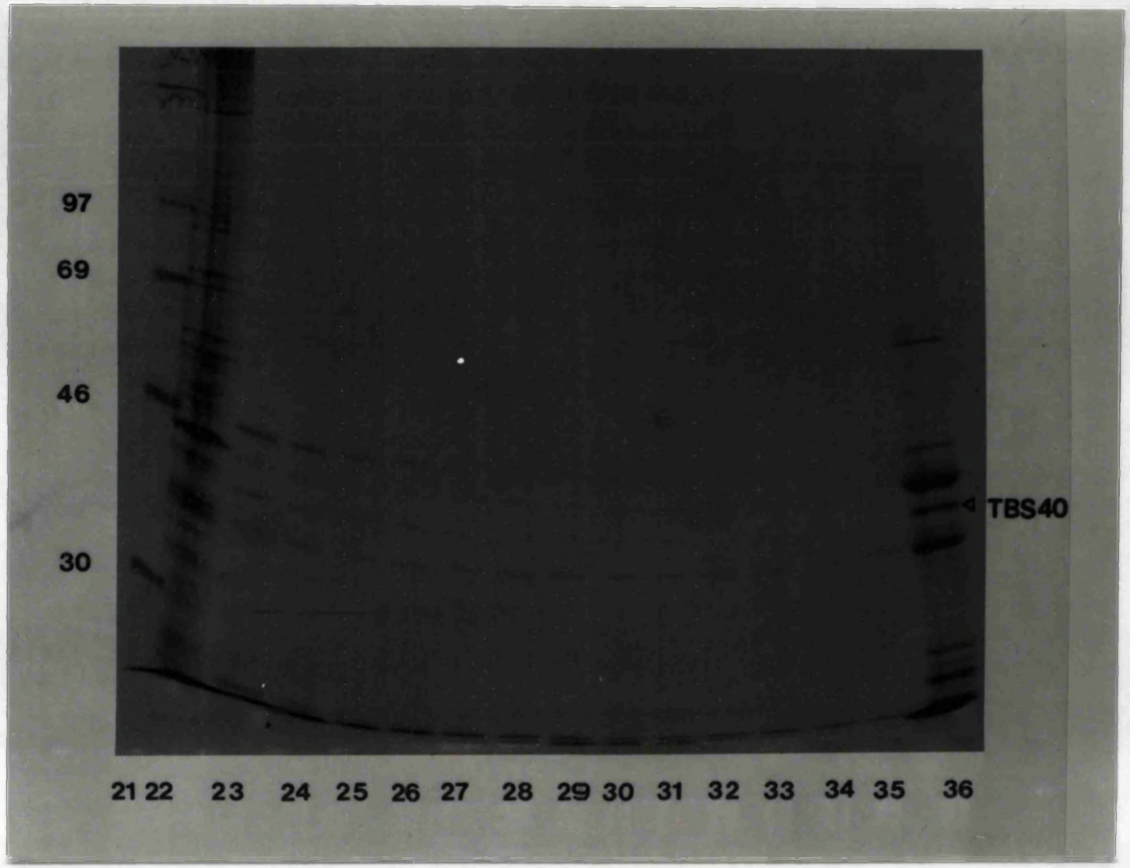
Fractions numbers

A



Tracks numbers

B



Tracks numbers

for later work. The following tracks show the proteins eluted by the pH. gradient. From fraction 17 (track 11) onwards a 40KD protein is seen with a maximum at fractions 21 and 25. Unfortunately the peptides forming the 40KD bands could not be digested by Staph. aureus V8 protease because the gel had been enhanced (it was found that after enhancement polypeptides did not generate clear proteolytic digestion patterns). This result confirms the existence of two proteins as shown by anion exchange chromatography at pH. 9.5. One elutes with a maximum in fraction 21 and the second with a maximum in fraction 25. It is likely that the protein which elutes in the void volume is similar to the protein eluting in fraction 21. A similar phenomenon is observed in the anion exchange chromatography at pH. 9.5. where the protein elutes in the void volume but some of it is retained by the column and is eluted when the percentage of buffer D reached 5% (see figures 3.20a, figure 3.21a for the Staph. aureus V8 digests and section 3.5.4.3.2).

3.4.5.5. 3rd PURIFICATION STEP: CONCLUSION.

The polypeptides eluted from the void volume of the 1ml Mono Q column at pH.8 were i.p. by TBS and the digest of the 40KD protein were indistinguishable from the TBS:40 as determined by the results of enzymatic and chemical digestion (figures 3.15, 16, 17 and 18).

Further purification of the void volume by anion exchange chromatography and chromatofocusing experiments proved the existence of two polypeptides determined from the results of Staph. aureus V8 protease digests. The digestion of these polypeptides by Staph. aureus V8 protease gives peptide maps which individually are different from the TBS:40 peptide map. Only the "COLUMN 40" is i.p. by TBS individually but the "VOID VOLUME 40" may be complexed to the "COLUMN 40" (protein:protein interactions) and i.p. with the "COLUMN:40".

It was decided to use anion exchange chromatography at pH. 9.5 and not electrofocusing for all further experiments. Modifications of the conditions used for anion

TABLE 3.6.

PURIFICATION OF THE 40KD PROTEINS IMMUNOPRECIPITATED BY TUMOUR BEARING SERA (TBS) IN Bn5T TUMOUR CELLS.

HOMOGENIZATON OF Bn5T CELLS.
Buffer : 20mM trisCl pH. 7.4, 20 mM NaCl..

|

CENTRIFUGE 10.000G, 10 min.
Retain supernatant.

|

Purification step:

1st Step. SALTING OUT USING AMMONIUM SULPHATE.
Retain 60%-80% fraction .

|

2nd Step. ANION EXCHANGE CHROMATOGRAPHY.
1 ml FPLC MonoQ column.

Proteins are loaded in buffer A (50 mM tris pH. 8) and eluted with a linear gradient of 50% buffer B (Buffer A + 1M NaCl).
The proteins of interest elute in the flow through.

|

3rd Step ANION EXCHANGE CHROMATOGRAPHY.
1 ml FPLC monoQ column.

Proteins are loaded in buffer C (20 mM ethanolamine pH. 9.5) and eluted with a step gradient of buffer D (Buffer C + 1 M NaCl).
3 ml with 2.5% buffer D.
3 ml with 5% buffer D.
3 ml with 7.5% buffer D.
19 ml of a linear gradient of 50% buffer D.
1 ml fractions are collected.

"VOID VOLUME 40"
elutes in the flow-through.

"COLUMN 40"
elutes in fraction 20.

exchange chromatography permitted the elution of the polypeptides in a reduced number of fractions. These modifications were impossible with electrofocusing because a fixed volume of buffer is required to produce the pH.gradient.

The purification method used in all further experiments is summarized in a flow chart, table 3.6.

Using anion exchange chromatography, the polypeptide eluting in the void volume and at low salt will be called "VOID VOLUME 40". The polypeptide eluting from 15% buffer D onward will be called "COLUMN 40" as previously stated.

3.5. PREPARATION FOR SEQUENCING.

After anion exchange chromatography at pH.9.5, the polypeptides were further purified to obtain amino acid sequence data. Two methods are generaly described to prepare polypeptides for N-terminus sequencing. High performance liquid chromatography (HPLC) with a reverse phase column and SDS-PAGE, followed by electoblotting onto a polyvinyl difluoride membrane (Problott*). The samples were sent to Dr G. Currie and Dr M. Cussak, Department of Geology, University of Glasgow. Protein sequencing was carried out using an ABI 477A pulse liquid protein sequencer and an ABI 420-H amino acid analyser complete with automatic hydrolysis head. This sequencer uses the chemistry developed by Edman (1950). Nine samples generated by digestion with Staph. aureus V8 protease were sent to Dr Keen of the SERC sequencing source, Department of Biochemistry, University of Leeds.

3.5.1. REVERSE PHASE HPLC.

The method recommended by the technical department of Applied Biosystems and by Professor Fothergill and Dr Dunbar (Department of Biochemistry, University of Aberdeen) was a final purification by High Performance Liquid Chromatography using a reverse phase column (RP-HPLC). The system I used was an Applied Biosystem 130A separation system with a Aquapore RP 300 C8, microbore column and a 50ul loop.

Reverse phase chromatography separates the proteins according to their hydrophobicity. The polypeptides were loaded on the column in 0,1% trifluoroacetic acic (TFA) and were eluted by a gradient of 0-70% acetonitrile. The polypeptides were recovered in a small volume of acetonitrile in 0.1% TFA. Both acetonitrile and TFA are volatile and do not interfere with the sequencing reaction, therefore the sample can be concentrated easily by evaporation to 30ul and applied to the sequencer. This method is not universaly applicable because the solvents

usually denature the polypeptides. The immunological reactivity is also usually destroyed. It was verified that extraction of Bn5T cells in 0.1% TFA and exposure to TFA for 30 minutes destroyed the immunogenic response as measured by immunoprecipitation of the 40KD by TBS.

3.5.2. ELECTROBLOTTING ON PVDF.

The proteins were separated by SDS-PAGE, and then electroblotted onto a PVDF membrane Problott* using 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffer at pH. 11.

This method may result in blockage of the N-terminus of the polypeptide by reaction with free radicals generated during the polymerisation of the gels. To minimize N-terminus blockage, there is a general agreement that gels should be cast at least a day in advance i.e. aged. Some authors advise pre-running the gel with "radical scavengers" (Speicher, 1989) such as thioglycolic acid with the addition of another "radical scavenger", glutathione to the tank buffer (Moos et al., 1989). Some authors do not use these precautions (Le Gendre and Matsudaira, 1989). I set the resolving gels at least a day in advance and I pre-ran the gels with 0.1 M thioglycollate for 2 hours at 20 milliAmp. in quarter strength resolving buffer.

A Bio-Rad Mini-Protean II electophoresis cell (mini-gel kit) was used for the isolation of the "VOID VOLUME 40". A Bio-Rad Mini Trans Blot Cell was used for electroblotting. Large gels used for the isolation of the "COLUMN 40" and for the separation of the fragments generated by Staph. aureus V8 protease digestion were cut to fit the size of the Mini Trans-Blot Cell.

The tank buffer used for electrophoresis contains glycine. The first experiments on sequencing polypeptides separated by this system and electroblotted on Problot* gave spurious peak of glycine in the first sequencing cycles (D. McNab personal communication). To avoid the use of glycine, two other gel systems were tried but found unsatisfactory. The system used by Moos et al. (1989)

uses the same buffer for the stacking and the resolving gels. The entire gel can therefore be pre-run with 10mM glutathione or 0.1mM thioglycolate 14 hours, at 10milliAmp.. The reagents used for the buffers were 2-2{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid, (TES); and 2[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol, (BisTris). The composition of the buffers was:

Constituent	Upper buffer	Lower buffer	Gel buffer
TES (g)	10.07		
1M HCl (ml)		50	21.6
BisTris (g)	23.66	13.10	10.31
Final volume (ml)	1000	1000	100
pH. (25°C)	7.25	5.9	6.61

The resolution of the buffer was poor and the "VOID VOLUME 40" and "COLUMN 40" could not be separated.

The system developed by Schragger and von Jaggow (1987) uses the following buffers :

Anode buffer : 0.2M tris HCl, pH. 8.9

Cathode buffer : 0.1M tris HCl
0.1M tricine, pH. 8.25, adjust with tricine if necessary.
0.1% SDS

Gel buffer : 3M tris HCl. pH. 8.45
0.3% SDS

This system is optimized for separation of protein of MW less than 20KD. Separation of the 40KD proteins took an excessive length of time, which resulted in a low AA yield (sequence dated 11/10, section 3.5.4.2).

The final system used a buffer where glycine was

replaced by tricine weight for weight in a Laemmli system (Laemmli 1970). The system gave good results in the purification of the 90KD polypeptide (M. Grassie personal communication) . In the case of the 40KD polypeptide the gel frequently showed distortion phenomena probably because the buffering power was lower. The buffering power is lower because the pKa2 of tricine is 8.15 as against 9.6 for glycine, and the molarity is lower since glycine has a MW = 76, whereas tricine has a MW = 179. The polypeptide was, nevertheless, separated (Figure 3.27) and sequenced.

The system resolved peptides well of the 90KD digested by Staph. aureus V8 protease (M. Grassie personal communication). Therefore this system was chosen to obtain internal sequence of the polypeptides.

To concentrate the polypeptide, three systems were used. Precipitation by 5% trichloroacetic acid, precipitation with 4 volumes of acetone and precipitation with 10 volumes of methanol after concentration on the Speedy Vac. Precipitation by 5% TCA was the most frequently used because of its speed and the possibility of precipitating the protein from concentrated salt solutions. It is a harsh method leading to losses, but it was previously successfully used for preparation of polypeptides for sequencing (Matsudaira, 1989). The other methods dilute the polypeptides which also leads to losses.

The polypeptides were separated on a 9% acrylamide gel. The polypeptides were electroblotted on Problott* with CAPS buffer pH. 11. After staining with Coomassie brillant blue R250 (Coomassie blue) the bands of interests were cut out and sent for sequencing.

3.5.3. AMINO ACID ANALYSIS.

AA analysis were performed in the department of Geology by Dr G. Currie and Dr M. Cussak with a ABI 920A analyzer module on aliquots of protein sent for sequencing on Problott*, as a preliminary analysis. The results presented in table 3.7. showed that the AA composition of the "VOID VOLUME 40" and the "COLUMN 40" are different. AA analysis

gave estimates of the amounts of polypeptide loaded on the sequencer (See sections 3.5.4.3. and 3.4.5.6.).

TABLE 3.7. RESULTS OF THE AA ANALYSIS OF THE "VOID VOLUME 40" AND THE "COLUMN 40".

PROTEIN	Void vol. 40	Column 40	Void vol. 40	Column 40
	Composition by molecular weight		picomoles by height of the peak	
Aspartic acid	28,7	24.7	176.70	259.78
Glutamic acid	91.3	34.7	561.93	366.18
Serine	14.5	23.4	89.56	247.10
Glycine	32.5	32.0	199.84	337.82
Histidine	2.9	9.9	18.39	105.06
Arginine	11.5	17.2	70.72	181.62
Threonine	10.5	17.1	64.44	180.25
Alanine	31.8	28.3	195.85	298.27
Proline	18.3	19.3	113.04	203.53
Tyrosine	6.4	10.4	39.88	109.50
Valine	31.7	34.0	195.16	358.35
Methionine		0.6		6.33
Cysteine		10.1		106.87
Isoleucine	10.3	29.6	106.86	311.97
Leucine	37.2	38.3	228.96	403.83
Phenylalanine	14.7	11.9	90.81	125.32
Lysine	14.9	27.0	91.88	285.20

When the "VOID VOLUME 40" was identified as the mitochondrial aspartate aminotransferase (See section 3.5.6), the published AA composition (Huynh *et al.* 1988) was compared to the result obtained. They were found to be different. Two peptides one matching the sequence of the fructose 1-6 diphosphate aldolase A and the other the sequence of the phosphoglycerate kinase were sequenced from the *Staph aureus* V8 digest of the "COLUMN 40" (See section 3.5.8). The AA composition of the two proteins was calculated from the published AA sequence (Ciccarese *et al.*, 1989; Joh *et al.*, 1985). It was compared to the result obtained for the "COLUMN 40" but the comparison was no help in distinguishing the two proteins sequenced.

3.5.4. N-TERMINUS SEQUENCE OF THE "VOID VOLUME 40" POLYPEPTIDE.

3.5.4.1. PURIFICATION USING RP-HPLC.

RP-HPLC was used successfully to prepare the "VOID VOLUME 40" for sequencing.

The Aquapore RP-300 column is a silica based column, and therefore unstable at a pH. above 8. The ethanolamine buffer pH. 9.5, was changed to buffer E (0.1% TFA in water), and each fraction of the void volume was concentrated using a Centricon 30 tube. The polypeptides were loaded on the column in buffer E and were eluted by a gradient of buffer F (70% acetonitrile in buffer E).

The 40KD polypeptide was eluted by 63 % of buffer F in a well individualised peak, after most of the other proteins. The peak, however, frequently showed a shoulder. It is known that acetonitrile can duplicate peaks (Kamp, 1986), but it also may be that the peak may not contain a pure polypeptide. To try to separate these peaks a shallower gradient of 45% to 75% buffer B was used . This failed to separate the peaks completely but it was retained because it reduced the contamination by trailing from other peaks.

The elution profile is presented in figure 3.25.

To confirm the purity of the polypeptide, an aliquot was loaded on a SDS-PAGE where it gave a clear 40KD band as seen in figure 3.26. The polypeptide was sent for N-terminus sequencing. The results of the sequencing experiments are presented in table 3.8.

Figure 3.25. Graph 3.

Graph showing the elution of polypeptides from the microbore reverse phase column. The polypeptides from the void volume of the anion exchange experiment at pH 9.5 were concentrated using a Centricon 30 and the buffer changed to buffer E (0.1 % TFA in water). The proteins were eluted from the column using buffer F (70 % acetonintrile in buffer E). The polypeptides were eluted by the following gradient.

time in min.	% of buffer F
0	0
3	0
8	45
45	75
48	100
53	100
55	0

The injection is shown by the bottom arrow (labelled "start") and the peak containing the "VOID VOLUME 40" is shown by the arrow at the left of the Graph.

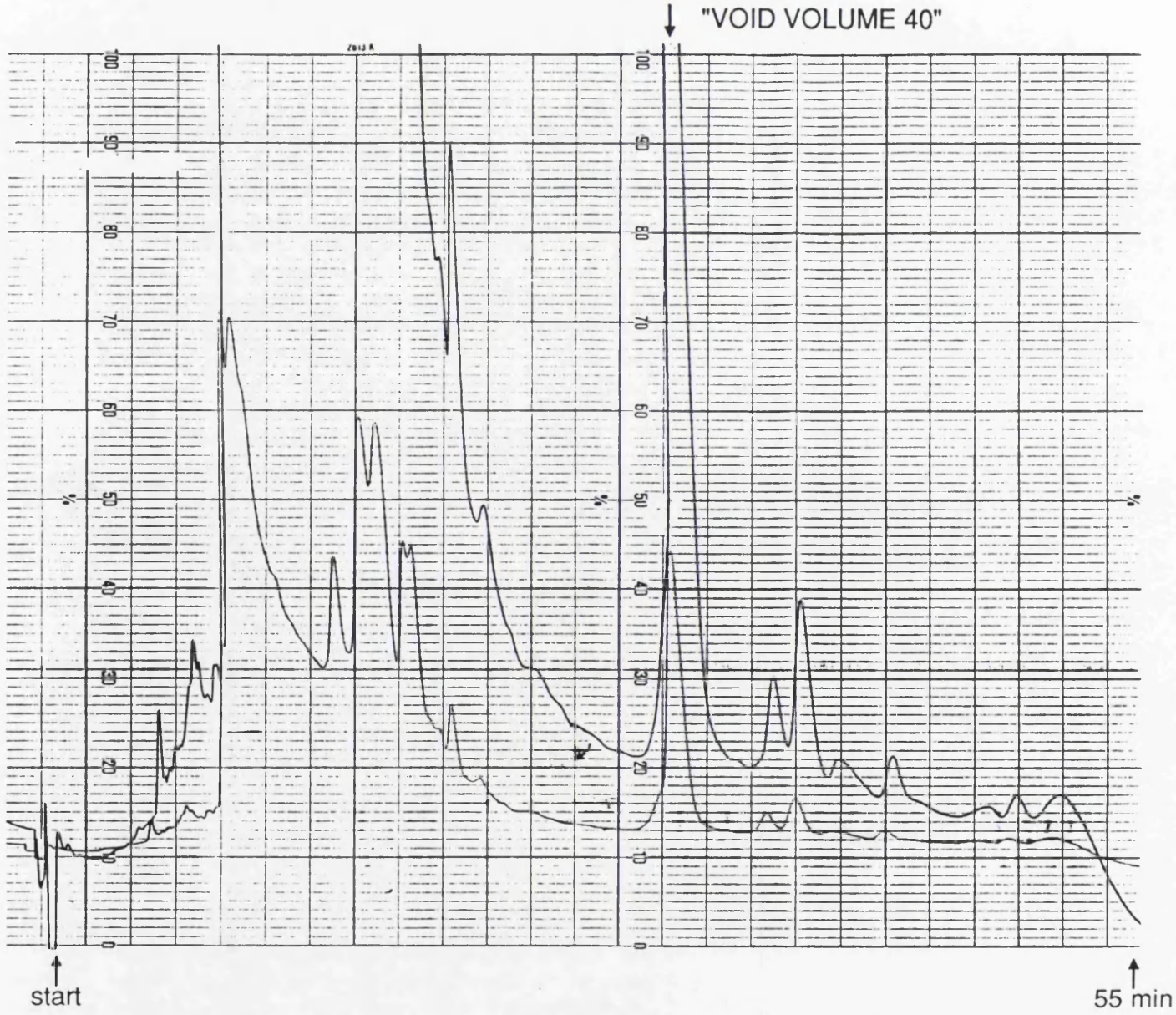

"VOID VOLUME 40"
start
55 min

Figure 3.26. Reverse phase HPLC.

The fractions of the void volume of the anion exchange chromatography experiment at pH. 9.5 were concentrated, the buffer changed to buffer E (0.1% TFA) and loaded onto the microbore reverse phase column. The peak eluting at 64% in buffer F (70% acetonitrile in buffer E) was saved and sent for sequencing. An aliquot was taken to be analysed on a 9% gel. The polypeptides were labelled with [^{35}S] L-methionine and visualized by autoradiography. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

Track 1 shows the MW markers.

Track 2 shows the "VOID VOLUMW 40" purified by RP-HPLC (labelled fplc).

Track 3 shows a control standard i.p (labelled i.p.).

The MW of the markers is at the left of the gel

The position of the "VOID VOLUME 40" is indicated by an an empty triangle at the left of track 2 and the TBS:40 by a filled triangle at the right of track 3.

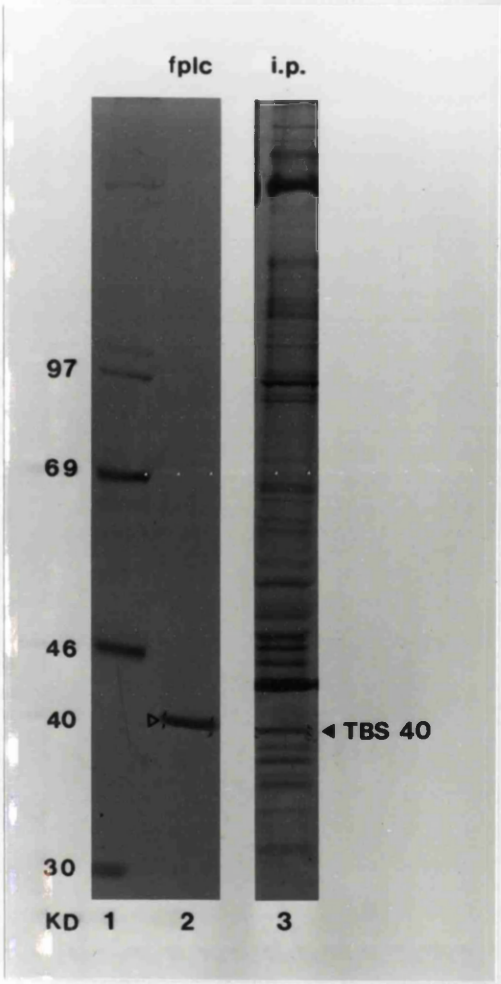

fplc
i.p.
97
69
46
40
30
KD
1
2
3
TBS 40

TABLE 3.8. AA SEQUENCE OF THE SAMPLES OF THE "VOID VOLUME 40" PURIFIED BY RP-HPLC.

Date	30/6		17/8		24/8	
AA N°		pmoles		pmoles		pmoles
1	SER	55	SER	25	ALA	20
2	SER	29	SER	24	-	
3	ASP	7	TRY	49	SER	2
4	SER	4	TRY	47	ARG	8
5	THR	135	THR	36	ARG	15
6	THR	98	HIS	9	ALA	
7	TRP	12	VAL	41		
8	TRP	11	GLU	10		
9	TRP	15	MET	39		
10	ASP	2	GLY	22		
11			PRO	25		
12			PRO	30		
13			ASP	10		
14			ARG	1		
15			ILE	13		

The theoretical initial yield were 42, 48 and 16 picomoles respectively.

3.5.4.2. PURIFICATION USING SDS-PAGE.

Figure 3.27 shows a Coomassie blue stained Problott* membrane. The polypeptides of a 1 ml sample of the void volume were divided in three and analysed on a 9% minigel together with a void volume sample of another experiment. The actin band of the Bn5T cell extract localizes the "VOID VOLUME 40", it migrates with a molecular weight just lower than actin (43KD). No TBS i.p. was run in parallel because it would not have helped in the localization of the 40KD protein.

Six samples were prepared using SDS-PAGE and electroblotting. The initial theoretical yields are shown in table 3.9.

Figure 3.27. Electroblot of the void volume of the anion exchange chromatography at pH, 9.5.

The fractions of the the void volume of the anion exchange chromatography at pH 9.5 were concentrated and loaded on a 9% SDS polyacrylamide mini-gel. After electrophoresis, the gel was electroblotted in CAPS buffer onto Problott* membrane, and the peptides visualized by Coomasie blue staining.

Track 1 shows a Bn5T cells polypeptides profile.
Tracks 2-5 shows aliquots of void volume fractions.
Track 6 shows the MW markers identified at the right of the gel.

The MW of the markers is at the left of the gel

The position of the "VOID VOLUME 40" is indicated by arrows.

The fraction analyzed in track 5 has not been purified in the same experiment as the other fractions. This explains the different intensity of the bands.

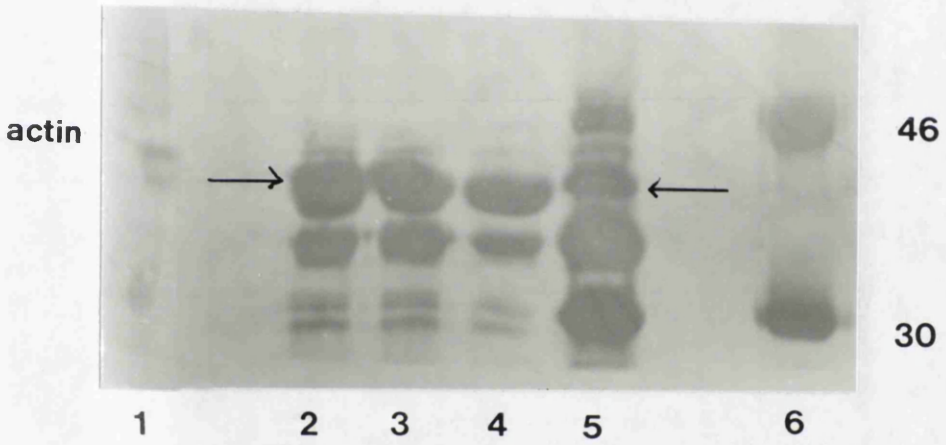

actin
46
30
1
2
3
4
5
6

TABLE 3.9. INITIAL SEQUENCING YIELD OF THE SAMPLES PURIFIED BY SDS PAGE.

Dates	Yields in pmoles
4/9	48
25/9	5
1/10	17
11/10	14
8/11	54
20/12	48

The three best results with the highest and most consistant AA yield are presented in table 3.10 AA are shown in brackets when computer analysis failed to give a result. The AA was then deduced from the direct analysis of the chromatograms by me.

TABLE 3.10. AA SEQUENCE OF THE SAMPLES OF THE "VOID VOLUME 40" PURIFIED BY SDS PAGE AND ELECTROBLOTTING.

Dates	4/9		8/11		20/12	
AA N°		pmoles		pmoles		pmoles
1	Ser	44			Ser	63
2	(Ser)	38			Ser	52
3	Trp	41			Trp	91
4	(Trp)	38			Trp	80
5	Thr	25			Thr	43
6	Lys	6			His (Ala)	2
7	Val	47			Val	80
8	Glu	21			Glu	39
9	Met	33			Met	65
10	Val(Gly)		Met(Gly)	114	Gly	93
11	(Pro)	38	Pro	106		
12	(Pro)	52	Pro	167		
13	(Asp)	18	Asp	81		
14	-		Thr	25		
15	Ile	26	Ile	77		
16	Ile	29	Leu	119		
17	Gly	39	Leu	133		
18	Val	34	Val	96		
19	Thr	13	Thr	45		
20	Val	32	Glu	64		
21			Ala	115		
22			Phe	84		
23			Lys	87		
24			-			
25			Asp	69		
26			Thr	45		

The data obtained after computer analysis were carefully scrutunized by me by eye to make sure that they were genuinly the result of the N-terminus sequencing of a polypeptide. Once some experience of the interpretation of sequencing data was acquired, early data were ignored because the yields were too low (less than 10 picomoles). To validate the data the following criteria were used.

The AA proposed was matched with the peak on the chromatogram. It was essential that the peak was significant,
its height was compared to the control to detect any erroneous yield. Moreover, its position relative to the DMP and DPT peak was checked to detect a possible shift in the chromatogram resulting in an erroneous identification.

The picomole amount of each AA proposed by the computer were compared to the values in the previous and in the following sequencing cycles. To be significant the value must be low in the previous cycle, and significantly lower in the following cycle. The transient increase of one peak is more important than the absolute pmoles value.

In addition to be sure that the data selected by the computer really represent the sequencing of a polypeptide the yield of each AA in the sequence should be grossly similar.

The following figures (3.28, 29 and 30) show three chromatograms of cycles 6, 7 and 8 from an experiment of 17/8/90. The valine derivative elutes at 19.17 min. Its amount rises from 7.27 pmoles in cycle 6 to 49.18 picomoles in cycle 7 to decrease to 21.98 pmoles in cycle 8.

The two strong peaks are N-dimethyl-N'-phenylthiourea (DMP) and N-N'diphenylthiourea (DPT) which elute at 8.85 min. and 20.75 min. respectively. They are by products of the Edman chemistry.

Figures 3.28, 29 and 30.

These three figures show the chromatograms of sequencing cycle 6, 7 and 8 respectively of the sequence of 17/8/90. The peak of valine appears in the cycle 6 and decreases in cycle 7.

The AA identified in cycle 6 is histidine 12.3 min.
7 is valine 19 min.
8 is glutamine 8.3 min.

The height of the peak and the values in picomoles are written under the chromatogram.

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

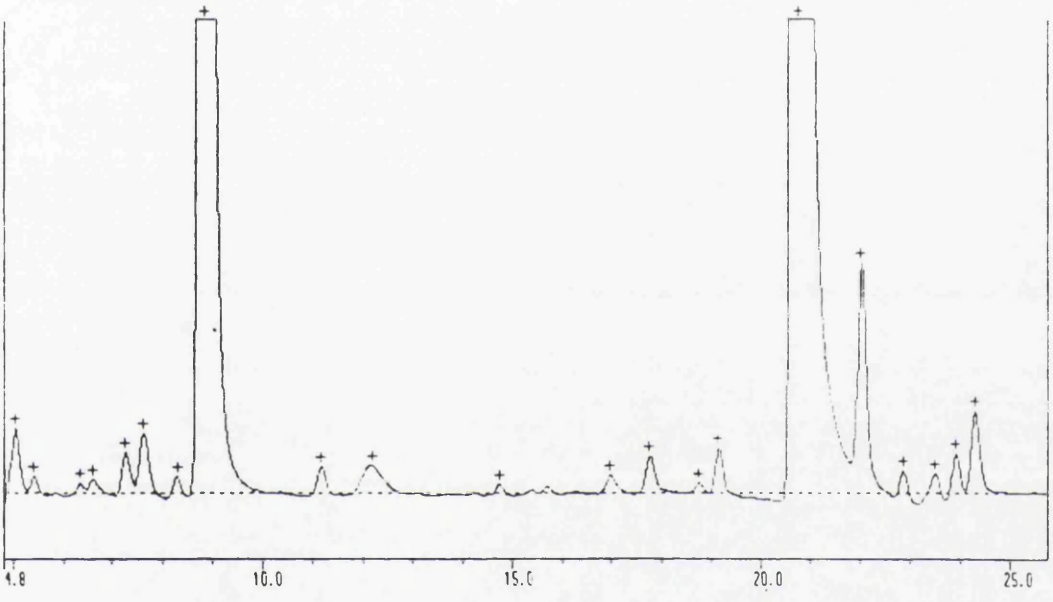
SAMPLE : LUC.170890
[Initiated 17 Aug 1990 2:17pm]

CYCLE SUMMARY :

Reaction cycle	: NORMAL	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 6 [17 Aug 1990 7:53pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 160890

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
ASP	5.05	5.07	3615	9.81	DPT	20.75	20.75	987570	1125.36
ASN	5.42	5.52	939	2.65	TRP	22.03	22.05	12852	29.62
SER	6.37	6.47	525	2.61	PHE	22.87	22.85	1269	4.04
GLN	6.63	6.75	783	2.79	ILE	23.50	23.45	1137	4.08
THR	7.28	7.40	2274	14.02	LYS	23.92	23.88	2280	4.93
GLY	7.65	7.75	3408	13.84	LEU	24.32	24.28	4593	15.21
GLU	8.32	8.47	909	2.77					
DMP	8.85	8.95	301809	1259.85					
ALA	11.18	11.28	1473	5.28					
HIS	12.18	12.30	1548	10.03					
TYR	14.75	14.82	507	1.57					
ARG	16.98	17.05	987	11.56					
PRO	17.78	17.80	2127	7.69					
MET	18.77	18.78	555	1.58					
VAL	19.15	19.17	2553	7.27					

Tabulation threshold : 999 uAU

- Applied Biosystems 477A Protein Sequencer Chromatogram Report -

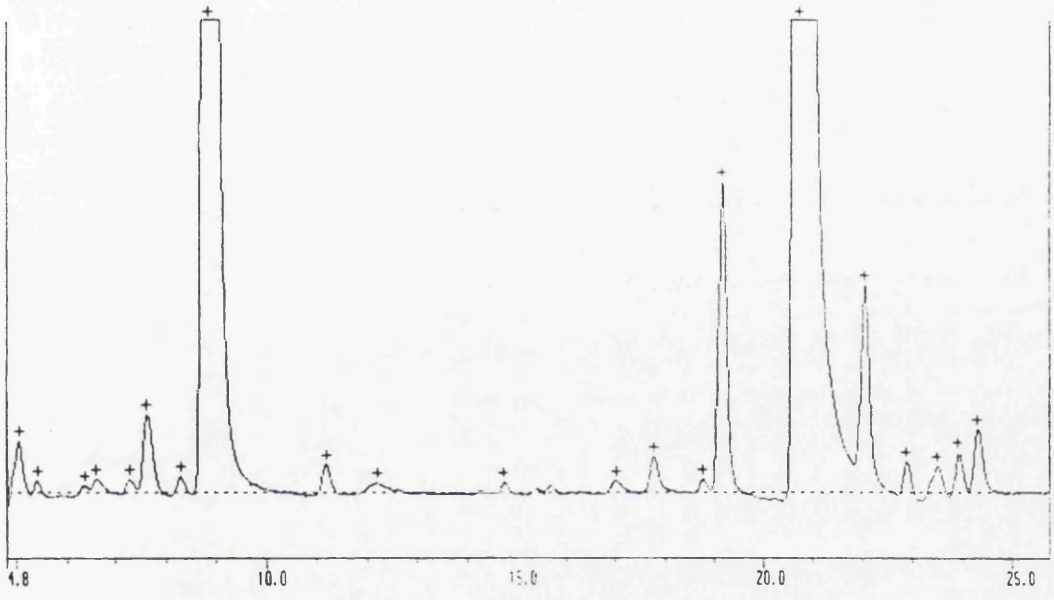
SAMPLE : LUC.170890
[Initiated 17 Aug 1990 2:17pm]

CYCLE SUMMARY :

Reaction cycle	: NORMAL	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 7 [17 Aug 1990 8:38pm] 2.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 160890

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
ASP	5.07	5.07	2874	7.80	DPT	20.77	20.75	1010157	1151.10
ASN	5.43	5.52	624	1.76	TRP	22.05	22.05	11550	26.80
SER	6.40	6.47	423	2.11	PHE	22.88	22.85	1716	5.46
GLN	6.63	6.75	771	2.74	ILE	23.52	23.45	1476	5.30
THR	7.30	7.40	720	4.44	LYS	23.93	23.88	2316	5.01
GLY	7.65	7.75	4329	17.58	LEU	24.33	24.28	3594	11.90
GLU	8.32	8.47	897	2.73					
DMP	8.85	8.95	278973	1164.52					
ALA	11.20	11.28	1596	5.72					
HIS	12.23	12.30	546	3.54					
TYR	14.77	14.82	552	1.71					
ARG	17.05	17.05	633	7.41					
PRO	17.80	17.80	2016	7.28					
MET	18.78	18.78	723	2.09					
VAL	19.17	19.17	17283	49.15					

Tabulation threshold : 999 uAU

– Applied Biosystems 477A Protein Sequencer Chromatogram Report –

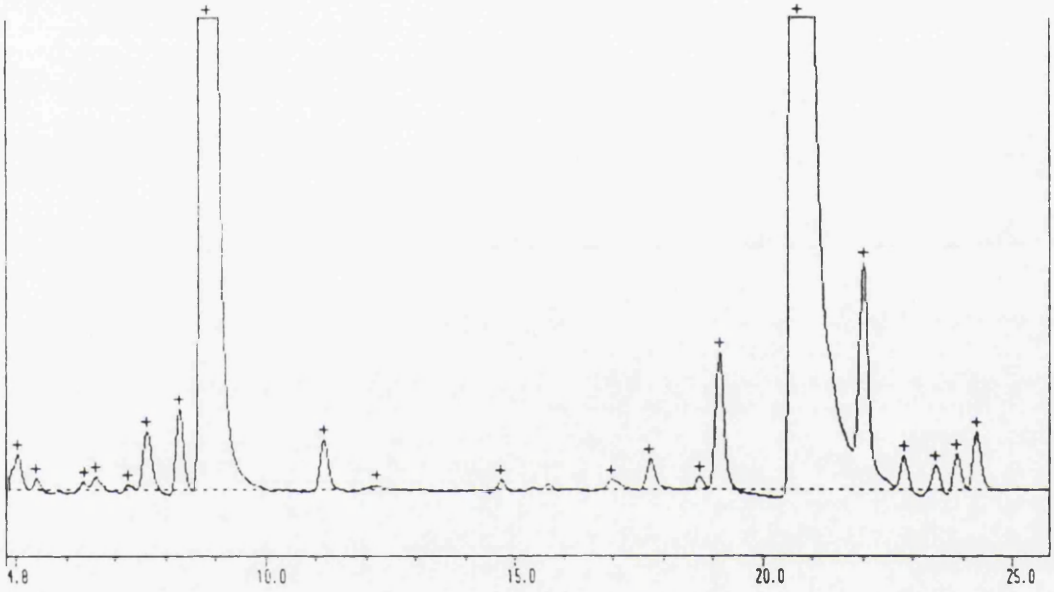
SAMPLE : LUC.170890
[Initiated 17 Aug 1990 2:17pm]

CYCLE SUMMARY :

Reaction cycle	: NORMAL	Data collect time	: 0.0 to 29.0 min
Conversion cycle	: NORMAL	Data interval	: 1.0 sec
Gradient	: NORMAL	Inject volume	: 50 of 150 uL

AMINO ACID # 8 [17 Aug 1990 9:22pm] 0.0100 AU

Baseline Corrected Data



Retention Time: Minutes

PEAK TABULATION : (100% injection) Calibration : 160890

Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol	Peak ID	R.Time (min)	C.Time (min)	Height (uAU)	Pmol
ASP	5.05	5.07	2043	5.54	DPT	20.72	20.75	1109241	1264.01
ASN	5.42	5.52	666	1.88	TRP	22.02	22.05	12807	29.71
SER	6.38	6.47	444	2.21	PHE	22.83	22.85	1908	6.07
GLN	6.63	6.75	723	2.57	ILE	23.48	23.45	1380	4.95
THR	7.28	7.40	303	1.87	LYS	23.88	23.88	2040	4.41
GLY	7.65	7.75	3267	13.27	LEU	24.28	24.28	3255	10.78
GLU	8.30	8.47	4566	13.89					
DMP	8.85	8.95	291546	1217.01					
ALA	11.18	11.26	2835	10.17					
HIS	12.23	12.30	258	1.67					
TYR	14.73	14.82	534	1.65					
ARG	16.98	17.05	555	6.50					
PRO	17.75	17.80	1758	6.35					
MET	18.75	18.78	735	2.09					
VAL	19.13	19.17	7725	21.98					

Tabulation threshold : 999 uAU

3.5.4.3. CONCLUSION OF THE N-TERMINUS SEQUENCING EXPERIMENTS OF THE "VOID VOLUME 40".

The AA sequence data were examined, The sequence obtained from samples purified by RP-HPLC dated 30/6 and 24/8 were disregarded because of the low and inconsistent AA yield. The sequence obtained on 17/8 was considered to be the sequence of the polypeptide because the AA yield remained throughout between 45 and 13 picomoles, a significant figure.

The AA sequence of the sample electroblotted on 20/9 had to be established by examination of the chromatograms, because a shift in the analysis of the cycle 10 incorrectly identified the peak of DPT as valine.

The sequence of the sample of 8/11 started at AA N°10 because of a technical problem on the sequencer which prevented identification of earlier AA.

A total of nine samples were sequenced, three prepared by RP-HPLC and six by electroblotting. The results of the four best sequences, selected because of their high and consistent AA yields are compared using the single letter code for AA, X denotes that the AA was undetermined.

		10	20
RP-HPLC		SSWWTHVEMG	PPDRI
Electroblotting	20/9	SSWWTKVEMG	PPDXIIGVTV
	8/11	X	PPDTILLVTE AFKXDT
	20/12	SSWWTHVEMG	

The sequence data confirm each other except for AA N°6, 14, 16, 17 and 20.

The AA 6 and 14 detected were different for the five other samples sequenced and the yields were much lower than for the other cycles. It was concluded that these AA could be either phosphorylated, glycosylated or might be cysteine. The cysteine residue must be modified to be

detectable with the HPLC procedure used with the sequencer (Dr M. Cussak, personal communication). Cysteine residue can be detected after S-pyridylation of the protein. S-pyridylation was carried out using the method developed by Amons (1987) but no cysteine was detected (Sequence dated 20/12, section 3.5.4.2).

The chromatograms of AA N°16 17 and 20 were examined, it was decided that AA 16 was most probably a leucine, AA N°17 a glycine and AA N°20 a glutamic acid residue.

The sequence was therefore most probably the following:

```
          10         20
SSWWTXVEMG PPDRILGVTE AFKXDT
```

The sequence was compared to the sequences of the NBRF data base using a VAX computer with the GCG package developed in the University of Wisconsin. It was found to be the N-terminus sequence of the rat mitochondrial aspartate aminotransferase (mAspAT), (The complete AA sequence of rat mAspAT is presented in section 3.5.5).

I was not confident that the rat mAspAT was the right protein. The intensity of the Coomassie blue staining of the bands sent for sequencing suggested that the sequencing yield was very low. The result obtained could represent the sequence of a minor protein co-migrating with a major protein whose N-terminus was blocked. N-terminus of eukaryotic protein blockage is a common occurence (Brown and Roberts, 1976). The N-terminus of the protein of interest could have been blocked during the electrophoresis or by modification.

Pinpointing accurately the "VOID VOLUME 40" polypeptide on a minigel was not very easy because of the large amount of material loaded. At the beginning an aliquot was run on a normal size gel. On the blot shown in figure 3.27, a Bn5T cell extract was run in parallel with the flow through fraction. The position of the actin band could be clearly seen and the position of the "VOID VOLUME 40" was thereafter deduced.

Two experiments were set up to estimate the efficiency of the gel system and the blotting technique.

To evaluate the potential recovery of AA, a quality control protein provided by Applied Biosystems was also put through the same experiments as the unknown samples. One hundred picomoles of beta lactoglobulin were loaded on a mini gel electrophoresed and blotted exactly as the "VOID VOLUME 40". The initial sequencing yield was 78.5 picomole, which is higher than expected from published data (Wilson and Yuan, 1989). Therefore the gel system used does not appear to block the N-terminus of proteins.

The amount of protein blotted was measured as part of the AA analysis. When the 4 bands, shown in figure 3.29, together with four other bands (not shown) were sent for N-terminus sequencing, an aliquot of a quarter of one band was used for AA analysis. The others bands were used for sequencing. The result of the sequencing experiment has already been presented (experiment dated 8/11, table 3.10, section 3.5.4.2); the yield of the first AA was 111 pmoles. The amount recovered from the AA analysis was 0.25ug of protein i.e. 6 pmoles. An exact quantification was not possible, because the amount of the protein blotted on the membrane was not necessarily the same for each band, but it indicates that approximatively 120 pmoles of purified 40KD proteins were loaded on the sequencer. According to Dr M. Cussak who sequenced the protein and supervised the AA analysis, the results showed that the recovery of AA by the sequencer was high. A percentage of recovery from PVDF membrane of 50% is usual (Wilson and Yuan, 1989).

The yield of the control protein and the AA sequencing showed that the rat mAspAT is not a contaminant. Further confirmation was obtained by internal AA sequence data.

3.5.5. INTERNAL SEQUENCE OF THE "VOID VOLUME 40".

As the N-terminus of the "COLUMN 40" was blocked (See section 3.5.6) it was digested by Staph. aureus V8 protease to obtain internal sequence data. The "VOID VOLUME 40" was

digested in the same experiment on a parallel track, and used as a control to distinguish between fragments of both 40KD proteins and fragments of the enzyme. Five peptides were sent to Dr Keen, Department of Biochemistry, University of Leeds, for sequencing. Some of these were peptides from the Staph. aureus V8 protease, but two fragments were not. They were matched by Dr Keen as fragments of the rat mAspAT. Their sequence is presented in Table 3.11.

TABLE 3.11. AA SEQUENCE OF TWO PEPTIDES GENERATED BY DIGESTION OF THE "VOID VOLUME 40" BY STAPH. AUREUS V8 PROTEASE.

	Peptide 3 (5KD): AA	pmoles	Peptide 4 (14KD): AA	pmoles
1	Ala	5	-	
2	Phe	8	Gln	5
3	Gly?	2	Leu	9
4	-		Pro?	1
5	Asp	10	Ile	2
6	Thr	5	Leu	5
7	Asn	4	Ile	2
8	Ser	2	-	
9	-		Pro	2
10	-		Leu	5
11	Met	4	Tyr	4
12	Asn	4	Ser	0
13	Leu	3	Asn	5
14	Gly	2	Pro	2
15	Val	3	Pro	2
16	Gly	1	Leu	1
17	Ala	3	Asn	1
18	Tyr	4	Gly	2
19	-		Ala	1
20	Asp	5	-	
21	Asp	4	Ile	0
22	Asn	2	Ala	0
23	Gly	1		
24	-			
25	Pro	0		
26	Tyr	0		
27	Val	1		
28	Leu	1		
29	Pro	0		
30	Ser	0		

The sequence of rat mAspAT was published by Huynh et al. (1980). It is presented here and compared with the

fragments sequenced. The N-terminus fragment sequenced (AA 1-26) is shown over the published sequence of the protein. The two internal peptides sequenced are shown underneath (AA 21-50 and AA 277-298). The strokes ¦ denote common AA.

```
    SSWWTXVEMG PPDXILGVTE AFKXDT
    ¦¦¦¦¦ ¦¦¦¦  ¦¦ ¦¦¦¦¦¦ ¦¦¦ ¦¦
  1 SSWWTHVEMG GPDPILGVTE AFKRDTNSKK MNLGVGAYRD DNGKPYVLPS
                           ¦¦¦ ¦¦¦¦   ¦¦¦¦¦¦¦¦ ¦ ¦¦¦ ¦¦¦¦¦¦
                           AFGXDTNSXX MNLGVGAYXD DNGXPYVLPS

 51 VRKAEAQIAG KNLDKEYLPI GGLADRCKAS AELALGENSE VLKSGRFVTV

101 QTISGTGALR VGASFLQRFF KFSRDVFLPK PSGGNHTEIA RDAGMQLEGY

151 RYYDPKTCGF DFSGALEDIS KIPEQSVLLL HACAHNPTGV DPRPEQWDEM

201 AAYVKKNNLF AFFDMAYQGF ASGDGNKDAW AVRHFIEQGI NVCLCQSYAK

251 NMGLYGERVG AFTVVCKDAE EAKRVESQLK ILIRPLYSNP PLNGARIAAT
                                 ¦¦  ¦¦  ¦¦¦¦¦¦ ¦¦¦¦¦ ¦¦
                               XQLP ILIXPLYSNP PLNGAXIA

301 ILTSPDLRQG WLQEVKGMAD RIGSMRTQLV SNLKKEGSSR NWQHITDQIG

351 MFCFTGIKPE QVERLTKEFS IYMTKDGRIS VAGVTSGNVG YLAHAIHQVT

401 K
```

The results match the published sequence of the rat mitochondrial aspartate amino-transferase well. There are, however, discrepancies for three amino-acids :

AA 11 a glycine is replaced by a proline.

AA 23 a lysine is replaced by a glycine in the fragment of internal sequence, but the identification given is only tentative, the recovered yield of glycine is only 2 pmoles (AA 3, peptide 3 (5KD)).

AA 280 a lysine is replaced by a proline.

3.5.6 N-TERMINUS SEQUENCE OF THE "COLUMN 40" POLYPEPTIDE.

Experiments using Coomassie blue stained gels showed that the "COLUMN 40" elutes in four fractions namely 19-23 with a maximum in fraction 20. It was not possible to isolate the "COLUMN 40" in a pure form from fraction 20 by

RP-HPLC. The resolution was insufficient, and the peak collected always contained contaminants when examined on SDS-PAGE (Data not shown). The polypeptide was therefore separated using SDS-PAGE. It was impossible to cut out the "COLUMN 40" protein by aligning it with a TBS:40 in a mini gel because the abundance of a contaminating protein overloaded the gel. Normal size gels (140x140 mm) of normal thickness (1.5 mm) were used. The "COLUMN 40" was separated on SDS-PAGE, electroblotted on Problott*, located using A Bn5T i.p. and a Bn5T cell extract and sent for N-terminus sequencing twice. The results are shown in table 3.12.

TABLE 3.12. RESULTS OF THE ATTEMPTS OF THE N-TERMINUS SEQUENCING OF THE "COLUMN 40".

Date	23/10		10/12	
AA N°	AA	pmoles	AA	pmoles
1	Trp	14	Ser	28
2	-		Thr	3
3	Arg	4	Val	4
4	Tyr	4	Glu	2
5	Phe	2	Tyr	3
6	Ala	6	Leu	12
7	Ser	6	Phe	8
8	Thr	3	Lys	2
9	Pro	3	Val	3
10	Pro	5	Ser/Lys	

The sequences are completely different, the initial yields were low (5 and 8 picomoles). One of the bands sent for AA sequencing the 10/12 was used for AA analysis. The amount of polypeptide recovered from the band used for AA analysis indicated that around 60 pmoles of polypeptide had been loaded on the sequencer. Thus it was concluded that the N-terminus was most probably blocked and the sequences obtained represented non specific background. The protein

had therefore to be fragmented to obtain internal sequence data.

3.5.7. ENZYMATIC DIGESTION WITH STAPH. AUREUS V8 PROTEASE.

The same method of enzymatic digestion with Staph. aureus V8 protease as used for identification of the FPLC:40 was again used; but with some modifications taking into account the experience gained from the sequencing of the U90 (M. Grassie, personal communication).

The stain used to visualize the bands contained only 10% methanol and 1% acetic acid to speed up destaining and facilitate electroelution from the gel slices. The gels were stained for only 15 min, and destained in the same solution minus stain until the bands were visible.
The bands excised were first dried to be packed into a normal sized gel; but the gel used was only 0.75 mm thick because electroelution proved to be difficult using 16.5% polyacrylamide gel of 1.5 mm thickness.

The pH. of the buffer was increased to 8, to decrease the number of fragments produced, because Staph. aureus V8 protease cleavage is restricted to glutamyl residues at pH.8 (Houmard and Drapeau, 1972).

Since the amount of polypeptide to be digested was considerably larger than for analytical TBS:40 digests, 50 ug of protease was used initially. However this was excessive, and it was later reduced to 10 or 15 ug.
The same amount of protease was run on a separate track in the gel, to detect the Staph. aureus V8 protease and any fragments generated although the technical department of the manufacturer (Sigma) indicated that the enzyme should not autodigest.

The gels were cast one day in advance, they were not pre-run and no "radical scavengers" (Thioglycolate or glutathione) were used. The concentration of the gels was 16.5%.

3.5.8. DIGESTION OF THE "VOID VOLUME 40" AND "COLUMN 40" INDIVIDUALLY.

This experiment was repeated three times. The fragments generated in the first two experiments were sent to the Department of Geology, University of Glasgow, but the levels were found to be too low for sequencing and Dr M. Cussak aborted the experiments.

The yield of peptides had to be increased to be successful. It was noticed that not only the peptide bands but also the whole track was stained with Coomassie Blue; this smear was attributed to protein degradation which could occur during the drying of the gel. To try to reduce protein degradation, in the third experiment the polypeptides were first analysed on 0.75mm thick gels instead of the normal 1.5mm gels. After staining the gel slices were equilibrated for 15 min. in the digestion buffer and stored at -70°C. The gel slice could then be inserted in the wells of the digesting gel without first drying the slice.

The "VOID VOLUME 40" and the "COLUMN 40" were digested in separate tracks. Twelve gel slices were loaded in each track. After staining the blot, five bands were cut out and sent for sequencing.

Figure 3.31. shows the result of the digestion of the two proteins blotted on Problott* and stained with Coomassie blue.

In one experiment [^{35}S] L-methionine labelled TBS:40 bands were loaded and digested in parallel, to compare the Coomassie blue stained and radio-active peptide map patterns. Unfortunately protein degradation prevented any significant comparison.

3.5.8.1. The "COLUMN 40"

The sequences of the peptides of the "VOID VOLUME 40" sent to Dr Keen Department of Biochemistry, University of Leeds have already been presented in section 3.5.5.

Five peptides of the "COLUMN 40" were sent to Dr Keen.

Figure 3.31. Electroblot of the digests of "VOID VOLUME" and the "COLUMN 40".

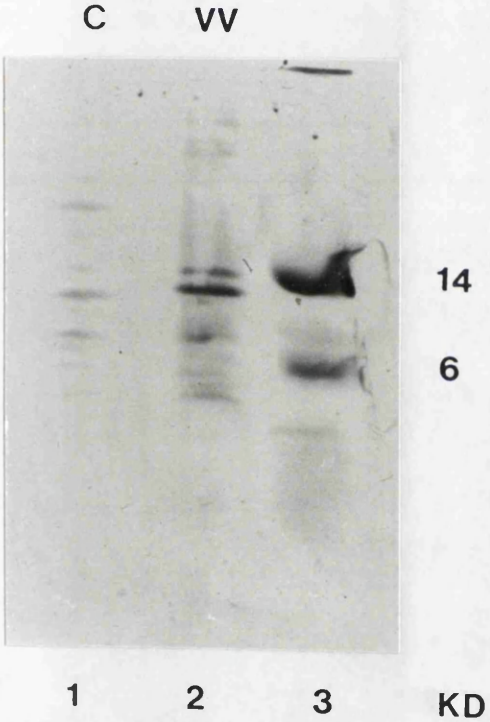
The bands containing the "VOID VOLUME 40" and the "COLUMN 40" were pooled from the fractions of two anion exchange experiments at pH.9.5 using cells from a total of 120 roller bottles. They were digested with 10ug of Staph. aureus V8 protease. The results were analysed on a 16.5% polyacrylamide gel. The polypeptides were electroblotted onto Problott* membranes and visualized by Coomasie blue staining.

Track 1 shows the digest of the "COLUMN 40" (labelled C).

Track 2 shows the digest of the "VOID VOLUME 40" (labelled VV).

Track 3 shows the MW markers.

The MW of the markers is at the right of the gel


C
VV
14
6
1
2
3
KD

Two fragments were successfully sequenced although the levels of AA were low. The results are presented in Table 3.13.

TABLE 3.13. AA SEQUENCE OF TWO PEPTIDES GENERATED BY DIGESTION OF THE "COLUMN 40" BY STAPH. AUREUS V8 PROTEASE.

	Fragment 3		Fragment 4	
	AA	pmoles	AA	pmoles
1	-		-	
2	Leu?	1	Asn	1
3	Pro?	1	Tyr	4
4	Tyr	2	Phe	2
5	Pro?	0	Ala	3
6	Ala	2	-	
7	Leu	1	Ala	2
8	Thr?	0	Leu	2
9	Asn	1	Glu	1
10	Glu	1	Ser	0
11	Gln	0	-	
12	-		Glu	1
13	Leu	0	-	
14	Glu	0	-	
15	Leu	0	Phe	1
16	Ala	2	Leu	1
17	Asp?	0	Ala	1
18	Ile	0	Ile	1
19	Ala	0	Leu	0
20			-	
21			-	
22			Ala	1
23			-	
24			-	
25			Ala	0
26			Asn?	1
27			-	
28			Ile?	0
29			Gln	0
30			Leu	0

The fragments were identified by Dr Keen as belonging to two different proteins . The fragment 3 matches the N-terminus of the rat fructose biphosphate aldolase A (Muscle specific), two of the 19 AA of this sequence are not identified. The fragment 4 matches the internal sequence of rat X-linked phosphoglycerate kinase (this gene is carried on the X chromosome), in this fragment 20 out of 30 AA were identified.

The AA sequence of the X-linked phosphoglycerate kinase (Ciccarese et al., 1989) and the fructose biphosphate aldolase A (Joh et al., 1985) have been published .

3.5.8.2. Fructose 1-6 biphosphate aldolase.

The published AA sequence of rat fructose biphosphate aldolase (Joh et al., 1985) is presented with the sequence of the fragment 3 written under the sequence of the protein (AA 1-19):

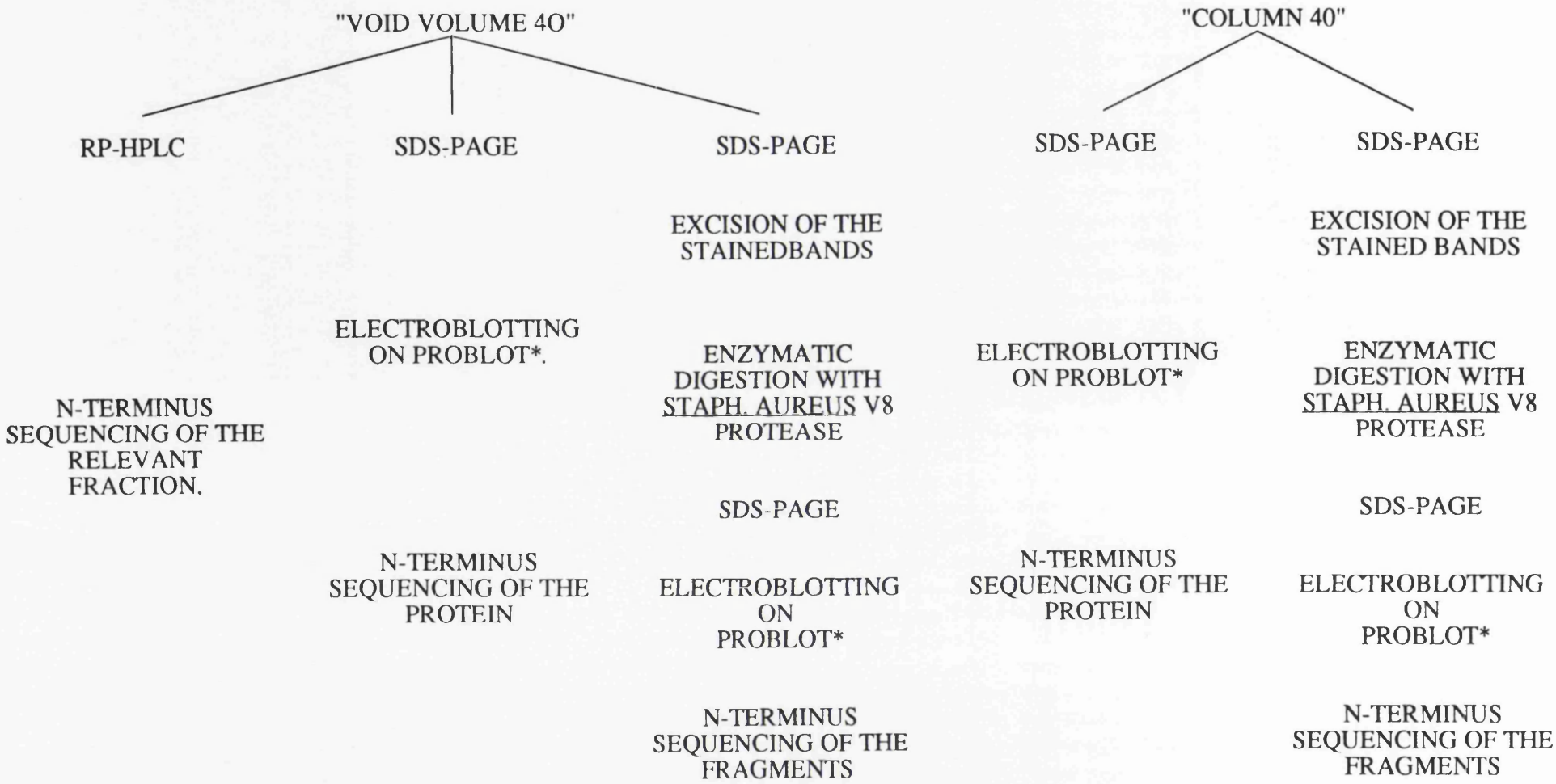
```
  1 PHPYPALTPE QKKELADIAH RIVAPGKGIL AADESTGSIA KRLQSIGTEN
    ||||||| | |  ||||||
    XLPYPALTNE QXLELADIA
 5I TEENRRFYRQ LLLTADDRVN PCIGGVILFH ETLYQKADDG RPFPQVIKSK

101 GGVVGIKVDK GVVPLAGTNG ETTTQGLDGL SERCAQYKKD GADFAKWRCV

151 LKIGEHTPSS LAIMENANVL ARYASICQQN GIVPIVEPEI LPDGDHDLKR

201 CQYVTEKVLA AVYKALSDHH VYLEGTLLKP NMVTPGHACT QKFSNEEIAM

251 ATVTALRRTV PPAVPGVTFL SGGQSEEEAS INLNAINKCP LLKPWALTFS

301 YGRALQASAL KAWGGKKENL KAAQEEYIKR ALANSLACQG KYTPSGQSGA

351 AASESLFISN HAY
```

Three AA of fragment 3 are different from the published sequence, AA N°2 leucine a tentative identification, AA N°8 asparagine and AA N°13 leucine. A total of 14 out of 19 AA of fragment 3 fit the published sequence.

3.5.8.3. Rat phosphoglycerate kinase.

The AA sequence of rat phosphoglycerate kinase (EC 2.7.2.3) is the following (Ciccarese et al., 1989)., the sequence of the fragment 4 is written underneath. Strokes show the similarity between the AA.

TABLE 3.14.
PREPARATION FOR SEQUENCING



```
  1 SLSNKLTLDK LDVKGKRVVM RVDFNVPMKN NQITNNQRIK AAVPSIKFCL

 51 DNGANSVVLM SHLGRPDGVP MPDKYSLEPV AAELKSLLGK DVLFLKDCVG

101 SEVENACANP AAGTVILLEN LRFHVEEEGK GKDASGNKVK AEPAKIDAFR

151 ASLSKLGDVY VNDAFGTAHR AHSSMVGVNL PQKAGGFLMK KELNYFAKAL
                                                 ¦¦¦¦ ¦¦
                                               XNYFAXAL
201 ESPERPFLAI LGGAKVADKI QLINNMLDKV NEMIIGGGMA FTFLKVLNNM
    ¦¦ ¦  ¦¦¦¦ ¦  ¦  ¦¦ ¦ ¦¦
    ESXEXXFLAI LXXAXXADXI QL
251 EIGTSLYDEE GAKIVKDLMT KAEKNGVKIT LPVDRVTADK FDENAKTGQA

301 TVASGIPAGW MGLDCGTESS KKYAEAVARA KQIVWNGPVG VFEWEAFARG

351 TKSLMDEVVK ATSRGCITII GGGDTATCCA KWNTEDKVSH VSTGGGASLE

401 LLEGKVLPGV DALSNV
```

Twenty AA out of 29 were identified in fragment 4, They all fit the published sequence of the rat phosphoglycerate kinase.

The amount of AA recovered is low but 14 and 20 AA have been identified and matched with the published sequences. This gives confidence in the accuracy of the identification.

3.5.9. SUMMARY OF THE SEQUENCING EXPERIMENTS.

Altogether these purification experiments summarized in table 3.14 identified three different AA sequences.

The N-terminus of "VOID VOLUME 40", the protein which is not i.p. by TBS, is identical to the N-terminus of the rat mitochondrial aspartate aminotransferase.

Two peptides were sequenced from the digestion of the "COLUMN 40", the protein which is i.p. by TBS, one is similar to the N-terminus sequence of rat fructose biphosphate aldolase and the second is very similar to internal sequences of rat phosphoglycerate kinase.

3.6. IMMUNOLOGICAL EXPERIMENTS.

The Staph. aureus V8 protease digest of the 40KD band eluting from the anion exchange chromatography experiment at pH. 9.5 showed that the FPLC:40 is made up of two proteins.

The "VOID VOLUME 40" was identified as a protein with sequence homologies to the rat mAspAT.

the "COLUMN 40" was digested with Staph. aureus V8 protease; two peptides were successfully sequenced one showed homology to the N-terminal sequence of rat fructose 1-6 diphosphate aldolase A and the other showed homology to the internal sequence of rat phosphoglycerate kinase-1.

3.6.1. IMMUNOPRECIPITATION EXPERIMENTS WITH AN ANTIBODY TO RAT MITOCHONDRIAL ASPARTATE AMINOTRANSFERASE.

The "VOID VOLUME 40", shown to have sequence homologies with the rat mAspAT, is not i.p. by TBS. Its apparent MW (40KD, as compared with the MW markers, fig 3.20 and 3.26) is less than the published result (44KD) (Huynh et al., 1980).

To test an immunological relationship between the "VOID VOLUME 40" and the mAspAT, a polyclonal antiserum against rat mAspAT was obtained from Professor Mattingly, Division of Molecular Biology and Biochemistry, University of Missouri-Kansas City. It was raised in goat, and recognized rat mAspAT in Western Blotting experiments (Mattingly et al. 1987). This antiserum was used in i.p. experiments.

Bn5T and RE cells radiolabelled with [^{35}S] L-methionine were extracted in RIPA buffer, incubated with 10ul of TBS or 10ul of mAspAT antiserum for 1 h.; 60ul of Pansorbin* was then added and the mixture was incubated for a further hour.

Figure 3.32 shows the result of the experiment.

TBS i.p. a 40KD protein from the Bn5T cell extract as expected (track 3).

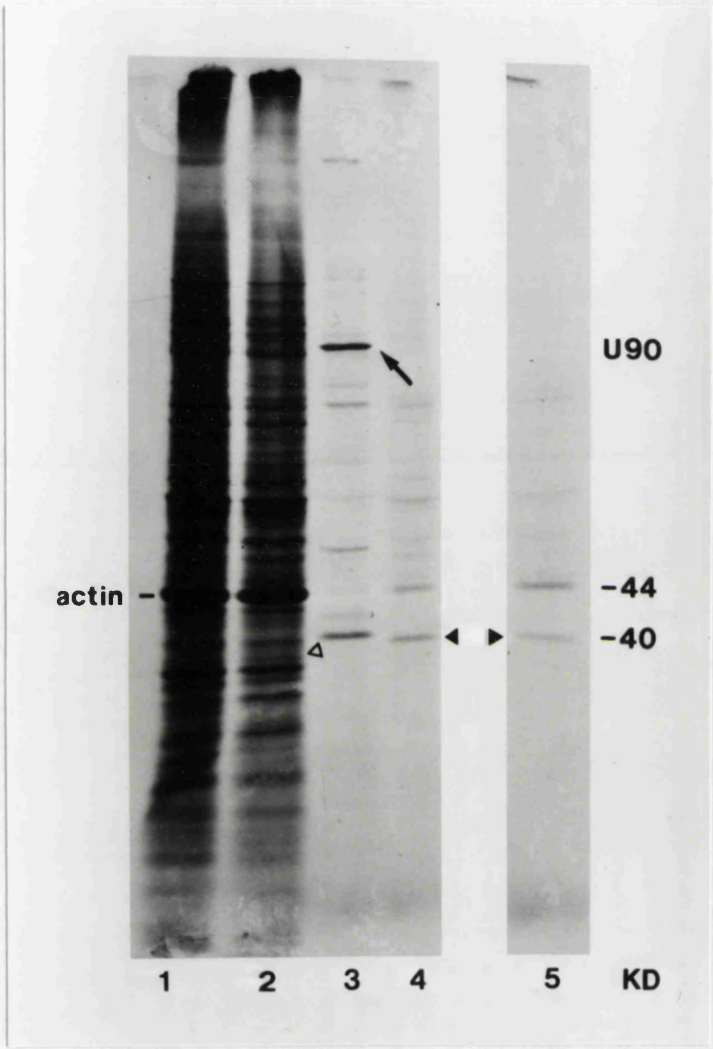
The mAspAT antiserum i.p. two proteins of approximate apparent MW 44KD and 40KD. The 40KD protein has an apparent

Figure 3.32. Immunoprecipitation of Bn5T polypetides by an antiserum specific of the rat mAspAT.

Autoradiograph of a 9% SDS polyacrylamide gel which shows i.p. experiments performed on [^{35}S] L-methionine labelled polypeptides extracted in RIPA buffer from Bn5T tumour cells with TBS and rat mAspAT antiserum. Although these samples were all run on the same gel only the relevant tracks were cut from the autoradiograph for photography.

Track 1 shows the Bn5T cell polypeptides profile.
Track 2 shows the RE control cell polypeptides profile.
Track 3 shows Bn5T cells i.p. with TBS.
Track 4 shows Bn5T cells i.p. with mAspAT antiserum.
Track 5 shows RE control cells i.p. with mAspAT antiserum.

The position of the TBS:40 is shown by an empty triangle. The mAspAT antiserum i.p. a polypeptide of an apparent MW slightly inferior to the TBS:40 in both Bn5T tumour cells and RE control cells shown by two filled triangles. The position of the U90 is indicated by an arrow.


actin
U90
-44
-40
1
2
3
4
5
KD

MW slightly lower than the TBS:40. Both the 44KD and the 40KD are present in the Bn5T and the RE cell extracts (tracks 4 and 5). The 44KD band is stronger in the RE track than in the Bn5T track and comigrates with a faint band in the TBS i.p. track and the strong bands labelled "actin" in the Bn5T and RE cell extract (tracks 1 and 2).

This antibody does not selectively i.p. a 40KD protein in Bn5T cells extract.

3.6.2. SLOT BLOT EXPERIMENTS.

The TBS:40 is increased by HSV-2 infection (Macnab et al. 1992). Slot blot experiments were set up to investigate if mAspAT expression was increased by HSV-2 infection. Bn5T cells were infected with HSV-2 HG52 (1 p.f.u./cells). The growth of one roller bottle of uninfected and infected cells were harvested in 2ml of WF buffer. The cell extracts were lysed with a Dounce homogenizer (50 strokes) followed by sonication as for the purification procedure. The protein concentration was measured and the Bn5T extract was diluted by a factor 2 to equalize the protein concentrations in Bn5T and RE cells extracts. Twelve doubling dilutions (1/2 to 1/4096) were made up and loaded on the slot blot manifold. After aspiration, the nitrocellulose was blocked in 3% gelatin in TBuS, probed with mAspAT antibody diluted 1/1000 in TTBuS at 37°C for 1h, washed three times with TTBuS, the blot was incubated 1 h. at RT with HRP conjugated rabbit anti-goat antibody after three washes with TTBuS, the chemoluminescence reagent was added.

Figure 3.33. shows that the HSV-2 infected extract gives a positive signal up to a titre of 1/1024 and the Bn5T extract a positive signal to a titre of 1/256. There is a difference of two doubling dilutions which is generally considered as the minimum increase to be significant.

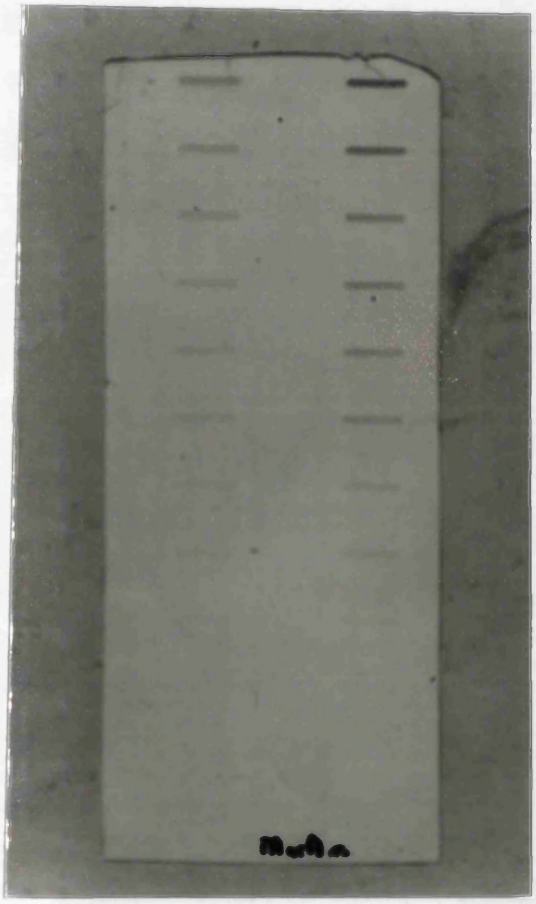
This result was repeated twice showing that HSV-2 infection significally increases the expression of the mAspAT as detected by this antibody.

Figure 3.33. Slot blot experiment.

Twelve doubling dilutions (1/2 to 1/4096) of Bn5T and HSV-2 infected Bn5T cell extracts were loaded on nitrocellulose and then blotted with an antibody specific for mAspAT. The reaction was visualized with the chemoluminescence reagent.

Track 1 contains the uninfected Bn5T extract doubling dilutions.

Track 2 contains the infected Bn5T extract doubling dilutions.



-1/2
-1/4
-1/8
-1/16
-1/32
-1/64
-1/128
-1/256
-1/512
-1/1024
-1/2048
-1/4096

1 2

3.6.3. WESTERN BLOTTING EXPERIMENT.

A Western blot experiment was set up with two aims.

1/ To further identify the proteins detected by the antibody specific to mAspAT in Bn5T cells extracts whose expressions were increased upon HSV-2 infection. The cell extracts were prepared as for the slot blot experiment.

2/ To test the immunological relationship between the two 40KD proteins purified ("VOID VOLUME 40" and "COLUMN 40") and the mAspAT.

A 0.75mm thick SDS-PAGE was loaded with:

Track 1: A sample containing the "VOID VOLUME 40".

Track 2: A sample containing the "COLUMN 40" (fraction 23 of an ion exchange chromatography experiment at pH. 9.5).

Track 3: An enlarged i.p. using 4.10^7 c.p.m. of Bn5T extracts and incubated 1 h. with 50ul TBS. the immune complexes were trapped by 100ul of pansorbin*.

The gel was cut to fit the size of a mini gel kit, the proteins were blotted onto nitrocellulose in CAPS buffer with 10% methanol pH. 11. The voltage was set at 50 Volts for 1h. 45 min.. The blot was probed with mAspAT antibody as described in section 3.6.2.. After development of the chemiluminescence reaction, the blot was exposed to detect the [^{35}S] L-methionine labelled proteins.

The chemiluminescence signal and the autoradiography of the blot are shown in figure 3.34a and b.

Figure 3.34a. is the chemiluminescence signal.

The "VOID VOLUME 40" is recognized by mAspAT antibody (track 1).

No protein is recognized in the column fraction 23 (track 2). Therefore the "COLUMN 40" is not recognized by

Figure 3.34. Immunoblotting with the mAspAT antiserum.

Two fractions of an ion exchange experiment at pH. 9.5 containing the "VOID VOLUME 40" and the "COLUMN 40", a control Bn5T i.p., were loaded onto a 9% polyacrylamide gel after completion of the electrophoresis, the polypeptides were blotted onto nitrocellulose.

The Western blotting experiment (figure 34a) and the autoradiographic control (figure 34b) are presented together.

Track 1 contains a fraction of the void volume and the "VOID VOLUME 40".

Track 2 contains the fraction 23 and the "COLUMN 40".

Track 3 shows the control Bn5T i.p.

The MW is indicated at the left of the gel,

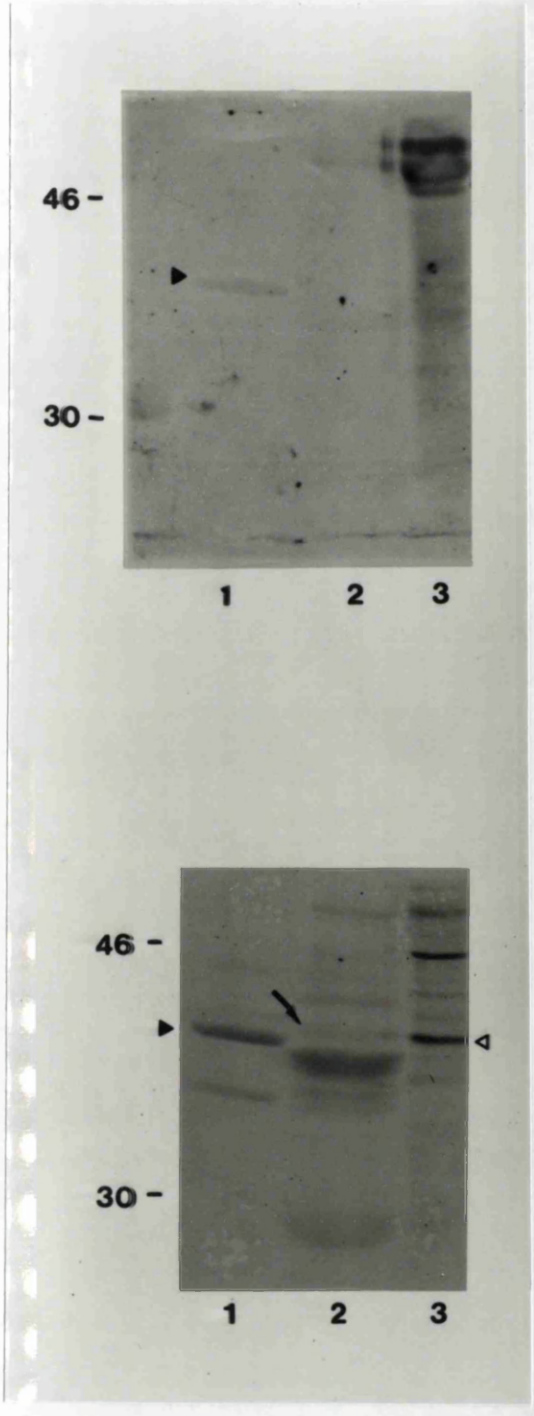
Figure 34a is the Western blotting experiment with an antibody raised against mAspAT.

The "VOID VOLUME 40"is shown by a filled triangle in track 1.

Figure 34b is the autoradiograph of the blot.

The "VOID VOLUME 40" is shown by a filled triangle in track 1. The column 40 is shown by an arrow in track 2 and the TBS:40 by an empty triangle in track 3.

A



B

mAspAT antibody.

Figure 3.34b is the autoradiography of the blot.

It confirms the presence of the "COLUMN 40" in track 2 (arrow), which comigrates with the TBS:40 in track 3 (empty triangle). The mAspAT in track 1 (filled triangle) has a lower apparent MW than the TBS:40.

This result shows that the "VOID VOLUME 40" is immunologicaly related to the mAspAT.

The "COLUMN 40" is not recognized by this mAspAT antibody.

3.7. SUMMARY OF THE RESULTS.

The attempt of raising monoclonal antibodies by immunizing mice against Bn5T cell lysates was a failure, but the screening experiments showed that mouse did not recognize the 40KD protein which TBS did.

Purification and sequencing experiments identified three rat proteins: mitochondrial aspartate aminotransferase, fructose 1-6 diphosphate aldolase and phosphoglycerate kinase. The relevance of these data to transformation will be addressed in the "Discussion".

The purified "VOID VOLUME 40", was related to mAspAT by its sequence, it was recognized in Western blotting experiment by an antibody specific to mAspAT. The "VOID VOLUME 40" was not i.p. by TBS but was part of a complex i.p. by TBS.

An antibody specific to rat mAspAT i.p. a 40KD protein in both RE and Bn5T cell, hence failing to detect a 40KD protein specificaly i.p. in Bn5T cells.

Slot blot experiments using doubling dilutions and the same antibody specific to mAspAT showed that the expression of mAspAT is increased by HSV-2 infection.

Two proteins, namely rat fructose 1-6 diphosphate aldolase and phosphoglycerate kinase were identified as components of the "COLUMN 40" by sequencing. The "COLUMN"

40 was always i.p. by TBS, but this was not checked for the samples sent for sequencing. It is also not known which of the two enzymes is i.p. by TBS.

Aldolase A and PGK are normal cellular proteins. Explanation for their i.p. in Bn5T cells and not in RE control cells and the relevance of these results to transformation will be discussed in the "Discussion" following. Further experiments to confirm these results will be suggested.

PART 4
DISCUSSION.

The aim of the project was to purify and obtain AA sequence for a polypeptide of 40 KD i.p. by TBS from a Bn5T cell extract but not from an RE control cell extract. Moreover infection with HSV increased the amount of polypeptide i.p.. The 40 KD polypeptide was characterized by its digestion pattern with the enzyme Staph. aureus V8 protease. The initial characterization was carried out on Bn5T cells and not on infected cells.

In these experiments TBS was found to i.p. more than one polypeptide. It was decided to purify and identify each polypeptide in turn and lastly to test the effect of HSV-2 infection on its expression. Two candidate polypeptides were purified and AA sequences belonging to three proteins were obtained namely, rat mitochondrial aspartate aminotransferase, rat phosphoglycerate kinase-1 and rat fructose 1-6 diphosphate aldolase A, but HSV-2 increased only the expression of the mAspAT as tested in slot blot experiments.

Attempts to raise antibodies in mice and rabbits were carried out. In rabbit there was no immunological response. To raise Mabs twelve mice were immunized against Bn5T tumour cells. The 90KD polypeptide i.p. by mouse antisera and the U90 i.p. by TBS were similar, in respect of the Staph. aureus V8 protease peptide map. By contrast the 40KD polypeptide i.p. by the serum of the mice and the TBS:40 had different peptide maps. Therefore, in this instance the immune system of the rat and the mouse recognized different 40KD proteins. Unfortunately attempts to raise Mabs against the 90KD and the 40KD polypeptide failed. Although the 40KD polypeptide did initially raise Mabs, these were unfortunately subsequently lost.

The discussion will be divided in three parts, the first part will cover specific points of the purification procedure, the second part will propose a mechanism for the different specificity of the mouse antisera and TBS and

the third part will review the biochemical properties of the proteins identified and discuss their identification as the TBS:40.

4.1. THE PURIFICATION PROCEDURE.

4.1.1. PRECIPITATION BY AMMONIUM SULPHATE.

This experiment confirmed that the TBS:40 and the U90 could be i.p. independently of each other (Macnab et al., 1985; Hewitt et al., 1991) and were not merely recognized as part of a complex. They were i.p. in different ammonium sulphate fractions, the U90 in both the extraction pellet and the 30% pellet, and the TBS:40 in the 70% supernatant.

Two 40KD proteins i.p. by TBS were separated in this experiment which were distinguished by their Staph. aureus V8 protease peptide map.

The 40KD polypeptide i.p. in the extraction pellet, 30% pellet and 70% pellet was i.p. independently from the U90 since no U90 was i.p from the 70% pellet. (figure 3.12). The 40KD in the extraction pellet was not studied further because its peptide map after digestion by Staph. aureus V8 protease was different from the peptide map of the TBS:40

However although most of the 40KD protein precipitated in the pellet is not soluble, a fraction of it may be soluble and be present in the 70% ammonium sulphate supernatant. Therefore the 40KD polypeptide in the pellet could be one of the polypeptides making up the final pattern of the i.p. TBS:40 band. (figure 3.13).

4.1.2. ANION EXCHANGE CHROMATOGRAPHY.

4.1.2.1. RESOLUTION AT pH.8.

The peptide maps of the TBS:40 and the FPLC:40 are undistinguishable (figures 3.16, 17, 18), as identitified by radiolabelled peptides detected by autoradiography.

4.1.2.2. RESOLUTION AT pH 9.5.

The Staph. aureus V8 protease digests showed that the FPLC:40 was made of two proteins (figure 3.21a and b). Comparison of the two peptide map patterns to all the peptide maps previously made suggested that the pattern of the "COLUMN 40" was highly similar but close to the pattern of the TBS:40. Because it is i.p. by TBS, it is probably the most intersting component of the TBS:40.

The "VOID VOLUME 40" is not i.p. by TBS and has a Staph. aureus V8 protease peptide map which differs considerably from the TBS:40 (see figures 3.21). Because the peptide maps of the TBS:40 and the FPLC:40 were undistinguishable, the "VOID VOLUME 40" had to be a component of the TBS:40. The "VOID VOLUME 40" identified as sequence related to the mAspAT may be i.p. by TBS from Bn5T cells extract as part of a complex, and the complex could have been disrupted as a result of this purification step. Therefore both proteins had to be purified and sequenced.

4.1.3. RP-HPLC.

The method used was adapted after consultation with the technical departmemt of Applied Biosystem and was described in the litterature as the method of choice to prepare proteins for N-terminus sequencing. However, the results obtained with RP-HPLC were disapointing. Out of the 3 samples sequenced, only one gave a good result. The other two were disregarded because of low or irregular levels of AA recovery. The reasons for the poor recovery is not known. No satisfactory explanation could be found in the literature. It is possible that RP-HPLC was degrading the "VOID VOLUME 40" since the protein became totally insoluble if dried, whereas this was not the case before separation on the RP-HPLC column.

4.2. HYPOTHESIS TO EXPLAIN THE DIFFERENT SPECIFICITY OF TBS AND MOUSE ANTISERUM.

The lack of reactivity of mice to proteins with a peptide map similar to the TBS:40 is surprising. Bn5T cells injected into a mouse act as a xenoantigen. Xenoantigens are expected to stimulate the immune response strongly. The lack of immune response of the mouse to the TBS:40 can be explained by the similarity of the rat and the mouse protein.

PGK-1 could be such a protein. The AA sequences of the rat and mouse PGK-1 differ by three AA. Could such a small difference in the AA sequence prevent an effective stimulation of the immune response? There is no answer to this in the current study but an attempt at raising antibody against another well conserved protein, aldolase A, suggests it is possible. A rabbit failed to make antibodies to human aldolase A (Okajima et al., 1990). Comparison of the published sequences of the rabbit and the human aldolases, using the GCG Bestfit programm, found only six different AA.

4.3. THE BIOCHEMICAL ROLE OF THE ENZYMES ISOLATED FROM THE 40KD BAND AND THEIR RELEVANCE TO TRANSFORMATION.

Aspartate aminotransferases are involved in the synthesis of AA from oxaloacetate and L-glutamate. Two forms of enzyme exist a cytosolic and a mitochondrial form (cAspAT and mAspAT).

The two enzymes fructose 1-6 diphosphate aldolase and phosphoglycerate kinase are part of the glycolytic cycle. The glycolytic pathway plays a critical role in the production of adenosine triphosphate and therefore in the energy metabolism .

4.3.1. FRUCTOSE 1-6 DIPHOSPHATE ALDOLASE.

Fructose 1-6 diphosphate aldolase, also called aldolase (EC 4.1.2.13) catalyses the following reaction:

fructose 1-6 diphosphate ⇌ dihydroxy acetone phosphate + glyceraldehyde 3-phosphate

Two types of this enzyme are known designated class I and class II. The enzymes of class I function by imino formation between the substrate and a catalyticaly essential lysine residue in the active site. The class II enzymes utilize a divalent metal ion.

The enzymes of eukaryotes generally fall into class I and are tetrameric (Mr 160.000). They consist of four identical subunits which have an apparent MW of 40KD, and which occurs as three isozymes namely A,B and C. The nucleotide and the AA sequences of the three aldolase isozymes have been determined (Tsutsumi et al., 1984; Joh et al.,1985; Kukita et al., 1988). The AA sequences of the isozymes are homologous especially in the region of the imine forming lysine residue (Reviewed by Perham, 1990). The different isozymes occur in different tissues.

Aldolase A is a foetal form of aldolase, it is present in large amounts in muscle and in much smaller amounts in kidney.

Aldolase B is found predominantly in kidney.

Aldolase C is found mainly in the brain and is composed of subunits C and A (reviewed by Asaka, 1983).

The peptide called fragment 3 was generated by digestion of the "COLUMN 40" with Staph Aureus V8 protease.The AA sequence of fragment 3 was found to be similar to the N-terminus of the rat aldolase A sequence. This result contradicts the previous result i.e. failure to obtain N-terminal sequences from the "COLUMN 40" (see section 3.5.6). Dr Keen who sequenced the protein could not give an explanation. The sequence of fragment 3 was compared with the sequences of aldolase A, B and C, to confirm the isozyme identification. The AA differences between aldolase A and aldolase B and C are shown below in bold characters. The AA of fragment 3 which do not fit the aldolase A sequence are underlined.

								?	?	10	
Fragment 3	-	Leu	Pro	Tyr	Pro	Ala	Leu	Thr	Asn	Glu	Gln -
Aldolase A	Pro	His	Pro	Tyr	Pro	Ala	Leu	Thr	Pro	Glu	Gln Lys
Aldolase B	**Ala**	His	**Arg**	**Phe**	Pro	Ala	Leu	Thr	**Ser**	Glu	Gln Lys
Aldolase C	Pro	His	**Ser**	Tyr	Pro	Ala	Leu	**Ser**	**Ala**	Glu	Gln Lys

		?				?	?	19	
Fragment 3		Leu	Glu	Leu	Ala	Asp	Ile	Ala	
	A	Lys	Glu	Leu	Ala	Asp	Ile	Ala	
	B	Lys	Glu	Leu	**Ser**	**Glu**	Ile	Ala	
	C	Lys	Glu	Leu	**Ser**	Asp	Ile	Ala	

? : querry AA.

The sequence of fragment 3 differs from aldolase A by two AA, AA 2 a leucine replaces a histidine, and AA 9 an asparagine replaces a proline, but in this case the asparagine is only a tentative identification. This result is not surprising, allelic variants of aldolase A have been reported previously (Joh et al., 1985).

In this region aldolase A differs from aldolases B by five AA, four of them identified, from aldolase C by four AA. Therefore the isoenzyme identification is not ambiguous.

Aldolase levels were investigated as possible markers of various cancers.

The high glycolytic activity of tumoral tisssues was discovered by Warburg (1930). High aldolase levels have been observed in rat tumour tissues (Sibley and Lehninger, 1955) and in the venous blood from a rat tumour (Warburg and Christian, 1943; Sibley and Lehninger, 1955), histologically classified as a sarcoma like the tumours induced by injection of Bn5T cells (J.C.M. Macnab, personal communication).

More recently elevated aldolase levels have been reported in the sera of patients with cervical cancer (Yeshorwardana and Sangita, 1985), ovarian cancer (Yeshorwardana and Singh, 1984) and in bronchial cancer

(Dallüge et al, 1984), but a raised aldolase level is also associated with chronic liver diseases and muscle diseases (Reviewed by Asaka et al., 1983).

An identification of the aldolase A as the 40KD protein recognized by TBS is compatible with some properties known for the TBS:40.

Aldolase A is a protein expressed at high level in embryo and in tumour tissues (Reviewed by Asaka et al., 1983) and is also capable of inducing auto-antibodies (Brown et al., 1984). These properties fit with the TBS:40 which is located in the cytoplasm and is detected by i.p. in early embryo cells (12 days), disappears at 15-19 days (J.C.M. Macnab, unpublished results) and is again detected by i.p. in all lines of transformed and immortalized cells tested (Macnab et al., 1985).

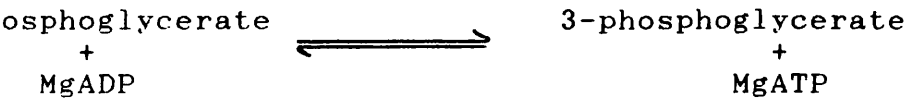
The Aldolase A monomer has a predicted MW of 39,125 which fits with the MW of the TBS:40.

The pI. of Aldolase A is 8.9, thus it would not be retained by a anion exchange column at pH.8. The published purification procedure for aldolase A is not however directly comparable (Reviewed by Horeker et al., 1970).

In contrast to the TBS:40, the bulk of aldolase A is precipitated by a 60% saturated ammonium sulphate solution. HSV-2 infection induces an 8 fold decrease of the expression of aldolase A as tested with doubling dilutions in a slot blot experiment (data not shown). Moreover, the sequencing of the N-terminus is difficult to conciliate with the N-terminus blockage of the "COLUMN 40". In conclusion, aldolase A is unlikely to be the "COLUMN 40", it may be a contaminating protein.

4.3.2. PHOSPHOGLYCERATE KINASE.

Phosphoglycerate kinase (PGK) is a monomeric enzyme which catalyzes the following reaction :



In mammals PGK is encoded by two genes, *pgk-1* and *pgk-2*. *Pgk-1* is X chromosome linked and is expressed in somatic cells (Vandeberg et al., 1976). *Pgk-2* is expressed only in meiotic and haploid male germ cells.

The peptide called fragment 4 was generated by the digestion of the "COLUMN 40" by Staph. aureus V8 protease. The AA sequence of fragment 4 was found similar to a region of the rat X-linked phosphoglycerate kinase (PGK-1) sequence.

The nucleotide and the AA sequences of PGK-1 and PGK-2 in mouse (Boer et al., 1989) and rat PGK-1 (Ciccarese et al., 1989) have been published. To make sure that the isozyme identification is correct, the sequence from the purified peptide obtained in this study (top line) is compared with the sequence of rat PGK-1 and of mouse PGK-2, the sequence of rat and mouse PGK-1 is the same for the region in which the polypeptide fits. The AA which differs in PGK-2 (from PGK-1) are written in bold characters. The sequence of the purified peptide starts at AA 193, and contains 30 AA.

										10			
Fragment 4	-	Asn	Tyr	Phe	Ala	-	Ala	Leu	Glu	Ser	-	Glu
PGK1 (rat)	Leu	Asn	Tyr	Phe	Ala	Lys	Ala	Leu	Glu	Ser	Pro	Glu
PGK2 (mouse)	Leu	**Asp**	Tyr	Phe	**Ser**	Lys	Ala	Leu	Glu	**Lys**	Pro	Glu
						?		20				
Fragment 4	-	-	Phe	Leu	Ala	Ile	Leu	-	-	Ala	-	-
PGK1	Arg	Pro	Phe	Leu	Ala	Ile	Leu	Gly	Gly	Ala	Lys	Val
PGK2	Arg	Pro	Phe	Leu	Ala	Ile	Leu	Gly	Gly	Ala	Lys	Val
				?		30						
Fragment 4	Ala	Asp	-	Ile	Gln	Leu						
PGK1	Ala	Asp	Lys	Ile	Gln	Leu						
PGK2	**Lys**	Asp	Lys	Ile	Gln	Leu						

The sequences of PGK-1 and PGK-2 differ by 4 AA which have been all identified in this fragment. Therefore the identification of fragment 4 as PGK-1 is not ambiguous

although the molar levels of AA in the purified peptide are low.

PGK-1 has been identified as the primer recognition protein 2 (PRP2). (Jindal and Vishwanatha, 1990). PRP are proteins important for the efficiency of DNA replication. Purified PRP is made up of two subunits PRP1 and PRP2 of MW of 36,000 and 41,000 on SDS gel.

Evidence from studies of SV40 DNA replication in vitro, has led to to the proposal that DNA polymerase delta is the leading strand replicase while DNA polymerase alpha functions as a lagging strand replicase. For polymerase alpha to function on the lagging strand it must be capable of extending an RNA primer of 6-9 bases utilizing a template of 40-300 bases of ssDNA, i.e. working on substrates with a low primer to template ratio. Polymerase alpha is very ineffective at using substrates where the primer to template ratio is low. PRP are cofactors of polymerase alpha which increase the efficiency of the enzyme (Jindal and Vishwanatha, 1990).

The two subunits of PRP are tightly bound, they co-elute from a MonoQ column at pH. 6.9 (Tsurimoto and Stillman, 1989). The i.p. of the two subunit of PRP could explain the existence of a 34KD polypeptide which is i.p. by TBS (Hewitt, 1988). A 34KD is also purified in the same experiment as the TBS:40, however, it does not co-elute with the TBS:40 from the 1ml Mono Q column at pH.8, but it can be i.p. by TBS in fraction 8 (see figure 3.21). The identification of such a 34KD protein has not been further pursued.

Published properties of PGK-1 suggests that it could be one of the constituents of the TBS:40.

PGK-1 showed properties similar to the TBS:40 in a published purification procedure (Hiremath and Rothstein, 1982). As for the TBS:40, PGK-1 was precipitated in a 54%-80% ammonium sulphate pellet, and it did not stick to an anion exchange resin at pH.8.

PGK-1 calculated molecular weight is 44,596 Daltons,

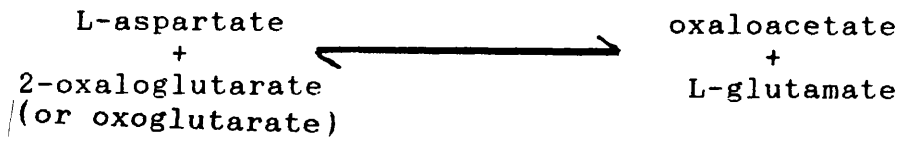
but it is reported to migrate faster in SDS gels (Mas et al., 1986). The apparent mobility of PGK-1 is variable in different rat tissues and minor isoforms exists (reviewed by VandeBerg, 1985). PGK-1 isoforms can be induced by transformation, (Tollefsbol and Gracy, 1980). Some isoforms could therefore be i.ped by TBS.

PGK-1 expression is decreased 8 fold, but not shut-off by HSV-2 infection as detected by slot blots (data not shown). Since the TBS:40 is made of several proteins, PGK-1 could be a component of the TBS:40 whose expression is not increased by HSV-2 infection. Moreover the slot blots experiment used measured the abundance of the proteins reacting with an antibody and not of an individual isoform.

A Western blotting experiment with the antiserum raised against PGK-1 could show an immunological relationship between the "COLUMN 40" and PGK-1.

4.3.3. RAT MITOCHONDRIAL ASPARTATE AMINOTRANSFERASE.

Aspartate aminotransferases are dimeric enzymes which catalyze the following reaction.



Aspartate aminotransferases use pyridoxal phosphate as co-enzyme. Both the cytoplasmic and the mitochondrial aspartate aminotransferases are synthesized in the cytoplasm. The mAspAT precursor migrates to the mitochondria, where it is cleaved to form the mature enzyme.

The rat mAspAT has been purified (Huynh et al., 1980) sequenced (Huynh et al., 1981) and the gene cloned (Mattingly et al., 1987). The subunit of the rat mAspAT has a predicted MW of 44,358 (Huynh et al., 1981), which matches its apparent MW on SDS-PAGE (Huynh et al., 1980).

The N-terminus sequence and the sequence from two internal peptides from the "VOID VOLUME 40" matched the published sequence of the rat mAspAT.

Two AA of the peptides sequenced differ from the published sequence of rat mAspAT. The glycine, AA 11, is replaced by a proline. This substitution was also observed in the clone of the mAspAT gene isolated and sequenced by Mattingly et al., (1987). The second difference is a lysine, AA 280, replaced by a proline but from my purification the identification of the proline was only tentative, because of the low level of AA recovered from the sequencer. These differences could be confirmed by cloning the gene and sequencing it.

Aspartate aminotransferases are the subject of considerable studies because they are markers of myocardial infarction and of liver diseases. No report of their involvement in cancer has been found in the literature. There is, however, extensive evidence of mitochondrial derangement in cancer but it is not clear whether these derangements are a factor initiating transformation or a consequence of it (Reviewed by Shay and Werbin, 1987). Mitochondria are the site of the cellular respiration. However the oxidative metabolism of cancer cells is impaired. Cancer cells can obtain approximately the same amount of energy from fermentation as from respiration, whereas normal cells obtain much more energy from respiration than from fermentation. Warburg (1956), therefore, proposed that an impairement of mitochondrial function was essential to the transformed phenotype. These results also showed that mitochondria were unimportant for the energy metabolism of cancer cells and genes coding mitochondrial proteins could become non-functional without modification of the phenotype of the cell, and therefore the importance of the modification of such genes is in doubt (Howard Jacobs, personal communication).

Mitochondrial proteins are also known to induce auto-antibodies. The proteins responsible have been studied in primary liver biliary cirrhosis. Mitochondrial aspartate aminotransferase has not been identified as one of the components of the mitochondrial autoantigens in the

published literature.

The biochemical properties of the purified mAspAT (Huynh et al., 1980) are compatible with the results presented in this thesis. The mAspAT is precipitated by ammonium sulphate in the 40%-80% fraction and it is not retained by an anion exchange column at pH.8.

The mAspAT was reported to be functional, associated with glutamate dehydrogenase, the alpha ketoglutarate dehydrogenase complex and the mitochondrial malate dehydrogenase (Fahien et al., 1989); the mAspAT associates with lipids and with a binding protein on the internal membrane of the mitochondrion (Teller et al., 1990). It is possible that TBS i.p. one of these proteins and the mAspAT as a consequence. The complex could be disrupted by the purification procedure. This explanation is compatible with the results of the i.p. experiments. The "VOID VOLUME" may be i.p. as a complex with the "COLUMN 40" at pH. 8. But co-precipitation of the "VOID VOLUME 40" and the "COLUMN 40" is not the only possible explanation. The "VOID VOLUME 40" could co-precipitate with another protein (see figure, 3.19).

The purification of the "COLUMN 40" was repeated using radiolabelled extracts. Four preparations using the growth of 60 roller bottles were used. One fraction of each preparation was used for an i.p. experiment with TBS to verify the presence of the "COLUMN 40". The experiment was carried out as described in section 3.5.8.. Six bands from this digest were sent for sequencing to Dr Keen. No sequence could be obtained from one of the bands sent because of insufficient material. More than one sequence was obtained from the other bands. Nine sequences having an initial yield of 10 to 50 picomoles matched the sequence of the mAspAT.

The internal peptides sequenced are shown underneath. The strokes ¦ denote common AA.

```
  1 SSWWTHVEMG GPDPILGVTE AFKRDTNSKK MNLGVGAYRD DNGKPYVLPS
                          !!!!!!!!!! !!!!!
                          AFGRDTNSKK MNLGV

 51 VRKAEAQIAG KNLDKEYLPI GGLADRCKAS AELALGENSE VLKSGRFVTV
                                     !!!!!!!! !!!!!!!!!!
                                     LALGENSE VLKSGRFVTV

101 QTISGTGALR VGASFLQRFF KFSRDVFLPK PSGGNHTEIA RDAGMQLEGY
    !!!!!!!!!
    QTISGTGAL

151 RYYDPKTCGF DFSGALEDIS KIPEQSVLLL HACAHNPTGV DPRPEQWDEM
                          !!!!!! !!!!!!!!!!           !
                          QSVLLL HACAHNPTGV           M

201 AAYVKKNNLF AFFDMAYQGF ASGDGNKDAW AVRHFIEQGI NVCLCQSYAK
    !! !!! !!! !!!!!!!
    AAVVKKKNLF AFFDMAY

251 NMGLYGERVG AFTVVCKDAE EAKRVESQLK ILIRPLYSNP PLNGARIAAT
                               !!!! !!!!!!!!!!
                               SQLK ILIRPLYSNP

301 ILTSPDLRQG WLQEVKGMAD RIGSMRTQLV SNLKKEGSSR NWQHITDQIG

351 MFCFTGIKPE QVERLTKEFS IYMTKDGRIS VAGVTSGNVG YLAHAIHQVT

401 K
```

In total 89 AA were identified. The sequences obtained are identical to the sequence of mAspAT except for AA 203 and 207. This result suggested that the "COLUMN 40" could be also related to the mAspAT.

4.4. CONCLUSION AND FUTURE WORK.

The "VOID VOLUME 40" is immunologically and sequence related to the mAspAT. The mechanism of the i.p. of the "VOID VOLUME 40" by TBS should be investigated.

HSV-2 infection increases the expression of mAspAT as detected in slot blot experiments. In common with most house keeping gene the mAspAT contains neither CAAT nor TATA boxes (Setoyama et al., 1990) therefore the mechanism of induction by HSV-2 is not by transactivation. Several mechanisms of induction of cellular genes by HSV have been documented (Reviewed by Everett, 1987). These includes transactivation by Vmw175 and Vmw110, the formation of

complexes of TAATGARAT with Vmw65 (Kemp et al. 1986), the induction by adsorption of the virus to the cell surface and induction without any protein synthesis (Patel et al., 1986). So far we have not investigated these mechanisms, but it is known that the TBS:40 activates at least seven and a half hour later than the U90 (Hewitt, 1988), and moreover the U90 is increased by those proteins which regulates protein synthesis (Macnab, personnal communication).

In the second experiment the sequences obtained from the "COLUMN 40" were matching the sequences of the mAspAT. A contamination of the "COLUMN 40" by the "VOID VOLUME 40" must be ruled out. Other methods of purification must be used such as chromatofocusing, cation exchange chromatography or two dimension gel electrophoresis. If the sequences obtained are identical to the mAspAT sequences, the relationship between the "COLUMN:40" and the mAspAT would be confirmed.

Finally which of the 40KD proteins is i.p. by the sera of cervical cancer patients could be determined. Its gene could be cloned and the entire protein sequenced. Its function could be investigated.

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