Studies of the Protein Kinases of α-Herpes Viruses

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ABBREVIATIONS

The abbreviations recommended by the Biochemical Journal in its instructions to authors (*Biochem. J.* **241** 1-17) have been used with the following additions:

anti-serum P	anti-serum raised against protein product of part of *pk* gene
anti-serum S	anti-serum raised against protein product of part of US3 gene
BHK	baby kidney hamster (cells)
BSS	balanced salt solution
CaM	calmodulin
C-terminus	Carboxy-terminus (of protein)
D-MEM	Dulbecco modified Eagle's minimum essential medium
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EBV	Epstein-Barr virus
EF-2	elongation factor-2
EGF	epidermal growth factor
eIF	elongation initiation factor
EHV-1	equine herpes virus type 1
ES	Earle's salts
G-MEM	Glasgow modification of Eagle's minimum essential medium

gp	glycoprotein
HMCV	human cytomegalovirus
HMG CoA	hydroxymethylglutaryl CoA
HPLC	high pressure liquid chromatography
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HSV-PK	protein kinase induced during infection with HSV-1
IE	immediate early (gene)
IPTG	isopropyl-thiogalactoside
MAP-2	microtubule associated protein-2
MEM	minimum essential medium
NP-40	Nonidet P-40
OD	optical density
oz	ounce
PBS	phosphate buffered saline
pfu	plaque forming units
PK	protein kinase
PMSF	phenylmethylsulphonyl fluoride
PP	protein phosphatase
PRV	pseudorabies virus
PRV-PK	protein kinase induced during infection with PRV
R_L	long repeat (region of genome)
R_S	short repeat (region of genome)
TBE	Tris, Borate, EDTA (buffer)
TE	Tris, EDTA (buffer)
TEA	Tetraethylammonia
TEMED	N, N, N', N-tetramethyl-ethylene diamine

TPA	12-O-tetra-decanoyl phorbol-13-acetate
U_L	unique long (region of genome)
U_S	unique short (region of genome)
VSV	vesicular stomatitis virus
VZV	varicella zoster virus
X-gal	5-bromo-4-chloro-3-indolyl-β-galactoside

SUMMARY

This work concerns the two similar protein kinase activities - HSV-PK and PRV-PK - which are found in cultured animal cells infected with herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PRV), respectively; and two genes, predicted from their sequence to encode protein kinases: US3 in HSV-1 and *pk* in PRV. The aims of this work were to determine the relationship between *pk* and PRV-PK, and to investigate the location and possible substrates of the protein kinases.

First, anti-sera were raised against the protein products of the US3 and *pk* genes in the form of fusion-proteins with β-galactosidase in *E. coli*. A 0.8kb *Sal* I fragment of the US3 gene and a 0.8kb *Pst* I fragment of the *pk* gene were separately cloned into appropriate sites in the β-galactosidase gene of the plasmid vector pUEX-2, and *E. coli* cells were transformed with the resulting recombinants. The fusion-proteins were induced, partially purified by gel exclusion chromatography, injected into rabbits and anti-sera were prepared.

PRV-PK was purified to homogeneity by successive chromatography on DEAE-cellulose, TSK phenyl-5PW, threonine-Sepharose and protamine-agarose. During the purification, protein kinase activity coeluted with material of 38kDa, found only in infected cells, which showed immunoreactivity towards the anti-serum raised against the protein product of the *pk* gene of PRV. This demonstrated that PRV-PK is encoded by the *pk* gene of PRV, and is consistent with previous work which had indicated that the US3 gene of HSV-1 encodes HSV-PK. This anti-serum also cross-reacted with a cellular protein of 34kDa which

was resolved from PRV-PK during chromatography on DEAE-cellulose. As a result of this cross-reactivity, the anti-serum was unsuitable for immunochemical studies which might have determined the location of the enzyme within intact infected cells.

The anti-sera were used to investigate the location of PRV-PK and HSV-PK within their respective virions. Purified virions were subjected to polyacrylamide gel electrophoresis and the proteins transferred to nitrocellulose. The anti-serum raised against the product of the US3 gene reacted with two proteins of 68kDa and 69kDa, as was also observed for extracts from infected cells. This indicated that HSV-PK is present within the virions of HSV. The anti-serum raised against the product of the gene, *pk*, reacted with a single protein of 38kDa, demonstrating that PRV-PK is present within the virions of PRV. This anti-serum also reacted with purified nucleocapsids and with virions which had been treated with the non-ionic detergent, NP-40, to remove the envelope of the virion. This implies that PRV-PK is associated with the nucleocapsid, which may be important in determining the physiological substrate(s) of the viral kinase.

Possible viral substrates of PRV-PK within PRV virions were investigated. Purified virions were incubated with $\gamma[^{32}P]ATP$, subjected to gel electrophoresis and the phosphorylated proteins detected by autoradiography. The major phosphorylated protein had a molecular weight of 112000. This protein was also phosphorylated when virions, in which the endogenous protein kinases had been inactivated by heat, were incubated with exogenous PRV-PK in the presence of $\gamma[^{32}P]ATP$, demonstrating that the 112kDa protein can be

a substrate for PRV-PK *in vitro*. When cells were incubated with [^{32}P]orthophosphate during infection *in vivo*, the 112kDa protein was also phosphorylated. This suggested that the 112kDa protein may be a physiological substrate of PRV-PK. However, at least three other distinct protein kinases have been identified in PRV virions, one of which, casein kinase II, can also phosphorylate this virion protein *in vitro*. When PRV virions were incubated with γ[^{32}P]ATP in the presence of 0.5M KCl, which inhibits casein kinase II but not PRV-PK, the phosphorylation of the 112kDa protein was inhibited. This implies that casein kinase II is responsible for most of the phosphorylation of the 112kDa protein in isolated virions *in vitro*, but does not exclude the possibility that the protein is a substrate for PRV-PK *in vivo*. However, immunochemical assays showed that PRV-PK is at least 100 times more abundant in the cytosol of infected cells than within virions, so that it seems more likely that the physiological role of PRV-PK involves the phosphorylation of proteins within the cytosol of infected cells.

An attempt was made to detect possible cellular substrates for HSV-PK by expressing the US3 gene constitutively in eukaryotic cells, after introducing it into the cellular genome. A 2.8kb fragment of DNA which included the promoter and coding region for US3 was cloned into the vector, Homer 5. The recombinants were transformed into 208F rat fibroblasts by calcium phosphate precipitation, and transformants selected by resistance to geneticin, which was conferred by the vector. Extracts from the transformed cell lines were subjected to chromatography on DEAE-cellulose and assayed for HSV-PK activity, but this could not be detected. Cell lines

expressing greater quantities of HSV-PK will be required before this approach to identify a possible cellular substrate can be executed.

Chapter 1

INTRODUCTION

This work concerns two related protein kinases: those encoded by the genome of the herpes viruses, Herpes Simplex Virus 1 (HSV-1) and Pseudorabies Virus (PRV). The interest in these viral enzymes stems from what is known about other protein kinases. Since cellular protein kinases are known to be involved in the regulation of many cellular processes, it seems likely that a viral protein kinase may similarly play a regulatory role during viral infection. The infection of cells with herpes viruses is a system potentially well suited to studying the effects of the activity of a particular protein kinase, since the viral protein kinases are specifically induced during infection, and this process brings about many changes in the infected cell that might well be mediated by the viral protein kinase.

In this Introduction, the characteristics of herpes viruses, their morphology, life cycle and the structure of their genome will first be presented. The activities and functions of some protein kinases in general will then be considered before discussing in more detail the discovery and characterisation of the protein kinases induced during infection of cells with HSV-1 (HSV-PK) and PRV (PRV-PK).

A. Herpes Viruses

The herpes viruses are a large group of DNA viruses which infect a wide range of animal hosts (Roizman & Batterson, 1985). Of particular interest are the five which infect humans, namely HSV-1, HSV-2, Varicella Zoster Virus (VZV), Epstein Barr Virus (EBV) and Human Cytomegalovirus (HCMV), and also those which infect domestic animals. e.g. PRV, which infects pigs. Herpes viruses can be subdivided into three groups (α, β, γ) according to their pathological behaviour. The α-herpes viruses, which include HSV-1, HSV-2, VZV and PRV, are characterised by having a variable host range and a relatively short reproductive cycle. Lytic infection results in the destruction of the infected cells. However these viruses may also produce latent infections in neurons. Reactivation can occur under conditions of stress to produce lytic infection. The β-herpes viruses, in contrast, have a restricted host range and a long reproductive cycle. Lytic infection frequently results in the enlargement of the infected cells. β-herpes viruses can also produce latent infections in secretory glands and kidneys. HCMV is a member of this group. The γ-herpes viruses, of which EBV is the most studied example, principally infect lymphoblastoid cells and frequently produce latent infections.

A. 1 The Structure of Herpes Virions.

All herpes virions share a characteristic structure in which there is a core, nucleocapsid, tegument and envelope (Widly *et al.*, 1960). The core consists largely of a single protein species around which the viral DNA is wound. This is enclosed within an icosahedral nucleocapsid which is formed from 162 capsomers. A number of other proteins, including some which have been identified as having enzymic activity, are also associated with the nucleocapsid. The tegument forms a layer of variable thickness between the nucleocapsid and the viral envelope, and contains most of the minor structural proteins of the virion. Finally, the envelope consists of a membrane acquired from budding through the cell's nuclear membrane. Specific viral glycoproteins are inserted into this membrane, at least some of which are known to be important in the interaction between virions and target cells.

A. 2 The Life Cycle and Latent Infection.

The life cycles of all α-herpes viruses appear to be similar, but that of HSV-1 has been studied most thoroughly. Adsorption onto the target cell is presumed to involve interaction between the viral glycoproteins and specific receptors on target cells, thus limiting the number of cell types which a particular virus can infect, and determining the host range of the virus (Vahne *et al.*, 1979). Penetration, which also involves at least one of the viral glycoproteins (gB in the case of HSV-1 : Sarmiento *et al.*, 1979), is

probably brought about by the fusion of the virion envelope with the host plasma membrane. The virion particle, lacking the envelope, is then transported to the nucleus where it sheds the nucleocapsid proteins, and the viral DNA enters the nucleus through the nuclear pores (Batterson *et al.*, 1983). Certain virion proteins also enter the nucleus, including, in HSV-1 (but not in PRV), a 65kDa protein required for the stimulation of transcription of viral genes (Batterson & Roizman, 1983; Campbell *et al.*, 1984; Dalrymple *et al.*, 1985).

The viral genes can be divided into three groups. These are the immediate-early (α), early (β) and late (γ) genes (Honess & Roizman, 1974). All the genes are transcribed by the cellular RNA polymerase II (Constanzo *et al.*, 1977), but certain viral proteins switch on and off the transcription of each group of genes. The α genes (of which there are five in HSV-1 : table I) only require the 65kDa (UL48 or $V_{mw}65$) viral structural protein for their expression. Thus, they can be transcribed in the presence of cycloheximide (Honess & Riozman, 1974), which inhibits protein synthesis. Under these conditions, the transcripts of the α genes accumulate, suggesting that the α genes are negatively regulated by the translation product of one of the α genes, and this has been shown to be α4 (IE 175, the product of the gene RS1 : DeLuca *et al.*, 1985). This protein is also required for the expression of the β genes (Preston, 1979; Preston, 1981). This latter class of genes can be subdivided into two groups, β1 and β2, according to when they are expressed. Expression of the β1 genes occurs very early during infection. The expression of both groups, however, reaches a maximum

5-7h after infection (Honess & Roizman, 1974). The products of the β genes include proteins involved directly or indirectly in DNA synthesis. eg. DNA polymerase, DNA-binding protein, thymidine kinase. The γ genes are expressed late in infection after the initiation of viral DNA synthesis, which is a stringent requirement for the expression of the γ2 genes (Conley *et al.*, 1981). Most of the γ genes encode structural proteins of the virion.

The viral genome is present within the virion as a linear double-strand of DNA of 120-240kbp. On entry into the nucleus of the infected cell, the DNA molecule becomes circularised. Replication then occurs by the rolling circle method to produce concatamers which are cleaved during their insertion into nucleocapsids (Jacob & Roizman, 1977; Jacob *et al.*, 1979).

Assembly of the virion occurs largely within the nucleus. The nucleocapsid proteins, having been translated on polyribosomes associated with the cytoskeleton, are transported into the nucleus where they form capsids into which the DNA is inserted (Biber-Hardy *et al.*, 1982; Ben-Ze'ev *et al.*, 1983). Newly-synthesised viral glycoproteins are inserted into the nuclear membrane in regions which become devoid of cellular proteins. Capsids then become enveloped by budding through the inner nuclear membrane at regions containing the viral glycoproteins (Darlington & Moss, 1968; Nii *et al.*, 1968). These virions probably proceed through the endoplasmic reticulum and then bud through this to form vesicles from which they are released by fusion with the plasma membrane (reverse pinocytosis). However, there is also evidence for the envelopment of nucleocapsids occurring by budding into cytoplasmic vacuoles and Golgi membranes (Smith &

Harvea, 1973). The importance of this pathway is unclear, but it may help to account for the presence of cellular cytosolic proteins (eg. casein kinase II : Introduction C) within mature virions.

The processes involved in latent infection and its reactivation are much less well understood. For HSV-1, they have been studied using the infection of mice, since it does not seem possible to produce latency *in vitro* with this virus. This is in contrast to EBV in which latency frequently occurs on infection of B lymphocytes in culture. The latter allows the processes occurring at the cellular level to be investigated, but is complicated by the fact that the infected cells are also induced to replicate indefinitely. One explanation that has been proposed to account for the failure to induce latency *in vitro* with HSV-1 is that the immune system may be involved in supressing productive infection *in vivo* (Roizman & Sears, 1987).

HSV-1 forms latent infections in ganglia adjacent to the sites of productive infection. Virions originating from lytic infection probably infect the nerve endings and are transported along the axon to the nucleus. Several hundred copies of the viral genome appear to be present in the nuclei of these cells, suggesting that the viral genome is replicated, probably by a cellular DNA polymerase. The viral DNA is no longer a single linear molecule (Rock & Fraser, 1983: Rock & Fraser, 1985), but it is unclear if it is maintained as an episome, as in the case of EBV, or whether it is a concatamer which is not integrated into the chromosome of the host cell (Mellerick & Fraser, 1987).

Two approaches have been used to try to identify the viral proteins involved in establishing and maintaining latency; the construction of mutant viruses from which one particular gene has been deleted, and the detection of viral genes expressed in cells which are harbouring latent infection. Deletion mutants lacking one of the following genes were still found to be able to form latent infections in mice (Meignier *et al.*, 1988): US3 which encodes the viral protein kinase, US4 which encodes the glycoprotein gG, US8 which encodes glycoprotein gE, US12 which encodes the α protein, α47 (IE 12), and US2 and US11 the functions of which are unknown. This suggests that these genes are not involved in establishing latency. In another study (Leib *et al.*, 1989) three deletion mutants of the α gene, α0 (IE 110 encoded by RL(1)), were found to be able to replicate in ganglia, but varied in their ability to establish latent infection and to permit its subsequent reactivation, suggesting that α0 plays a role in both the establishment and reactivation of latency. The second approach has identified a new transcript in latently infected cells which has not been detected in productive infections (Stevens *et al.*, 1987; Spivack & Fraser, 1987; Wagner *et al.*, 1988). It has been suggested that the transcript may not be translated since it is predominantly present in the nucleus. Although the function of the transcript is not known, these observations suggest that it may be involved in latency.

The switch from latency to productive infection has been studied for EBV since with this virus it occurs spontaneously at a low frequency *in vitro*. The expression of the gene BZLF-1 can induce lytic infection in 50% of cells harbouring latent infection. The

product of this gene is a transactivator of some EBV promoters and hence probably acts to induce the genes involved in lytic infection (Countryman & Miller, 1985; Takada *et al.*, 1986; Chevallier-Greco *et al.*, 1986; Grogan *et al.*, 1987). This suggests that the suppression of the expression of BZLF-1 is involved in establishing latent infection. The phorbol ester, 12-O-tetra-decanoyl phorbol-13-acetate (TPA), is also able to activate latent infections (Faggioni *et al.*, 1986). This suggests that the normal inducer of reactivation to lytic infection in humans may be an inducer of the hydrolysis of phosphatidyl-inositol, for example a hormone or growth factor. It seems likely that the processes involved in the reactivation of latent infection are similar for HSV-1 and EBV, but there is much that is still not understood about latency and its reactivation.

A. 3 The Viral Genome.

Herpes viruses have double-stranded linear DNA genomes of 120-240kbp which encode about 70 or more proteins. They can be divided into five groups according to the structure of their genomes, which consist of unique and repeated sequences (Roizman, 1981). Only the three arrangements which are relevant to the viruses to be discussed here will be presented (fig 1. 1). These consist of two regions containing unique sequences, U_S (unique short) and U_L (unique long). In HSV-1 and HSV-2, U_L is flanked by a sequence (R_L) which is present in the opposite orientation at either end of U_L. Another repeated sequence (R_S) flanks U_S (Sheldrick & Berthelot, 1974; Wadsworth *et al.*, 1975). Both unique sequences together with their flanking

repeated sequences can invert relative to each other, giving rise to four isomers of the genome which are present in equal frequency within any preparation of the virus (Hayward *et al.*, 1975). The genome of VZV also has such an arrangement. In PRV, U_S is similarly flanked by a repeated sequence in opposite orientations, but U_L is only flanked by a variable number of shorter direct repeats (Stevely, 1977; Ben-Porat *et al.*, 1979). Only two isomers of the PRV genome exist since only U_S can undergo inversion. The genome of EBV exists only as one isomer. A variable number of direct repeats are present at either end of the genome while U_S and U_L are separated by a number of larger tandemly repeated sequences (Given & Kieff, 1979; Kintner & Sugden, 1979; Hayward *et al.*, 1980). Despite these differences in the overall arrangement of the genomes, it has been found that the arrangement of genes within the genomes are similar, especially for viruses from the same group (eg. HSV-1 and VZV).

The homology between the genomes of different herpes viruses was initially investigated by measuring the cross-hybridisation between genomic DNAs. This showed that HSV-1 and HSV-2 were more closely related to each other than to either VZV or PRV, and that PRV and VZV were not closely related either (Kieff *et al.*, 1972; Ludwig *et al.*, 1972). In another study (Davison & Wilkie, 1983), the arrangement of homologous regions in different α-herpes viruses was compared by investigating to which restriction fragments from one viral genome, a particular restriction fragment from another viral genome could hybridise. In this way Davison & Wilkie showed that HSV-1 and HSV-2 are colinear, that the homologous regions of equine herpes virus type 1 and VZV are colinear, and that PRV is colinear to a particular

isomer (I_L) of HSV-1, except for an inversion of the region containing the first 40% of the genome (genome units 0.1 to 0.4). These early conclusions have been substantiated and were extended by the comparison of genomes for which the complete nucleotide sequence has been determined ie. the α-herpes viruses HSV-1 (McGeoch *et al.*, 1985; McGeoch *et al.*, 1988a) and VZV (Davison & Scott, 1986), and the γ-herpes virus, EBV (Baer *et al.*, 1984). These were found to consist of 152260bp, 124884bp and 172282bp respectively.

The genome of HSV-1 encodes a total of 70 genes, of which 56 are in the U_L region, 12 are in the U_S region, and the terminal repeated regions each contain one gene, both of which are therefore present twice within the genome (McGeoch *et al.*, 1988a). These latter two genes encode the α proteins, α0 (IE 110) and α4 (IE 175). All the genes except RL(1), UL1 and UL15 lack introns. Although all the genes are transcribed from one or more distinct promoter, only 29 of the termination sites for transcription are unique to the transcript of one gene. The other 19 termination sites are shared by two or more transcripts. Thus, many of the mRNAs encode more than one protein, although it is likely that only the first open reading frame of each transcript is translated.

Something is known about the functions of the protein products of about half of the genes encoded by HSV-1. These can be divided into a number of groups according to what is known of their properties. There are five α (immediate-early) genes (table I), three of which appear to be essential for productive infection ie. α0 (IE 110), α4 (IE 175) and α27 (IE 63). These are transactivators of viral transcription. Seven genes encode the glycoproteins which are

inserted into the viral envelope (table II). Another seven genes have been shown to be essential and sufficient for DNA replication (table III). These include the viral DNA polymerase, components of the helicase-primase complex and three DNA-binding proteins. The genome also encodes a number of enzymes which are involved in nucleotide metabolism (table IV). These are dispensable for growth in culture. The other enzymes which the genome is known to encode are an exonuclease, and a protein kinase, the product of gene US3 (Purves *et al.*, 1987b), which is discussed further below. From the sequence of UL13, it is possible that this gene encodes a second protein kinase (Chee *et al.*, 1989; Smith & Smith, 1989). Two virion regulatory proteins have been identified (table V): the protein product of UL41 brings about the initial inhibition of host protein synthesis (Kwong *et al.*, 1988), while UL48 encodes $V_{mw}65$, the virion protein which is the activator for expression of the α (immediate-early) genes (Batterson & Roizman, 1983; Campbell *et al.*, 1984; Dalrymple *et al.*, 1985). Six proteins, defined by molecular weight, have been identified as components of the nucleocapsid. The genes encoding two of these have been identified (table V): UL19 encodes one of the major structural components (Costa *et al.*, 1984), and UL26 encodes a surface component which may be involved in the assembly of virions (Davison & Scott, 1986b; Preston *et al.*, 1983). There remain seven genes which are known to encode virion proteins, but which have not yet been correlated with the known structural proteins.

Comparison of the amino acid sequences predicted for the proteins encoded by the genes of the U_L region of HSV-1 with those predicted for VZV showed that for most of the genes in HSV-1 there is an

equivalent gene in VZV, and that it is located at the same position within both genomes (McGeoch *et al.*, 1988a). In the U_L region, the VZV genome was found to have five genes without equivalents in HSV-1 ie. genes 1, 2, 13 (thymidylate synthase), 32, and 57. There are two genes in the U_L region of HSV-1 without equivalents in VZV ie. UL45 and UL50. The U_S region of HSV-1 contains six genes without equivalents in VZV ie. US2, US4, US5, US6 US11 and US12, but all the genes in the U_S region of VZV have equivalents in HSV-1. Another difference is that in VZV, gene 61, the equivalent of HSV-1, αO (IE 110), is present within the U_L region rather than R_L and hence only has one copy within the genome.

A comparison of the predicted amino acid sequences for the genes of VZV and EBV identified seven having strong homology, eight having moderate homology and 15 having weak homology (Davison & Taylor, 1987). All the homologous genes are located within the U_L region of both genomes. However, the positions of the homologous genes within the U_L region are not directly equivalent, in contrast to the situation regarding HSV-1 and VZV. The U_L region can be divided into three sections, which are orientated ABC in VZV but CA'B in EBV (where A' is the inverse orientation of A). Thus, the U_L region of the γ-herpes virus, EBV, is more distantly related to the U_L region of the α-herpes viruses than are the equivalent regions of HSV-1 and VZV to each other. No homology has been detected between the proteins encoded by the U_S region of EBV and those of VZV or HSV-1, or indeed that of the β-herpes virus, HMCV (Weston & Barrell, 1986).

The U_S region of HSV-1 and its flanking repeated sequence contains 13 distinct open reading frames (fig 1.2) (McGeoch *et al.*,

1985). The corresponding region of VZV contains seven distinct genes, all of which have counterparts in HSV-1 (Davison & Scott, 1986). With the exception of US1, these genes are located in the same order in the two viruses.

Part of the U_S region of PRV has been sequenced. Most of the U_S region lies within the *Bam* HI-7 fragment of the genome (fig 1.3). This was found to contain five open reading frames which encode four glycoproteins: gX (Rea *et al.*, 1985), gp50 (Petrovskis *et al.*, 1986a), gp63 (Petrovskis *et al.*, 1986b), gI (Petrovskis *et al.*, 1986b) and an 11kDa protein (Petrovskis & Post, 1987). The glycoprotein gX corresponds to glycoprotein gG in HSV-2 (encoded by the US4 gene), but does not show any homology to the protein encoded by US4 in HSV-1 (McGeoch *et al.*, 1987). The other four proteins correspond to the products of US6, US7, US8 and US9 in HSV-1 respectively (fig 1.2).

B. Protein Kinases

Protein kinases are enzymes which catalyse the transfer of a phosphate group from a nucleotide, usually ATP, to the hydroxyl group of a seryl, threonyl or tyrosyl residue of a protein. Although several protein kinases are now known in prokaryotes, it is in eukaryotes that there appear to be a large number of different protein kinases. To date about one hundred different mammalian protein kinases have been identified, but it has been suggested that there may be as many as 1000 eukaryotic protein kinases (Hunter, 1987). This multiplicity appears to be a feature of eukaryotes, and may be highly significant in eukaryotic evolution in that the processes in which protein kinases are implicated, such as complex cell differentiation and the response to hormones, do not occur in prokaryotes.

B. 1 Classification of Protein Kinases.

Protein kinases differ from each other in a number of different properties which can be used to define and classify them. These include i) molecular structure, ii) substrate specificity for the amino acid which is phosphorylated and the range of proteins phosphorylated, iii) the mechanism of activation, and iv) tissue distribution. One classification scheme involves the division of protein kinases into two groups according to whether they phosphorylate seryl and threonyl, or tyrosyl residues, with a further

division on the basis of their mechanism of activation eg. cAMP-dependent protein kinase, or their protein substrate specificity eg. glycogen synthase kinase 3.

B. 2 Sequence Motifs of Protein Kinases.

Most protein kinases are found to consist of at least two domains, one of which is the catalytic domain, whereas another may be involved in the regulation of the catalytic activity, and often binds an allosteric activator eg. cGMP, Ca^{2+}. Like many other families of related enzymes, the amino acid sequences of protein kinases are found to share significant homology within their catalytic domains, and this presumably reflects their similar catalytic mechanisms. This homology was first observed by Barker & Dayhoff (1982), who found that the amino acid sequences of the catalytic subunit of the cAMP-dependent protein kinase and the tyrosine protein kinase, $pp60^{src}$, shared a significant number of identical amino acids. This was subsequently shown to be a more general phenomenon by the extensive homology found between the cAMP-dependent protein kinase and the cGMP-dependent protein kinase (Takio *et al.*, 1984), and between these and the γ subunit of phosphorylase *b* kinase (Reimann *et al.*, 1984).

The amino acid sequences or predicted amino acid sequences are now known for over fifty protein kinases. These include both serine/threonine and tyrosine protein kinases of both vertebrate and invertebrate origin, some of which are homologues from different species. From a comparison of these sequences, Hanks *et al.* (1988)

defined eleven regions of homology which are separated by regions of much lower homology. Six of these consist of sequences with 2-8 amino acids which are conserved to varying degrees (fig 1.4). The other regions of homology consist of a single amino acid which is highly conserved, flanked by less highly conserved amino acids, or a sequence of amino acids which undergo conservative changes (eg. conserving their hydrophobic or hydrophilic character). The latter are thus more difficult to define and use as diagnostic sequences for identifying protein kinase genes.

The motif G-G--G-V, in subdomain I, was originally thought to be totally conserved, but with the sequencing of more protein kinases, only the second glycine residue has been found to remain totally conserved. A similar motif is also present in other proteins which bind nucleotides, including several for which the crystal structure has been determined (eg. adenylate kinase ; Von Zabern *et al.*, 1976; which has the motive G----GK). From this, it is suggested that the motif is probably located in the fold between two α-helices and is part of the nucleotide binding domain.

The lysine residue in subdomain II is probably present in all protein kinases. In chemical modification studies of several protein kinases with the ATP analogue, p-fluorosulphonylbenzoyl 5'-adenosine, the conserved lysine residue has been shown to be modified (Zoller *et al.*, 1981; Hashimoto *et al.*, 1982). This, together with the observation that mutation of this residue can abolish kinase activity (Snyder *et al.*, 1985; Kamps & Sefton, 1986), suggests that this lysine is involved in the catalytic mechanism of protein kinases.

There are two motifs which can be of use in differentiating between tyrosine and serine/threonine protein kinases. Within domain VI there is a sequence, AAR, which, although not totally conserved, appears to be characteristic of tyrosine protein kinases, except those related to *src*, in which, in most cases, it is replaced by RAA. The equivalent sequence in serine/threonine protein kinases is considerably more diverse. The second motif is a sequence within subdomain VIII which can be diagnostic of tyrosine protein kinases: P-(K/R)W(T/M). Although such motifs can be of use to distinguish between tyrosine and serine/threonine protein kinases, the overall homology of tyrosine protein kinases to one another is sufficiently great that genes encoding tyrosine protein kinases are probably most effectively identified by comparing the sequence of the whole catalytic domain to that of known tyrosine protein kinases.

The identification of these motifs from the amino acid sequences or the conceptual translations of the nucleotide sequences of known protein kinases has had considerable predictive value. A number of genes for which the product was unknown have been identified as probably encoding protein kinases on the basis of their predicted amino acid sequences. These include many retroviral oncogenes and their corresponding cellular proto-oncogenes, eg. *c-mos* (Van Beveren *et al.*, 1981), and the products of a number of genes encoding regulators of the cell cycle in yeast and other eukaryotic organisms eg. *cdc2* (Hindley & Phear, 1984), *wee1*$^+$ (Russel & Nurse, 1987a), *nim1*$^+$ (Russel & Nurse, 1987b). In several cases, this has been substantiated (eg. Simanis & Nurse, 1986).

Of particular relevance here is the fact that this approach identified the US3 gene of HSV-1 and its homologues in HSV-2 and VZV as putative protein kinase genes (McGeoch & Davison, 1986). The predicted amino acid sequences for the three proteins can be divided into two sections which probably reflect their functional domains. The C-terminal domain shows about 45% identity between HSV-1 and VZV, except for the extreme C-terminal region, and contains sequence motifs characteristic of the catalytic domain of protein kinases (Leader & Purves, 1988). The N-terminal domain shows little apparent homology between HSV-1 and VZV and also varies in size between the different enzymes. Its purpose is thus unclear. The way in which it was established that the US3 gene does encode a protein kinase is described in a later section (Introduction C).

B. 3 The Activities of Protein kinases

The specificity of protein kinases appears to range from those which are highly specific, only being known to phosphorylate one or a few proteins (table VI), to others which have a much broader specificity (table VII). In the case of the latter, the specificity is probably largely defined by the amino acids around the serine, threonine or tyrosine on the target protein. Such specificities have been investigated using peptides as substrates, and are shown in table VII. Substrate recognition for protein kinases with much narrower substrate specificities probably depends on secondary and tertiary structural features of the substrate proteins as well as the primary structure. What proteins are phosphorylated by a particular

protein kinase *in vivo* may also be determined by the localisation of a protein kinase to a particular tissue or subcellular compartment. This is thought to be important for tyrosine protein kinases.

Many protein kinases are known to undergo autophosphorylation (table VIII). In many cases this appears to be functionally significant. Autophosphorylation of many serine/threonine protein kinases results in activation of the protein kinase or reduces the dependence of the enzyme on allosteric activators. For some of the oncogenic tyrosine protein kinases, autophosphorylation is important in the activation of the protein kinase and transforming activity (eg. $pp60^{v\text{-}src}$: Smart *et al.*, 1981). However, there are other enzymes for which autophosphorylation appears to have no effect, and hence may not occur under physiological conditions (eg. casein kinase II; Dahmus, 1981).

B. 4 Functions of Protein Kinases.

Protein kinases are believed to play an important role in the regulation of many cellular processes by bringing about the phosphorylation of key regulatory proteins. Such processes include the regulation of metabolic pathways, muscle contraction, neurotransmitter release, protein synthesis, cell proliferation, the activation of cells such as platelets and mast cells, and the regulation of the cell cycle. The processes which are better understood are those involving a protein kinase for which the substrate(s) is known. Most of these protein kinases have a limited substrate specificity (table VI). However, protein kinase C has been

implicated in a number of processes, although the substrates involved have not been conclusively identified (Nishizuka, 1986). Most of the tyrosine protein kinases and some serine protein kinases have been identified as proto-oncogenes (table IX). This implies that they are involved in the regulation of cell proliferation, but since their substrates have not been identified (other than the enzymes themselves) it is not known how they exert their effects. Some of these proto-oncogenes are receptors for hormones and growth factors eg. EGF and PDGF. The binding of the ligand activates the tyrosine protein kinase activity, and this has been shown in the case of the insulin receptor to be important for signal transduction, but how this is brought about is unknown (Ellis *et al.*, 1986; Chou *et al.*, 1987).(table X)

B. 5 Protein Phosphatases.

Although eukaryotic protein kinases thus appear to be important in many cellular processes, the protein phosphatases which oppose the actions of the protein kinases may well be equally important in these. Compared with protein kinases, there appear to be a more limited number of protein phosphatases. For serine/threonine protein phosphatases, four different classes of enzyme have been described, three of which have broad substrate specificities. The properties of these enzymes are shown in table XI. The broad and overlapping substrate specificities of PP-1, PP-2A and PP-2C make it difficult to determine their relative importance in the dephosphorylation and hence the regulation of particular substrates *in vivo*. However, they

show differences in cellular location, which probably relate to substrate specificity (Cohen, 1988). Thus the two major active forms of PP-1 in muscle, PP-1_G and PP-1_M, are particulate, being associated with glycogen and myosin, respectively. PP-1_G consists of the catalytic subunit associated with a glycogen-binding subunit responsible for the association of the enzyme complex with glycogen (Strålfors *et al.*, 1985). A similar targeting subunit is probably also associated with PP-1_M. In contrast, PP-2A and PP-2C are mainly cytosolic.

PP-1 and PP-2B are known to be highly regulated in response to the second messengers Ca^{2+} and cAMP. PP-1 is inhibited by the inhibitor protein, inhibitor-1, which is activated by cAMP-dependent protein kinase (Huang & Glinsmann, 1976; Nimmo & Cohen, 1978). This probably serves to amplify the response to hormones such as glucagon, which act via the activation of protein kinases. The glycogen-binding subunit of PP-1_G can also be phosphorylated by cAMP-dependent protein kinase (Strålfors *et al.*, 1985; Caudwell *et al.*, 1986), which results in the dissociation of PP-1_G from glycogen (Hiraga & Cohen, 1986) and facilitates its inactivation (Strålfors *et al.*, 1985). The catalytic subunit of PP-2B is activated by association with the Ca^{2+}-binding subunit of PP-2B, or calmodulin. Both interactions require Ca^{2+}, which thus effectively regulates this enzyme. PP-2B has a much narrower substrate specificity than the other three protein phosphatases (Ingebritsen & Cohen, 1983a) and appears to be specific for proteins which regulate other protein phosphatases (eg. inhibitor-1 (Ingebritsen *et al.*, 1983a) and the related inhibitor, dopamine and cAMP-regulated phosphoprotein (DARPP)) and

protein kinases (eg. the regulatory subunit of cAMP-dependent protein kinase (Blumenthal *et al.*, 1986)). It may thus be involved in a protein phosphatase cascade system whereby the activation of PP-2B by Ca^{2+} results in the inactivation of inhibitor-1 and hence the activation of PP-1.

The importance of the protein phosphatases has been further demonstrated by Haystead *et al.* (1989), who have shown that the potent tumour promoter, okadaic acid, acts by being an inhibitor of two protein phosphatases, 1 and 2A. It stimulated increased phosphorylation in intact adipocytes and in the latter mimicked the effects of glucagon on glycogenolysis and gluconeogenesis. However, although the importance of protein phosphatases is now apparent, they are, as yet, less well understood than protein kinases.

C. Protein Kinases and Viral Infection.

The infection of eukaryotic cells with many viruses has been found to result in the phosphorylation of proteins of both viral (Wilcox *et al.*, 1980) and cellular origin (Kennedy *et al.*, 1981). In principle, these phosphorylations might be catalysed either by protein kinases which are associated with the virions or by protein kinases induced or activated during infection.

Protein kinase activities have been found to be present within the virions of many enveloped viruses eg. vesicular stomatitis virus (VSV ; Clinton *et al.*, 1982), RNA tumour viruses (Weiss & Faras, 1983), influenza virus (Kamata, 1977) and also some non-enveloped viruses eg. polio virus (Lackmann *et al.*, 1987). Such enzyme activities have been detected by incubating pure virions in the presence of $\gamma[^{32}P]$ATP, and examining the radioactive labelling of virion proteins or exogenous substrates such as protamine, casein or histones. Some of these virion protein kinases have been characterised with respect to protein substrate specificity, requirements for divalent cations, and whether ATP and GTP may be used as the donor for the phosphoryl residue. From such studies it has become apparent that many of the protein kinases present within virions have very similar catalytic properties to known cellular protein kinases and thus probably are cellular enzymes which have become incorporated within the virion during its assembly. For example, a protein kinase associated with the virion of VSV has been

shown conclusively to be the cellular protein, pp60^{src} (Clinton *et al.*, 1982). The protein kinase can be immunoprecipitated from VSV virions with anti-serum which specifically recognises pp60^{src}.

The protein kinase activities associated with the virions of α-herpes viruses were investigated by Lemaster & Roizman (1980) and Stevely *et al.* (1985). Lemaster & Roizman showed that virions of HSV-1 and HSV-2 contained endogenous protein kinase activity which was able to phosphorylate a number of virion proteins. Stevely *et al.* showed that the endogenous protein kinase activity of HSV-1 and PRV virions could be attributed to multiple protein kinases. PRV was found to contain at least four different protein kinases, two of which could use casein and two which used protamine as a substrate. Further characterisation of the former protein kinases suggested that they were the cellular protein kinases, casein kinase I and II. The properties of one of the protamine protein kinases suggested that it might be protein kinase C, also of cellular origin. It is unclear whether this incorporation of cellular enzymes into virions is functionally significant or occurs adventitiously during the assembly of the virion.

Protein kinases of viral origin (ie. encoded by the viral genome) could also be present within virions. In order to prove that a protein kinase is of viral origin, it is necessary to identify the viral gene which encodes it. As has been discussed above, protein kinase genes can be tentatively identified from their sequences. Since the genomes of most well-studied viruses have been sequenced in their entirety, it should be quite easy to determine whether there is a putative protein kinase gene present within their genome. So far only

the genomes of α-herpes viruses have been shown conclusively to encode a viral protein kinase ie. the US3 gene of HSV-1 and its homologues in HSV-2 and VZV (McGeoch & Davison, 1986).

Many acutely oncogenic retroviruses are also known to encode protein kinases (Sefton, 1985). Indeed, the genes encoding many of the cellular tyrosine protein kinases were initially identified as the cellular homologues of oncogenes carried by particular oncogenic retroviruses. Although these protein kinases are encoded in viral genomes, they are not true viral protein kinases in that they are acquired from the cellular genome by the retroviruses. Since there is a limit to the size of the viral genome, the cellular gene is usually acquired at the expence of part of the normal retroviral genome, thus giving rise to a defective virus which is unable to replicate or undergo productive infection alone (Gross, 1983). The recent cellular origin of such protein kinases is evident from the nucleotide sequences of the genes, which are almost identical to that of the cellular homologues (Takeya & Hanafusa, 1983).

The protein kinases of HSV-1 and PRV

The protein kinase activity which is the main concern of the work described here, was first observed in the cytoplasm of baby hamster kidney fibroblasts (BHK cells) infected with PRV (Mcgarvey, 1981). It was found to appear 4h after infection and increased in amount up to 10h after infection (Katan *et al.*, 1985). This activity was purified 100-fold by Katan *et al.* and characterised. It was found to phosphorylate serine residues of basic proteins, showing a

preference for protamine over mixed histones. It was specific for ATP as the phosphate donor and could not utilise GTP. The activity was refractory to molecules known to affect the activity of other protein kinases: cAMP, cGMP, Ca^{2+}/calmodulin, Ca^{2+}/phospholipid, double-stranded RNA and heparin. A distinguishing feature of the enzyme was that it retained its activity in salt concentrations up to 0.8M KCl.

Purves *et al.* (1986b) detected an analogous protein kinase activity in BHK cells infected with HSV-1. The kinetics of the appearance of both protein kinases were similar to those of the viral DNA polymerase. For both protein kinases, the activity induced in infected cells was related to the multiplicity of infection, and viruses which had been subjected to ultra-violet radiation did not induce the protein kinase activity. Infection with a temperature-sensitive mutant of HSV-1 induced the protein kinase when grown at the permissive temperature but not at the restrictive temperature. This evidence, together with the observation of different chromatographic properties for the protein kinases induced by HSV-1 and PRV, suggested - but did not prove - that these enzymes were encoded by the viral genomes.

The substrate specificities of the protein kinases induced by infection with HSV-1 (HSV-PK) and PRV (PRV-PK) were investigated using peptide substrates and compared to those of other protein kinases (Purves *et al.*, 1986a). Both protein kinases could phosphorylate seryl and threonyl residues and showed a preference for peptides with arginine residues on the N-terminal side of the seryl/threonyl residue, but were not able to phosphorylate similar

peptides in which ornithine replaced the arginine. Peptides with arginyl residues only on the C-terminal side of the seryl residue were also inefficient substrates. This specificity contrasted to that of protein kinase C, which can phosphorylate peptides having arginyl residues on either side of the seryl residue and also peptides in which arginine is replaced with ornithine. The peptides phosphorylated by PRV-PK and HSV-PK could also be phosphorylated by cAMP-dependent protein kinase, but the reaction preferences were different for the latter. The fact that the substrate specificities of both enzymes appears to be very similar suggests that PRV-PK and HSV-PK are related proteins despite their different chromatographic properties.

Purves *et al.* (1987a) purified PRV-PK to homogeneity at a specific activity of approximately 1000nmol phosphate mg^{-1}, min^{-1}. Analysis of the purified enzyme by gel electrophoresis under denaturing conditions revealed a single band corresponding to a molecular weight of 38kDa, and this band became labelled when the pure enzyme was incubated with $\gamma[^{32}P]ATP$ in the absence of other substrates. The isoelectric point of the phosphorylated species was approximately 4.9.

HSV-PK was purified 100-fold (Frame *et al.*, 1987b). The resulting material consisted of two proteins of 65kDa and 68kDa, of which only the latter could be labelled when this material was incubated with $\gamma[^{32}P]ATP$. This could either be due to autophosphorylation, as with PRV-PK, or to phosphorylation of the 68kDa protein by another protein kinase (eg. the 65kDa protein) in the preparation.

Two approaches were used to determine the genetic origin of the protein kinase induced by infection with HSV-1, both of which were possible because the sequence of part of the genome of HSV-1 was known. This allowed the US3 gene to be identified as a candidate for encoding a viral protein kinase (McGeoch & Davison, 1986). The first approach involved raising anti-sera against an oligopeptide consisting of the predicted 8 amino acids at the C-terminus of the US3 gene (Frame *et al.*, 1987). In immunoblots, this anti-serum was found to react with the 68kDa band of the highly purified protein kinase. Although this showed that the 68kDa band was the product of the US3 gene, it was still formally possible that the 65kDa band contained the HSV-PK activity. The second approach involved the use of a mutant virus which lacked the US3 gene (Purves *et al.*, 1987b). This mutant virus could infect BHK cells effectively, as assessed by the yield of virus, but did not induce HSV-PK. Although it was still possible that the US3 gene might have been only indirectly involved in the induction of the protein kinase activity, taken together, these two pieces of work strongly supported the conclusion that HSV-PK is the product of the gene, US3.

During the course of the present work, a protein kinase gene, *pk*, equivalent to the US3 gene of HSV-1, was identified within the *Bam* HI-10 fragment of the PRV genome and sequenced (G. Zhang *et al.*, 1989 : fig 1.3). The gene was predicted to encode a protein of 334 amino acids and thus appeared to be of approximately the correct size to encode the 38kDa protein kinase, PRV-PK.

The aims of this work were four-fold: first, to determine if the putative protein kinase gene, *pk*, identified in the *Bam* HI-10 fragment of the genome of PRV encodes the protein kinase (PRV-PK) induced during infection of cells with PRV, and hence to determine if PRV-PK is of viral origin; second, to investigate the location of the product of the *pk* gene in both infected cells and within virions; third, to identify possible physiological substrates of both protein kinases, which might help shed light on the function of these enzymes in infection of cells with α-herpes viruses; and fourth, to determine if expression or over-expression of HSV-PK, in the absence of other viral proteins, could cause malignant transformation or other phenotypic changes to cells in culture.

Figure 1.1
Schematic diagram showing the arrangement of the genomes of HSV-1, PRV and EBV.

UL represents unique long region.
US represents unique short region.
Shaded areas represent large repeats, with the arrows indicating the orientation of the repeat.
Open boxes in EBV represent direct repeats of a 2kbp sequence.
Vertical lines represent short terminal reiterations (direct repeats).
Numbers indicate the molecular mass of region in kDa.
(Roizman & Batterson, 1985).

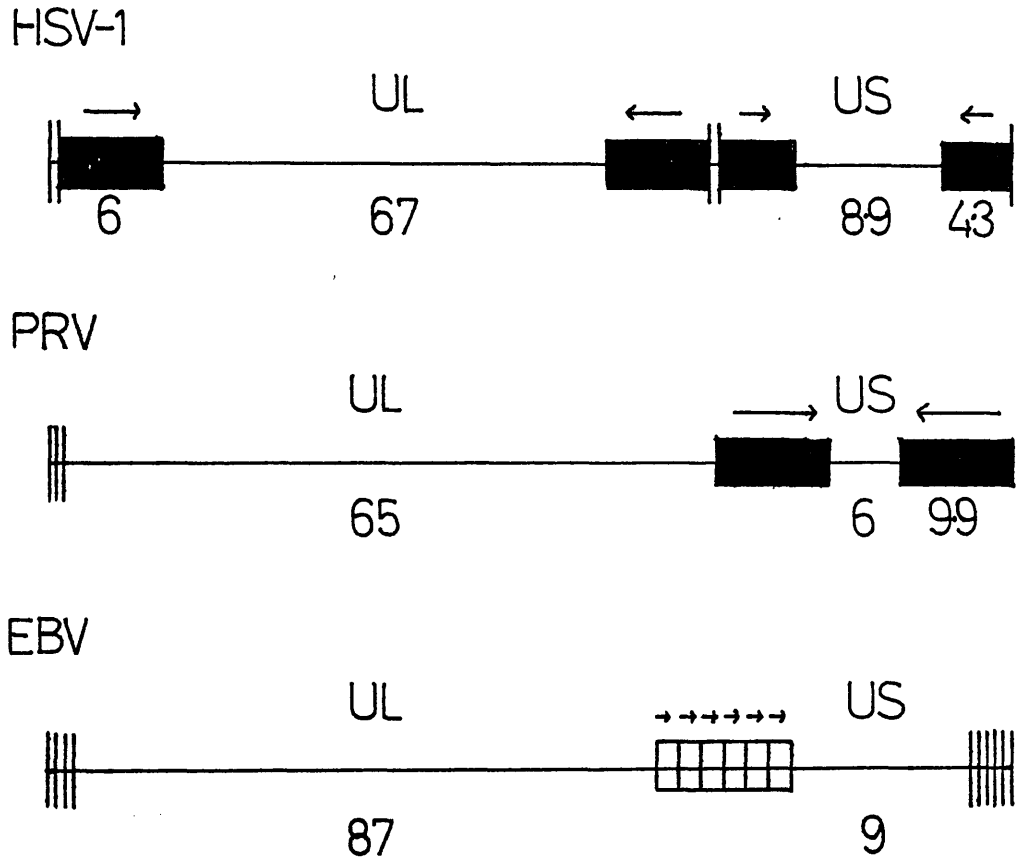

HSV-1
UL
US
6
67
89
43
PRV
UL
US
65
6
99
EBV
UL
US
87
9

Figure 1.2
Schematic diagram of U_s and R_s regions from the genomes of PRV, HSV-1 and VZV, showing homologous genes.

Arrows indicate the direction of transcription of the genes.
In HSV-1, the open reading frames of US10 and US11 overlap.
(Rea *et al.*, 1985; Davison & McGeoch, 1986; Petrovskis *et al.*, 1986a; Petrovskis *et al.*, 1986b; Petrovskis & Post, 1987)

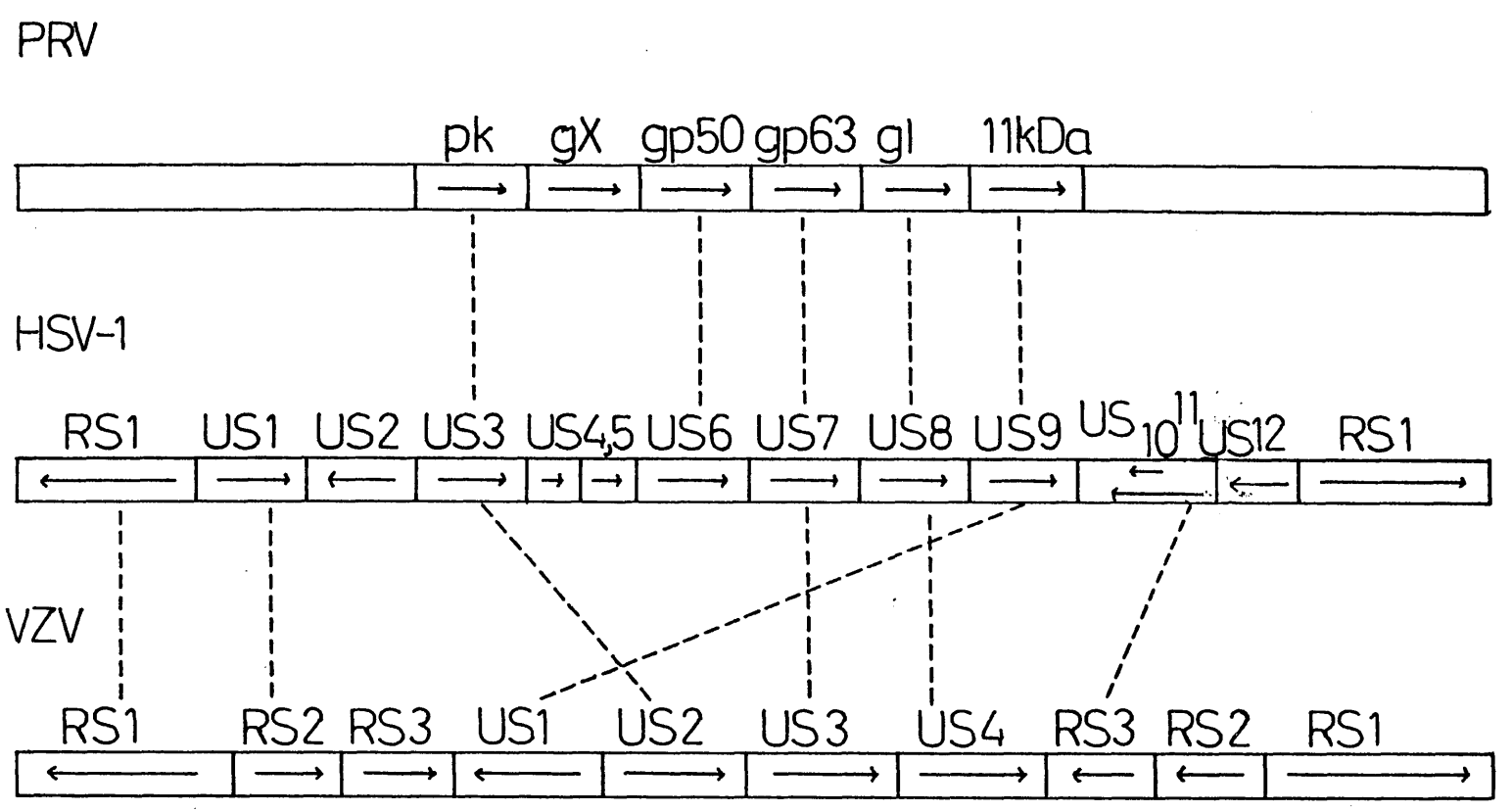
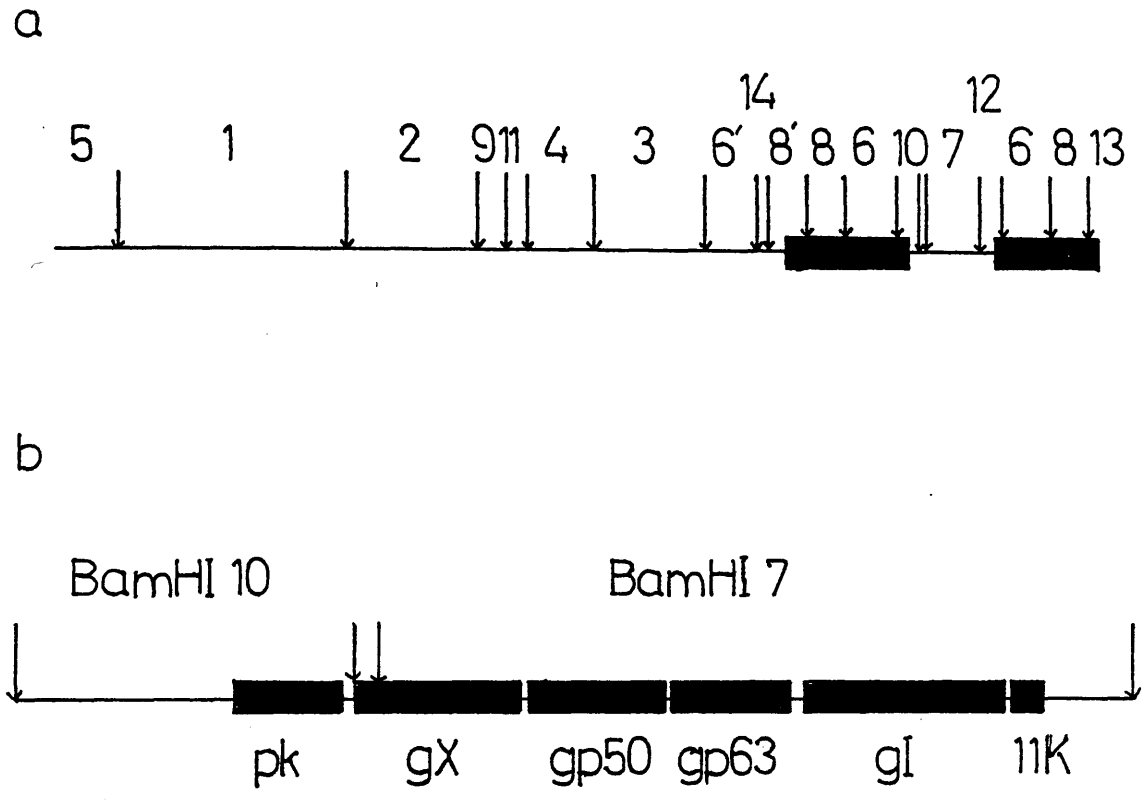

PRV
pk
gX
gp50
gp63
gI
11kDa
HSV-1
RS1
US1
US2
US3
US4,5
US6
US7
US8
US9
US10,11
US12
RS1
VZV
RS1
RS2
RS3
US1
US2
US3
US4
RS3
RS2
RS1

Figure 1.3
a) Schematic diagram showing the *Bam* HI cleavage sites in the PRV genome.

The cleavage sites are marked as arrows and the resulting fragments are numbered according to their size when subjected to electrophoresis on agarose gels. The inverted repeats of the genome are indicated by shaded boxes (Petrovskis *et al.*, 1986).

b) Schematic diagram of *Bam* HI fragments 7 and 10 of the PRV genome.

Shaded boxes represent the positions of the genes which have been identified. (There is also a small *Bam* HI fragment of 0.2kb between fragments 7 and 10.)
(Rea *et al.*, 1985; Petrovskis *et al.*, 1986a; Petrovskis *et al.*, 1986b; Petrovskis & Post, 1987)


a
14
12
5
1
2
9
11
4
3
6'
8'
8
6
10
7
6
8
13
b
BamHI 10
BamHI 7
pk
gX
gp50
gp63
gI
11K
scale for b
——=1kbp

SUBDOMAIN		SHARED SEQUENCE MOTIF
I		G - G - - G - V
II		A - K
VI	ser/thr	h r D l K - - N
	tyr (src family)	H R D L R A A N
	tyr (others)	H R D L A A R N
VII		D F G
VIII	ser/thr	a p E
	tyr	P - (K/R) W - a p E
IX		D - W (S/A) - G

Figure 1.4
Shared sequence motifs of the conserved subdomains within the catalytic domain of protein kinases. (Hanks *et al.*, 1988)

The single-letter amino acid code is used. Upper case letters which are underlined represent residues which are completely conserved in all known protein kinases. Upper case letters which are not underlined represent highly conserved residues or residues which undergo conservative changes. Lower case letters represent moderately conserved residues or residues which are highly conserved but can undergo non-conservative changes.

Table I
IMMEDIATE-EARLY PROTEINS OF HSV-1

references:

1) Everett, 1984
2) Perry *et al.*, 1986
3) Preston, 1979
4) Preston, 1981
5) Post & Roizman, 1981
6) Everett, 1986
7) Longnecker & Roizman, 1986
8) Mavromara-Nazos *et al.*, 1986

Table II
GLYCOPROTEINS OF HSV-1

references:

1) Pellett *et al.*, 1985
2) Little *et al.*, 1981
3) Frink *et al.*, 1983
4) Watson *et al.*, 1982
5) Longnecker & Roizman, 1986
6) Neidhardt *et al.*, 1987
7) Longnecker & Roizman, 1987
8) Weber *et al.*, 1987
9) Gompels & Minson, 1986
10) Longnecker *et al.*, 1987

Table III
PROTEINS OF HSV-1 ESSENTIAL AND SUFFICIENT FOR DNA REPLICATION

references:

1) Wu *et al.*, 1988
2) McGeoch *et al.*, 1988b
3) Quinn & McGeoch, 1985
4) Parris *et al.*, 1988

Table I

IMMEDIATE-EARLY PROTEINS OF HSV-1

gene	IE designation	α designation	function	reference
RL(1)	IE 110	α0	transactivation	1, 2
RS(1)	IE 175	α4	transactivation	3, 4
US1	IE 68	α22	? dispensable	5
UL54	IE 63	α27	transactivation	6
US12	IE 12	α47	? dispensable	7, 8

Table II

GLYCOPROTEINS OF HSV-1

gene	protein	abundance	function	reference
UL27	gB	major	essential	1, 2
UL44	gC	major	dispensable	3
US6	gD	major	essential	4
US8	gE	major	dispensable	5, 6
US4	gG	minor	dispensable	7, 8
UL22	gH	minor	essential	9
US7	gI	minor	dispensable	10

Table III

PROTEINS OF HSV-1 ESSENTIAL AND SUFFICIENT FOR DNA REPLICATION

gene	protein	reference
UL5	component of helicase-primase complex	1, 2
UL8	component of helicase-primase complex	1, 2
UL9	origin-binding protein	1, 2
UL29	major DNA-binding protein	3
UL30	DNA polymerase	3
UL42	? binds double-stranded DNA	1, 2, 4
UL52	component of helicase-primase complex	1, 2

Table IV
OTHER ENZYMES ENCODED BY THE GENOME OF HSV-1

references:

1) Kit & Dubbs, 1963
2) Wagner *et al.*, 1981
3) Preston *et al.*, 1984
4) McLauchlan, 1983
5) Preston & Fisher, 1984
6) Fisher & Preston, 1986
7) Worrad & Caradonna, 1988
8) Draper *et al.*, 1986
9) Purves *et al.*, 1987b
10) Chee *et al.*, 1989
11) Smith & Smith, 1989

Table V
GENES ENCODING VIRION PROTEINS OF HSV-1

references:

1) Costa *et al.*, 1984
2) Davison & Scott, 1986b
3) Preston *et al.*, 1983
4) Kwong *et al.*, 1988
5) Campbell *et al.*, 1984
6) Dalrymple *et al.*, 1985

Table IV

OTHER ENZYMES ENCODED BY THE GENOME OF HSV-1

gene	enzyme	function	reference
UL23	thymidine kinase	dispensable	1, 2
UL39 and 40	ribonucleotide reductase	dispensable	3, 4
UL50	dUTPase	dispensable	5, 6
UL2	uracil-DNA glycosylase	dispensable	7
UL12	exonuclease	essential	8
US3	protein kinase	dispensable	9
UL13	? protein kinase	?	10, 11

Table V

GENES ENCODING VIRION PROTEINS OF HSV-1

gene	protein	reference
UL19	VP5, major structural component of virions	1, 2
UL26	VP22a, surface component of virions	3
UL41	protein which shuts off host protein synthesis	4
UL48	$V_{mw}65$, major tegument protein which activates expression of immediate-early genes	5, 6

Table VI
EUKARYOTIC SERINE/THREONINE PROTEIN KINASES HAVING NARROW SUBSTRATE SPECIFICITIES

references:

1) Pickett-Gies & Walsh, 1986
2) Stull *et al.*, 1986
3) Nairn & Greengard, 1987
4) Nairn *et al.*, 1985
5) Ryazanov, 1987
6) Blenis *et al.*, 1987
7) London, *et al.*, 1987
8) Reed & Yeaman, 1987
9) Randle *et al.*, 1987
10) Frank & Buzney, 1975
11) Benovic *et al.*, 1986
12) Hardie *et al.*, 1989
13) Emki *et al.*, 1980
14) Hemmings *et al.*, 1981

Table VI

EUKARYOTIC SERINE/THREONINE PROTEIN KINASES HAVING NARROW SUBSTRATE SPECIFICITIES

PROTEIN KINASE (PK)	SUBSTRATES	REGULATION	REF
phosphorylase *b* kinase	phosphorylase *b*	activated by Ca^{2+}, ↓ATP, ↑ADP	1
myosin light chain kinase	myosin light chain	activated by Ca^{2+}/CaM	2
Ca^{2+}/CaM-dependent PKI	synapsin I (site I), protein III	activated by Ca^{2+}/CaM	3
Ca^{2+}/CaM-dependent PKII	glycogen synthase, synapsin I (site II), tryptophan hydroxylase, MAP-2	activated by Ca^{2+}/CaM	4
Ca^{2+}/CaM-dependent PKIII	EF-2	activated by Ca^{2+}/CaM	5
S6 kinase	ribosomal protein S6	? activated by mitogens	6
haem-regulated PK	eIF-2α	inhibited by haem	7
ds RNA-dependent PK	eIF-2α	activated by ds RNA	7
pyruvate dehydrogenase kinase	pyruvate dehydrogenase	activated by ↑acetyl CoA and NADH, ↓ATP, high [pyruvate]	8
branched chain α keto acid dehydrogenase kinase	branched chain α keto acid dehydrogenase	inhibited by ADP, acetoacetyl CoA, branched chain keto acids	9
rhodopsin kinase	rhodopsin		10
β adrenergic receptor kinase	β adrenergic receptor (occupied by agonist)		11
AMP-PK	hydroxymethylglutaryl CoA reductase. acetyl CoA carboxylase	AMP, phosphorylation by kinase kinase	12
glycogen synthase kinase 3	glycogen synthase, PP-inhibitor 2 cAMP-dependent PK R_{II}		13 & 14

Table VII
EUKARYOTIC SERINE/THREONINE PROTEIN KINASES
HAVING BROAD SUBSTRATE SPECIFICITIES

references:

1) Beebe & Corbin, 1986
2) Nishizuka, 1986
3) Ahmad *et al.*, 1984
4) Pendergast & Traugh, 1985

The one letter amino acid code is used, with X indicating any amino acid.

Table VII

EUKARYOTIC SERINE/THREONINE PROTEIN KINASES HAVING BROAD SUBSTRATE SPECIFICITES

PROTEIN KINASE	REGULATION	SUBSTRATE SPECIFICITY	EXAMPLES OF SUBSTRATES	REF
cAMP-dependent protein kinase	cAMP	RRXS	phosphorylase kinase, acetyl CoA carboxylase, myosin light chain kinase	1
cGMP-dependent protein kinase	cGMP (nM) cAMP (μM)	basic-PS-basic		1
protein kinase C	Ca^{2+} + diacylglycerol	basic residues on N- or C-side of S/T	EGF receptor, acetyl CoA carboxylase	2
casein kinase I	?		glycogen synthase, amino-acyl tRNA synthase	3, 4
casein kinase II	inhibited by heparin, 2,3 diphospho-glycerate, activated by polyamines	acidic amino acids on C-side of S/T	glycogen synthase, acetyl CoA carboxylase, DNA topoisomerase II	1

Table VIIIa

PROTEIN KINASES KNOWN TO UNDERGO AUTOPHOSPHORYLATION

SERINE/THREONINE PROTEIN KINASES

references:

1) Rangel-Aldao & Rosen, 1976
2) Todhunter & Purich, 1977
3) Hoffmann & Flockerzi, 1983
4) Aitken *et al.*, 1984
5) Hallenbeck & Walsh, 1983
6) Lai *et al.*, 1987
7) Kikkawa *et al.*, 1982
8) Gross & Mendelewski, 1978
9) Berry & Samuel, 1985
10) Ahmad *et al.*, 1984
11) Dahmus, 1981
12) Lee *et al.*, 1982

Table VIIIa

PROTEIN KINASES KNOWN TO UNDERGO AUTOPHOSPHORYLATION

PROTEIN KINASE (pk)	SITE OF AUTO-PHOSPHORYLATION	EFFECT OF AUTO-PHOSPHORYLATION	REF
SERINE/THREONINE PROTEIN KINASES			
cAMP-dependent pk	R_{II} subunit, ser 95	↓reassociation with C-subunit	1 &2
cGMP-dependent pk	thr 58	↑affinity for cAMP, no effect on affinity for cGMP	3 &4
phosphorylase *b* kinase		activation	5
Ca^{2+}/CaM-dependent pk II	α and β subunits	activity independent of Ca^{2+}/CaM	6
protein kinase C		↑affinity for Ca^{2+} and diacylglycerol	7
haem-regulated pk	3-5 sites	activation	8
ds RNA-dependent pk		activation	9
casein kinase I		no effect	10
casein kinase II		no effect	11
rhodopsin kinase		?	12

Table VIIIb
PROTEIN KINASES KNOWN TO UNDERGO AUTOPHOSPHORYLATION

TYROSINE PROTEIN KINASES

references:

1) Smart *et al.*, 1981
2) Cooper *et al.*, 1986
3) Tornqvist & Avruch, 1988
4) Tornqvist *et al.*, 1988
5) Cooper *et al.*, 1981
6) Stern *et al.*, 1986
7) Meckling-Hasen *et al.*, 1987
8) Weinmaster *et al.*, 1983
9) Reynolds *et al.*, 1982

Table VIIIb

PROTEIN KINASES KNOWN TO UNDERGO AUTOPHOSPHORYLATION

PROTEIN KINASE (pk)	SITE OF AUTO-PHOSPHORYLATION	EFFECT OF AUTO-PHOSPHORYLATION	REF
TYROSINE PROTEIN KINASES			
pp60$^{v\text{-}src}$	major site tyr 416	activation of protein kinase and trans-forming activity	1
pp60$^{c\text{-}src}$	major site tyr 527	activation of protein kinase and trans-forming activity	2
insulin receptor	6 sites, most important: tyr 1146, 1150 and 1151	activation of pk	3, 4
EGF receptor		?	5
neu protein		?	6
v-fgr protein	tyr 553 (equivalent to tyr 416 of *v-src*)	activation of pk	7
v-fps protein	tyr 1073 (equivalent to tyr 416 of *v-src*)	activation of pk	8
v-abl protein	tyr 514 (equivalent to tyr 416 of *v-src*)	?	9

Table IX
PROTEIN KINASES INVOLVED IN THE REGULATION OF CELL GROWTH

references:

1) Hanks *et al.*, 1988
2) Hunter & Cooper, 1986
3) Van Beveren *et al.*, 1981
4) Koenen *et al.*, 1988
5) Beck *et al.*, 1987
6) Dunphy & Newport, 1988
7) Russel & Nurse, 1987a
8) Russel & Nurse, 1987b

Table IX

PROTEIN KINASES INVOLVED IN THE REGULATION OF CELL GROWTH

TYROSINE PROTEIN KINASES	FUNCTION	REF
src family: protein products of *src, yes, fgr, lyn,* *lck, fyn, hck*	regulation of cell proliferation	1, 2
receptors for hormones and growth factors: EGF receptor, *neu* protein, insulin receptor, IGF-1 receptor, CSF-1 receptor, *kit* protein, PDGF receptor, *ros* protein, *met* protein, *ret* protein	response to hormones and growth factors	1, 2
SERINE PROTEIN KINASES		
mos/mil/raf protein	regulation of cell proliferation	3, 4 & 5
CDC28/cdc2/MPF/histone HI kinase	required for transitions in the cell cycle: interphase to G1, G2 to mitosis	6
wee1$^+$ protien	negative regulation of cdc2 protein	7
nim1$^+$ protein	negative regulation of *wee1*$^+$ protein	8

Table X
FUNCTIONS OF EUKARYOTIC SERINE/THREONINE PROTEIN KINASES

references:

1) Pickett-Gies & Walsh, 1986
2) Kamm & Stull, 1985
3) Stull *et al.*, 1986
4) Nairn & Greengard, 1987
5) Nairn *et al.*, 1985
6) Palfrey *et al.*, 1987
7) Blenis *et al.*, 1987
8) London *et al.*, 1987
9) Reed & Yeaman, 1987
10) Randle *et al.*, 1987
11) Strasser *et al.*, 1986
12) Sitaramayya & Liebman, 1983
13) Hardie *et al.*, 1989
14) Cohen, 1986
15) Cohen, 1985
16) Beebe, 1986
17) Nishizuka, 1986

Table X

FUNCTIONS OF EUKARYOTIC SERINE/THREONINE PROTEIN KINASES

PROTEIN KINASE	FUNCTION	REF
phosphorylase *b* kinase	activates glycogen phosphorylase, which catalyses glycogen breakdown	1
myosin light chain protein kinase	smooth muscle and non muscle: phosphorylation of myosin light chain is essential for the activation of myosin ATPase by actin	2
	skeletal and cardiac muscle: phosphorylation of myosin light chain; not essential, but modulates contraction	3
Ca^{2+}/CaM-dependent protein kinase I	phosphorylation of synapsin I, which is involved in neurotransmitter release	4,5
Ca^{2+}/CaM-dependent protein kinase II	phosphorylation of synapsin I and MAP-2, which are involved in neurotransmitter release	5
Ca^{2+}/CaM-dependent of protein kinase III	phosphorylation of EF-2, causes inhibition protein synthesis	6
S6 kinase	involved in cell growth	7
haem-regulated protein kinase	phosphorylation of eIF-2α, causes inhibition of translation	8
ds RNA-dependent protein kinase	phosphorylation of eIF-2α, causes inhibition of translation	8
pyruvate dehydrogenase kinase	inhibition of pyruvate dehydrogenase	9
branched chain α keto acid dehydrogenase kinase	inhibition of branched chain α keto acid dehydrogenase, causing inhibition of oxidation of leucine, isoleucine and valine	10

Table X (continued)
FUNCTIONS OF EUKARYOTIC SERINE/THREONINE PROTEIN KINASES

Table X (continued)

FUNCTIONS OF EUKARYOTIC SERINE/THREONINE PROTEIN KINASES

PROTEIN KINASE	FUNCTION	REF
β adrenergic receptor kinase	phosphorylation of receptor, desensitises receptor towards adrenalin	11
rhodopsin kinase	phosphorylation of rhodopsin prevents association with $G_{\alpha}GTP$ which prevents activation of cGMP phosphodiesterase	12
AMP-dependent protein kinase	inactivation of acetyl CoA carboxylase and HMG CoA reductase, causes inhibition of fatty acid and cholestrol synthesis	13
glycogen synthase kinase 3	inactivation of glycogen synthase causing inhibition of glycogen synthesis	14
cAMP-dependent protein kinase	regulation of: glycogen synthesis/glycogenolysis gluconeogenesis/glycolysis fatty acid synthesis/lipolysis cholestrol ester hydrolysis catecholamine synthesis aromatic amino acid degradation	15 &16
protein kinase C	release of hormones and neurotransmitters, activation of T and B lymphocytes, muscle contraction and relaxation	17

Table XI
CHARACTERISTICS OF PROTEIN PHOSPHATASES

reference:

Ingebritsen & Cohen, 1983

Table XI

CHARACTERISTICS OF PROTEIN PHOSPHATASES

protein phosphatase	abundance	substrate specificity	regulators
PP-1	high	broad	inhibitor-1 and -2, glycogen synthase kinase 3
PP-2A	high	broad	unknown
PP-2B	low	narrow eg. inhibitor-1, cAMP-dependent pk R_{II} subunit	Ca^{2+} and CaM
PP-2C	high	broad	Mg^{2+}

Chapter 2

MATERIALS AND METHODS

A. Materials

1. Radiochemicals

The following radiochemicals were obtained from **Amersham International plc**, England.

$\gamma[^{32}P]$ATP as TEA salt in stabilised aqueous solution at 10mCi/ml and 5000Ci/mmol, $[^{32}P]$orthophosphate in dilute HCl pH 2.3 carrier free 10mCi/ml.

2. Fine chemicals

Fine chemicals were AnalaR Grade supplied by **BDH Chemicals Ltd** or **Formachem (Research International) Ltd**, with the exception of those listed below.

The following were obtained from **Amersham International Ltd**, England.

Rainbow protein molecular weight markers (pre-stained proteins with molecular weights of 200kDa, 96kDa, 69kDa, 46kDa, 30kDa, 21.5kDa, 14.3kDa).

The following, a product of Toyo Soda Manufacturing Co. (Japan) was obtained through **Anachem**, Luton.

TSK phenyl-5PW column.

The following were obtained from **Bethesda Research Laboratories** or **New England Biolabs.**

restriction endonucleases, low melting point agarose (ultra pure)

The following was obtained from **Biorad Laboratories**

Biogel P60

The following was obtained from **Calbiochem-Behring.**

Aquacide (type II)

The following were obtained from **Difco Laboratories**, West Molesey, Surrey, England.

bacto agar (0140-01), bacto tryptone (0123-01), nutrient broth (0003-02), tryptose phosphate, yeast extract (1880-17)

The following were obtained from **Eastman Kodak Co**, USA.

N, N, N', N'-tetramethyl-ethylene diamine (TEMED), Bromophenol Blue

The following were obtained from **Gibco Biocult**, Scotland.

heat-inactivated horse serum, new-born calf serum, Dulbecco modified Eagle's minimum essential medium (D-MEM x 10), Glasgow modification of Eagle's minimum essential medium (G-MEM x 10), 7.5% $NaHCO_3$ pH7.4, L-glutamine x 100 (200mM), penicillin/streptomycin (10000IU/ml penicillin, 10000UG/ml streptomycin), trypsin (0.25%), MEM amino acids without L-glutamine x50, MEM vitamins x 100

The following was obtained from **Fuji Photo Film Co.**, Ltd,

Fuji X-ray film

The following was obtained from **Pharmacia**, Sweden.

aminohexyl (AH)-Sepharose 4B

The following was obtained from **Schleicher & Schuell**, Anderman & Co Ltd, Surrey, England.

nitrocellulose membrane filters

The following was obtained from **Scottish Antibody Production Unit**, Law Hospital, Carluke, Lanarkshire.

horse radish peroxidase-coupled donkey anti-rabbit immunoglobulin G

The following were obtained from **Sigma Chemical Co**

agarose type II, alkaline phosphatase-conjugated goat anti-rabbit IgG, ampicillin, Brij 58, 5-bromo-4-chloro-3-indolyl phosphate (p-toluidine salt), chloramphenicol, 4-chloro-1-napthol, Coomassie Blue G 250 and R 250, DNase I (bovine pancreas), diethylpyrocarbonate, hydrogen peroxide, lysozyme, nitroblue tetrazolium, N-laurylsarcosine, NP-40, phenylmethylsulphonyl fluoride, protamine-agarose, protamine sulphate (salmine), RNAase A (bovine pancreas), Sephacryl S300, L-threonine, Tween 20

The following were obtained from **Whatman Biochemicals Ltd**

DEAE-cellulose (DE-52), 3MM filter paper, 3MM filter discs

3. Cell lines

BHK 21/C13 : baby hamster kidney fibroblasts (MacPherson & Stoker, 1962)

208F : rat fibroblasts from D. Spandidos, Beatson Institute for Cancer Research, Garscube Estate, Bearsden, Glasgow. (Quade, 1979)

4. Viruses

PRV was originally obtained from Kaplan & Vatter (1959) via W. S. Stevely.

HSV-1 Glasgow strain 17 was from J. MacNab, MRC Virology Unit, Institute of Virology, University of Glasgow.

HSV-1 R7041, a mutant in which the US3 gene is deleted (Purves *et al.*, 1987b), was a gift from B. Roizman.

5. Bacteria

The strain of *E. coli* used was JM109 which has the genotype:

*rec*A1, Δ *lac pro. end* A1, *gyr* A96, *thi*-1, *hsd* R17, *sup* E44, *rel* A1/F', *tra* D36, *pro* AB$^+$, *lac* Iq, *lac* z Δ M15.

(Yanish-Perron *et al.*, 1985)

6. Plasmids

The plasmids used are described below.

pIC 20H is a vector with multiple cloning sites, flanked by two *Hin*d III sites, all of which occur within *lac z*. This allows colour selection of recombinants, (Marsh *et al.*, 1984).

pUEX1-3 are a family of vectors which allow expression of a fragment of DNA as a β-galactosidase fusion-protein in *E. coli* . (Bressan & Stanley 1987).

Homer 5 is a vector allowing the expression of eukaryotic genes from their own promoter but with the expression enhanced by the SV 40 enhancer sequence. This vector also allows selection for transformants by geneticin resistance. (Spandidos & Anderson, 1987)

pRB 425 consists of the *Hin*d III - *Sst* I fragment (bases 862-5337) of HSV-1 F strain cloned into pUC 18. This was a gift from B. Roizman (Longnecker & Roizman, 1987).

pPRVB 10 consists of the *Bam* HI fragment 10 of PRV cloned into pUC 18 and was a gift from G. Zhang (Petrovskis *et al.*, 1986).

B. Methods

1. *E. coli* culture

L-broth: 10g bactotryptone, 5g yeast extract, 5g NaCl in 1l water

L-Agar : 15g agar in 1l L-broth

ampicillin stock: 5mg/ml stored at -20°C

Hammersmith stabs: 0.9g nutrient broth, 0.75g agar, 0.5g NaCl in 100ml water, plus 1ml of 10mg/ml thymidine

M9 salts: 6g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 1g NH_4Cl in 500ml water

Minimal Agar: 500ml M9 salts, 500ml 3% agar, 1ml 1M $MgSO_4$, 1ml 0.1M $CaCl_2$, 1ml 1M thiamine-HCl, 5ml 40% glucose

IPTG stock: 20mg/ml in water, stored at 4°C

X-gal: 20mg/ml in dimethylformamide, stored at -20°C

E. coli JM109 was maintained on minimal agar plates.

Transformed *E. coli* colonies were grown on ampicillin plates ie. 30µg/ml ampicillin in L-agar, or in liquid culture with 20µg/ml ampicillin in L-broth.

Transformed *E. coli* colonies were stored as Hammersmith stabs at room temperature.

1.1 Preparation of competent cells for transformation

An overnight culture was inoculated from a single colony of JM109 from a minimal agar plate. 40ml of L-broth were inoculated with 2ml of the overnight culture and incubated at 37°C for 3h. The cells were then harvested by centrifugation at 2000rpm for 10min and resuspended in 20ml of pre-chilled 50mM $CaCl_2$. After 20min, the cells were centrifuged as before and then resuspended in 4ml 50mM $CaCl_2$. These were suitable for use for up to 7 days after they had been prepared and were stored at 4°C.

1.2 Transformation of *E.coli* with plasmid DNA

0.1-1ng DNA was added to 100µg competent cells and incubated on ice for 30min. The mixture was then incubated for 2min at 37°C, plated onto an ampicillin plate, and incubated at 37°C overnight.

For colour selection of recombinants in pIC 20H, the plates were spread with 30µl X-gal and 30µl IPTG before plating with the transformation mixture. Colonies containing recombinants were white, while those containing pIC 20H were blue.

2. Preparation of Plasmid DNA

2.1 Small scale plasmid "mini-prep"

Single colonies from a transformation were streaked onto ampicillin plates so as to cover half a plate. (A master plate was also prepared.) This was incubated at 37°C overnight.

The bacteria were scraped from the plate with a loop and resuspended in 1ml of a solution containing 8% sucrose, 50mM Tris-HCl

pH8, 50mM EDTA, 5% Triton X-100. 10µl of 10mg/ml H_2O lysozyme were added and mixed before incubating at 95-100°C for 7min. The tubes were then centrifuged for 15min and 0.6ml of the supernatant was transferred to another tube. 2µl 1mg/ml H_2O boiled RNAase A was added and incubated for 15min at 37°C. 1µl diethylpyrocarbonate was then added and the tube was incubated for a further 10min at 65°C. After this, 0.24ml 5M ammonium acetate and 0.54ml isopropanol was added. The tubes were mixed and left in dry ice for 5min, before centrifuging for 10min to precipitate the DNA. The precipitated DNA was then washed with 0.3M ammonium acetate, 70% isopropanol, dried by lyophilisation and dissolved in 30µl TE buffer (Methods 3).

2.2 Large scale plasmid "mini-prep"

Single colonies from a transformation were inoculated into 10ml of L-broth + ampicillin (Methods 1) and incubated overnight at 37°C. 1ml of this culture was then used to inoculate 50ml L-broth which was incubated overnight at 37°C.

The cells were harvested by centrifugation for 10min at 2000rpm and resuspended in 5ml 25% sucrose, 50mM Tris-HCl pH7.5. 1ml lysosyme (40mg/ml H_2O) was added and incubated for 10min on ice. 2ml 0.5M EDTA pH8 was added and incubated for a further 20min. 0.6ml 10% NP-40 was added and the tubes inverted to mix well. The cell debris was sedimented by centrifuging for 25min at 30000rpm in a T150 rotor. The supernatant was then extracted twice with equal volumes of phenol: chloroform: isoamylalcohol (25:24:1) and 100µl 10mg/ml H_2O boiled RNAase A added. After incubating for 30min at 37°C, a further phenol: chloroform: isoamylalcohol extraction was

performed and the DNA precipitated by adding 0.1 volumes 3M sodium acetate and 0.5 volumes isopropanol, and leaving at room temperature for 1h. The DNA was then sedimented by centrifuging for 25min at 10000rpm. After washing the DNA with 100% ethanol it was dried in a lyophiliser and resuspended in approximately 500µl TE buffer (Methods 3).

2.3 Large scale plasmid preparation

25ml of L-broth + ampicillin (Methods 1) was inoculated with a single colony containing the plasmid, usually obtained from a master plate (Methods 2.1), and incubated overnight.

Two flasks of 800ml L-broth were inoculated with 5ml of the overnight culture and grown till they gave an OD_{280} reading of 0.8. Chloramphenicol (25mg/ml ethanol) was added to give a final concentration of 165µg/ml and incubation was continued overnight.

The cells were harvested by centrifugation at 5000rpm for 10min, resuspended in 9.5ml 50mM glucose, 10mM EDTA, 25mM Tris-HCl pH8, and 0.5ml 40mg/ml H_2O lysozyme added. After incubating on ice for 30min, 20ml of a solution containing 0.2M NaOH, 1% SDS were added and incubated for 5min on ice. Then 15ml 3M sodium acetate pH4.8 were added and left on ice for 1h. The cell debris was removed by centrifugation for 30min at 30000rpm in a Ti60 rotor. Isopropanol (0.6 volume) was added to the supernatant which was left for 10min at room temperature before centrifuging for 15min at 8000rpm. The DNA precipitate was dissolved in 30ml TE buffer and 28.9g CsCl + 1.8ml 10mg/ml H_2O ethidium bromide added. The solution was clarified by centrifugation for 30min at 1500rpm, and transferred to a VTi50 tube,

which was heat-sealed, and centrifuged overnight at 50000rpm in a VTi50 rotor at 20°C.

The band of DNA was recovered by piercing the tube with a syringe needle (21G), and transferred to a VTi65 tube, which was heat-sealed and centrifuged overnight at 65000rpm in a VTi65 rotor at 20°C.

The DNA was recovered in a similar manner and the ethidium bromide removed by mixing the solution containing the DNA with an equal volume of isoamylalcohol, separating the aqueous and organic layers by centrifuging for 2min in a microfuge and removing the organic layer. This was repeated 4 or 5 times. The DNA was diluted two-fold with TE buffer and precipitated with 100% ethanol. After dissolving in TE buffer, the yield was estimated by measuring the A_{260}. 50µg/ml DNA gives an A_{260} reading of 1.0.

3. Manipulation of DNA

Solutions

TE buffer: 10mM Tris-HCl pH8, 1mM EDTA

Restriction enzyme buffers:

Low salt : 10mM Tris-HCl pH7.4, 10mM $MgSO_4$, 1mM DTT

Medium salt : 10mM Tris-HCl pH7.4, 10mM $MgSO_4$, 1mM DTT, 50mM NaCl

High salt : 10mM Tris-HCl pH7.4, 10mM $MgSO_4$, 1mM DTT, 100mM NaCl

Ligase buffer: 40mM Tris-HCl pH7.6, 10mM $MgCl_2$, 1mM DTT

Alkaline phosphatase buffer: 50mM Tris-HCl pH8, 10mM $MgCl_2$

3.1 Restriction endonuclease digestions

Digestions were carried out in a volume of 20-200µl (depending on the amount of DNA) using the appropriate restriction endonuclease buffer and an excess of the desired enzyme (ie. more than 1 unit/µg DNA, but not exceeding 0.1 of the total reaction volume) for 2hr at the temperature recommended by the manufacturers.

3.2 Deproteinisation with phenol

Protein was removed from restriction digestions by adding an equal volume of phenol saturated with TE buffer, mixing and centrifuging in order to separate the organic and aqueous layers. The aqueous layer was collected and the process repeated. Residual phenol was removed by a similar extraction proceedure using ether in place of phenol.

3.3 Ethanol precipitation of DNA

DNA was precipitated by adding 2 volumes ethanol and 0.1 volumes 3M sodium acetate. This was either left at -20°C overnight, or left in dry ice for 30min. The precipitate was recovered by centrifugation, and then dried in a lyophiliser before redissolving in TE buffer.

3.4 Treatment of vector DNA with alkaline phosphatase

After digestion of vector DNA with restriction endonuclease (Methods 3.1), deproteinisation with phenol (Methods 3.2), and ethanol precipitation (Methods 3.3), the DNA was redissolved in 20µl phosphatase buffer (Methods 3). 15U alkaline phosphatase were added

and the mixture was incubated for 30min at 37°C. The DNA was again deproteinated with phenol (Methods 3.2), precipitated with ethanol (Methods 3.3) and redissolved in TE buffer.

3.5 Ligation

Ligations were performed in a volume of 30µl using about 1µg each of vector and insert DNAs. These were incubated overnight at 15°C with 3µl ligase buffer x10, 3µl 5mM ATP and 1 unit T4 ligase. 15µl were used in a transformation.

4. Electrophoresis

4.1 Electrophoresis of DNA in Agarose gels

TBE buffer : 10g Tris, 5.5g boric acid, 0.93g EDTA in 1l water to give a final pH of 8.3

1% agarose gel: 0.6g agarose type II, 60ml TBE buffer, 3µl 10mg/ml ethidium bromide

1% low melting point agarose gel: 1.2g low melting point agarose, 120ml TBE buffer, 6µl 10mg/ml ethidium bromide

Sample loading buffer: 0.5ml glycerol, 0.5ml TBE buffer, 5µl 10mg/ml bromophenol blue

a) Standard 1% agarose gels

DNA samples were mixed with 0.1 volume sample buffer and loaded onto an agarose gel. Electrophoresis was carried out in TBE buffer with

50µl 10mg/ml H_2O ethidium bromide per litre, at 70V. The DNA was visualised by illumination on an ultra-violet transilluminator. Photographs were taken using a Polaroid camera using a Polaroid film type 665.

b) Low melting point agarose gels

DNA samples were prepared and loaded onto the gel as for the standard agarose gel, and electrophoresis was carried out in TBE buffer containing 0.5mg/l ethidium bromide, at 30V. The DNA was visualised by illumination on an ultra-violet transilluminator.

c) Elution of DNA from low melting point agarose gels

The desired band was cut from the gel and placed in a tube along with 200µl TE buffer and incubated at 65°C for 10min, before extracting with phenol saturated with TE buffer which had been warmed to 37°C. The phenol was removed from the resulting aqueous layer by extraction with ether and the DNA precipitated with ethanol (Methods 3.3).

4.2 Electrophoresis of Proteins on Polyacrylamide gels

Electrophoresis buffer : 14.4g glycine, 6g Tris, 1g SDS in 1l water, giving a final pH of 8.3

Sample loading buffer : 500µl 0.5M Tris-HCl pH 6.7,
250µl 2-mercaptoethanol,
250µl 20% SDS, 2ml glycerol,
50µl 0.1% bromo-phenol blue

Polyacrylamide gel:

	stacking gel	10% separating gel	12.5% separating gel
30% acrylamide in H_2O	2.5ml	13.3ml	16.6ml
water	8.7ml	16.5ml	13.4ml
20% SDS	0.075ml	0.2ml	0.2ml
TEMED	0.005ml	0.018ml	0.018ml
10% ammonium persulphate	0.5ml	0.5ml	0.5ml
1.5M Tris-HCl pH8.8		10.0ml	10.0ml
0.5M Tris-HCl pH6.7	3.8ml		

Unless otherwise stated, 10% polyacrylamide gels were used.

4.2a Coomassie Blue staining

Coomassie Blue protein staining solution:

0.25% w/v Coomassie Brilliant Blue R 250, 45% methanol, 10% acetic acid, 45% water

Destaining solution:

45% methanol, 10% acetic acid, 45% water

Protein samples were added to an equal volume of sample loading buffer and boiled for 3min before loading onto the gel. Electrophoresis was performed for 3-4h at 50mA and 300V in electrophoresis buffer. The gel was then soaked in staining solution

for 1h at room temperature and then in several changes of destaining solution.

4.2b Silver staining

solutions:

fixative : 30% methanol, 10% acetic acid, 60% water

reducer : 30ml ethanol, 65ml water, 2.5ml 4M sodium acetate. The pH was adjusted to 6.0 and 100mg sodium thiosulphate added.

silver reagent : 0.1% w/v silver nitrate in water plus 25µl/100ml 37% w/v formaldehyde

developer : 2.5% w/v sodium carbonate in H_2O plus 50µl/100ml 37% w/v formaldehyde

stop solution : 1% acetic acid in water

The gel was soaked in fixative for 30min and then reducer for 30min. After washing with water several times, the gel was soaked in silver reagent for 30min and washed with water before adding the developer. Once the bands had developed, the reaction was stopped by adding the stop solution.

4.2c Acetone precipitation of proteins

10 volumes of acetone were added to the protein solution and left at 4°C overnight. The precipitate was sedimented by centrifugation at 10000rpm for 15min. Protein samples containing a high concentration of salt were dialysed against 10mM Tris-HCl pH7.0 before acetone precipitation.

5. Cell Culture

Culture media

D-MEM growth medium:

450ml water, 50ml D-MEM x10, 25ml 7.5% $NaHCO_3$ pH7.4,
5ml L-glutamine x100, 5ml penicillin/streptomycin,
50ml new-born calf serum

G-MEM growth medium:

400ml water, 50ml G-MEM x10, 12ml 7.5% $NaHCO_3$ pH7.4,
8ml L-glutamine x100, 4ml penicillin/streptomycin,
50ml new-born calf serum, 40ml tryptose phosphate broth

cell freezing media : 1) 8% DMSO, 20% new-born calf serum,
72% D-MEM growth medium
2) 10% sterile glycerol, 25% new-born calf
serum, 65% G-MEM growth medium

Cell lines 208F, Ka-1, Ka-2, Kb-1 and Kb-2 were grown in D-MEM growth medium and stored in DMSO freezing medium.
BHK cells were grown in G-MEM growth medium and stored in glycerol freezing medium.

5.1 Storage and recovery of cells

Cells were stored by freezing in liquid nitrogen. $5x10^6$ cells were trypsinised (by adding sufficient trypsin to cover the cells and

incubating for 1-5min at 37°C, and harvested by centrifuging for 5min at 1000rpm. The supernatant was poured off and the cells resuspended in 1ml freezing medium, and transferred to a biofreeze vial. These were then frozen by wrapping in cotton wool and placing in a polystyrene box at -70°C overnight. They were then transferred to liquid nitrogen.

Cells were recovered from liquid nitrogen by thawing rapidly in a water bath at 37°C. The cells were then sedimented by centrifuging for 5min at 1000rpm, resuspended in medium and added to a 25cm² plastic flask containing 5ml medium, and 5% carbon dioxide.

5.2 Growth of cells in culture

Cells were grown in plastic flasks or glass roller bottles (80oz). 208F cells and the cell lines derived from these cells (Ka-1, Ka-2, Kb-1, Kb-2) were grown in D-MEM growth medium. BHK cells were grown in G-MEM growth medium. For all cells, carbon dioxide was added to the flasks or roller bottles to give a final concentration of 5%. The table below shows the number of cells used to seed flasks and roller bottles to achieve confluence in 2-3 days.

size of flask	inoculum of cells
25cm²	$1x10^6$
75cm²	$2x10^6$
150cm²	$3-4x10^6$
80oz roller bottle	$20-30x10^6$

When confluent, the cells were trypsinised by adding sufficient trypsin to cover the cells and incubating for 1-5min at 37°C. 5-25ml growth medium were then added and the cells counted using a haemocytometer before using them to seed further flasks.

5.3 Measurement of the growth of 208F cells and derived cell lines.

The growth of cells was measured over 10 days using either a Coulter counter or a haemocytometer to count the number of cells. Twenty two 60mm Petri dishes were seeded with $6x10^4$ cells in 3ml D-MEM growth medium. These dishes were incubated at 37°C in an atmosphere of 5% carbon dioxide for the course of the experiment. On alternate days, half the medium was removed and replaced with fresh D-MEM growth medium. Each day, all the cells from one dish were harvested for counting. All the medium was removed and 1ml of trypsin added. The dish was incubated for approximately 5min at 37°C until the cells were released from the plastic. For counting with the Coulter counter, 1ml D-MEM growth medium was added and the cells transferred to a tube for counting. The dish was washed with 1ml PBS (Methods 8.2) and the washings together with a further 3ml PBS were added to the tube to give a final volume of 6ml. The cells were then counted three times with the Coulter counter and an average taken. Readings for the same sample varied by 0.3% - 0.5%. For counting with a haemocytometer, the volume was not increased above 1ml. A drop of the cell suspension was placed on the haemocytometer and counted.

6. Infection of BHK cells with PRV

6.1 Infection for preparation of virus stocks

Eight roller bottles (80oz) of BHK cells which were approximately 80% confluent were infected with 0.02pfu/cell (50µl virus stock/roller bottle) in 100ml G-MEM growth medium but with only 1% new-born calf serum (Methods 5), and incubated for a further 24h by which time the cells were becoming detatched from the glass. The roller bottles were shaken to detatch all the cells and the medium poured off. This was stored in 500ml plastic bottles at -70°C and used as virus stocks for subsequent infections.

6.2 Infection for preparation of purified virions

Twenty roller bottles of confluent BHK cells were infected with 5pfu/cell (5ml virus stock/roller bottle) in 20ml G-MEM growth medium with 1% new-born calf serum (Methods 5). After 1h, this medium was poured off, and a further 20ml medium was added. The infection was continued for 18-24h, before harvesting (Methods 7.1).

6.3 Infection for preparation of purified PRV-PK

30-40 roller bottles of confluent BHK cells were infected with 20pfu/cell (20ml virus stock/roller bottle). After 1h, this medium was poured off and replaced with 20ml fresh G-MEM growth medium with 1% new-born calf serum (Methods 5). The infection was continued for 8h, before harvesting (Methods 8.2a).

7. Preparation and extraction of PRV virions

7.1 Preparation of Pseudorabies Virions (Stevely, 1975)

phosphate buffer : 1mM Na_2HPO_4/KH_2PO_4 pH 7.4

15% w/w sucrose in phosphate buffer

30% w/w sucrose in phosphate buffer

40% w/w sucrose in phosphate buffer

8M urea

Tris buffer: 10mM Tris-HCl pH 7.2, 0.15M NaCl

20% SDS in H_2O

1mg/ml DNase I in H_2O

10% Brij 58 in H_2O

18-24h after infection of BHK cells with PRV (Methods 6.2), the cells were scraped with a plastic scraper into the medium and harvested by subjection to centrifugation at 2000rpm for 10min.

Mature virions which had been released from the cells were obtained by subjecting the supernatant from the first centrifugation to a second centrifugation at 12000rpm for 2.5h. This precipitate was resuspended in phosphate buffer and layered onto a linear gradient of 15-40% sucrose in SW28 centrifuge tubes. After centrifugation at 20000rpm in a SW28 rotor for 1hr at 4°C, the band of virions was harvested using a syringe needle (21G) inserted through the tube. Urea was added to a concentration of 0.5M and the solution was sonicated in a sonicator bath (Kerry Pulsitron 55) for 5-10sec to

facilitate the release of the virions from any cellular material. A weight of sucrose equal to that of the solution containing the virions was added to give a 50% w/w solution. A sucrose step-gradient was then constructed by layering 40% and 30% sucrose solutions upon the 50% sucrose containing the virions. After centrifugation at 25000rpm for 18h in a SW28 rotor at 4°C, the band of virions was harvested again as above. The virions were diluted at least 2-fold in phosphate buffer and precipitated by centrifugation at 40000rpm for 18h at 4°C in a Ti50 rotor. This precipitate was then resuspended in a minimum volume of phosphate buffer (50-200µl).

Mature virions and nuclear capsids were isolated from the sedimented cells. The cells were washed 2-3 times in PBS (Methods 8.2) and resuspended in an equal volume of the hypotonic phosphate buffer. After leaving for 10min for the cells to swell, they were homogenised with 20 strokes in a teflon/glass homogeniser. This was then subjected to centrifugation at 2000rpm for 10min to sediment the nuclei. Virions were isolated from the supernatant using two sucrose gradients as described above.

Nucleocapsids were isolated from the sedimented nuclei. These were washed twice in PBS (Methods 8.2) and resuspended in Tris buffer. SDS was added to a final concentration of 0.5% and DNase I was added to give a final concentration of 50µg/ml and the mixture was incubated for 10min at 25°C. Brij 58 was then added to a final concentration of 0.5% and urea was added to give a concentration of 0.5M. This mixture was then subjected to centrifugation at 2000rpm for 10min and the

supernatant collected. Nucleocapsids were isolated from the supernatant in a manner similar to that described above using the two sucrose gradient steps.

7.2 Extraction of protein kinase activity from PRV Virions

Method 1 : A suspension of virions, to which 10% NP-40 was added to give a final concentration of 1%, was incubated at 25°C for 30min and then subjected to centrifugation in a microfuge at 13000rpm for 5min. The precipitate was resuspended in 10mM Tris-HCl pH7.5 to restore the original volume. (Lemaster & Roizman, 1980)

Method 2 : 1M NaCl was added to a suspension of virions to give a final concentration of 0.6M and NP-40 was added to give a concentration of 10%. This was incubated for 30min at 25°C and then diluted 10-fold with 10mM Tris-HCl pH7.5 before centrifugation and resuspension as for method 1. (Stevely *et al.*, 1985)

8. Assay and isolation of PRV-PK

8.1 Protein kinase assay (using protamine as substrate)

ATP mixture: The appropriate volumes of 1M $MgCl_2$ and 5mM ATP were mixed to give 10mM $MgCl_2$ and 0.05mM ATP as final concentrations in the assay. 0.5-2µCi $\gamma[^{32}P]$ATP were added per assay to give a specific activity of 0.08-0.33µCi/nM.

Substrate mixture: The appropriate volumes of 1M Tris-HCl pH 7.5, 2M KCl, 2-mercaptoethanol and 10mg/ml protamine sulphate were mixed to give 20mM Tris-HCl pH7.5, 50mM KCl, 1% v/v 2-mercaptoethanol and 0.8mg/ml protamine sulphate as final concentrations in the assay.

10% w/w trichloroacetic acid in H_2O

5% w/w trichloroacetic acid in H_2O

40µl of the material to be assayed for enzyme activity were incubated with 20µl ATP mixture and 60µl substrate mixture for 30min at 30°C. All the reaction mixture was then spotted onto Whatman 3MM filter discs which were then dropped into 10% trichloroacetic acid (10ml per filter). The filters were washed once with 10% trichloroacetic acid, twice with 5% trichloroacetic acid and once with ethanol, for 15min each. All the washings with trichloroacetic acid were performed at 4°C. The radioactivity was then measured as Cherenkov radiation in a scintillation spectrometer.

8.2 Isolation of viral PRV-PK (Purves *et al.*, 1987a)

PBS-A : 10g NaCl, 0.25g KCl, 1.44g Na_2HPO_4, 0.25g KH_2PO_4 in 1l water, pH adjusted to 7.4 with concentrated HCl

PBS-B : 0.25g $CaCl_2$ in 1l water

PBS-C : 0.25g $MgCl_2$ in 1l water

PBS : 8:1:1 mixture of PBS-A:PBS-B:PBS-C

RSBE : 10mM KCl, 1.5mM magnesium acetate, 1mM EGTA, 10mM Tris-HCl pH7.5

Med K : 25mM Tris-HCl pH7.5, 125mM KCl, 5mM magnesium acetate, 5mM 2-mercaptoethanol

PMSF : 20mg/ml in ethanol

buffer A : 20mM Tris-HCl pH7.5, 1mM EDTA, 1mM EGTA, 10mM 2-mercaptoethanol, 10% glycerol

buffer B : 1M ammonium sulphate in buffer A

a) Preparation of post-ribosomal supernatants from infected and uninfected BHK cells

The cells were harvested by scraping them into the medium followed by centrifugation for 10min at 2000 rpm. The sedimented cells were then washed twice with PBS, before resuspending in an equal volume of RSBE + 40µg/ml PMSF. After leaving on ice for 5min, the cells were homogenised with 20 strokes in a glass-teflon homogeniser. 0.11 volumes Med K x10 were added and the cell debris removed by centrifugation at 10000rpm for 30min. This supernatant was further centrifuged for 2.5h at 40000rpm in a Ti50 rotor at 4°C to sediment the ribosomes.

b) DEAE-cellulose chromatography of protein kinases in post-ribosomal supernatants from tissue culture cells

The post-ribosomal supernatant was dialysed overnight against buffer A + 40μg/ml PMSF.

i) Analytical Chromatography

A 1 x 5cm column of DEAE-cellulose (DE-52) was set up and equilibrated with buffer A. The flow rate was 12ml/h. A post-ribosomal supernatant prepared from cells harvested from 4-6 roller bottles was loaded at 12ml/h, and the column was washed with 12ml buffer A. Elution was then performed with a 60ml gradient of 0 - 0.4M KCl in buffer A. 60 fractions of 1ml were collected.

ii) Preparative Chromatography

This was performed as for analytical chromatography but using a column of 1.6 x 6cm and a flow rate of 80-90ml/h. A post-ribosomal supernatant prepared from cells harvested from 30-40 roller bottles was loaded onto the column which was washed with buffer A. Protein was eluted with a similar 500ml gradient, and 70 fractions of 7ml collected. The fractions containing PRV-PK activity were stored at -70°C until the next stage of the purification.

c) Hydrophobic-interaction chromatography on TSK phenyl-5PW

The material from six DEAE-cellulose preparations (derived from 4-5 x 10^{10} infected cells) was allowed to thaw at 4°C and concentrated 10-15 times in Aquacide II over 36h. The concentrated material was dialysed for 12h against several changes of buffer B. The dialysed

material (35-40ml containing 90-100mg protein) was applied by repeated injection to a 7.5 x 0.75-cm TSK phenyl-5PW column, pre-equilibrated with buffer B, and linked to an HPLC system. The column was washed at 1ml/min with buffer B until all unbound protein had eluted, and then eluted with 60ml of a linear gradient of 1.0 - 0M ammonium sulphate in buffer A lacking glycerol, at a flow rate of 1ml/min, and then with a further 20ml of the buffer at the final concentration of 0M ammonium sulphate. Fractions (1ml) were collected, and those containing PRV-PK were combined and subjected immediately to the next stage of the purification.

d) Threonine-Sepharose chromatography

L-threonine was coupled to AH-Sepharose-4B essentially according to Kikkawa *et al.* (1986), care being taken to maintain the pH between 5 and 6 during the coupling reaction. After extensive washing with distilled water, a 7 x 1cm column of the chromedium was prepared and equilibrated with buffer A. The PRV-PK from TSK phenyl-5PW (20-25 ml containing 5-6mg protein) was applied directly (ie. without dialysis) to the column which was washed with 25ml buffer A at a flow rate of 30ml/h, 5ml fractions being collected. The flow rate was then decreased to 15ml/h and the column eluted with 50ml of a linear gradient of 0 - 0.4M KCl, followed by 25ml 0.4M KCl, all in buffer A. PRV-PK was then eluted from the column with 50ml of a linear gradient of 0.4 - 1.0M KCl in buffer A, 2.5ml fractions being collected. Finally, the column was washed with 15ml 1.0M KCl. The fractions containing the bulk of the enzyme activity eluted at approximately 0.7M KCl. They were then combined and diluted with an equal volume of

buffer A to decrease the ionic strength to a value which would allow immediate further chromatography without prior dialysis.

e) Protamine-agarose chromatography

The diluted PRV-PK (25-30ml containing 0.7-0.8mg protein) was applied to a 3 x 1cm column of protamine-agarose, pre-equilibrated with buffer A. After washing with 20ml buffer A, the column was eluted with 12-ml steps of 0.5M, 0.8M and 1.0M KCl at a flow rate of 12ml/h, 1.2ml fractions being collected. PRV-PK eluted at 0.8M. The fractions containing PRV-PK were stored at -70°C.

f) The specific activity of the final material from the purification of PRV-PK

This was calculated for the major peak of the purified enzyme. The specific activity of $\gamma[^{32}P]$ATP in a standard assay was 1µCi/12nmol ATP. 1000cpm is equivalent to 1.4nCi. In a standard assay, 40µl of this fraction (0.8µg protein) gave 2.6×10^5cpm in a 30min incubation. This is equivalent to 0.36µCi, which is equivalent to the incorporation of 4.3nmol phosphate. Since 1 unit of enzyme activity is defined as that which produces 1nmol phosphate in 1min, this is 0.14 units. The specific activity is thus 180 units/mg. This is one-fifth of the best results obtained by Purves *et al.* (1987a), but is comparable with the results obtained in other preparations of the enzyme. The lower value is probably due to greater inactivation of the enzyme during the preparation. Also, the measurement of the amount of protein at such low concentrations is not very accurate and could easily be out by a factor of two.

9. Phosphorylation of viral proteins

9.1 Phosphorylation of viral structural proteins *in vitro*

a) Endogenous protein kinase activity

This was carried out by incubating virions in the standard kinase assay (Methods 8.1) but in the absence of protamine and using 2µCi $\gamma[^{32}P]$ATP per assay which gives a specific activity of 1µCi/nM.

b) Exogenous protein kinase activity

Endogenous virion protein kinases were inactivated by incubation of virions at 85°C for 3min. This material was used as a substrate in the standard kinase assay as above (Methods 9.1a) with various exogenous protein kinases.

The reactions were stopped by adding 50µl sample loading buffer (Methods 4.2) and boiling for 3min. The samples were then ready for electrophoresis (Methods 4.2a).

9.2 Detection of the phosphorylation of proteins during infection of cells

G-MEM growth medium (Methods 5) without tryptose phosphate and containing 10% new-born calf serum

G-MEM growth medium (Methods 5) without tryptose phosphate and containing 1% new-born calf serum

BSS x10 : 6.8g NaCl, 0.4g KCl, 0.2g $MgSO_4$, 0.14g NaH_2PO_4, 0.39g $CaCl_2$, 1.5ml phenol red in 100ml water (sterile)

ES x10 : 5.4ml MEM amino acids without L-glutamine x50, 2.7ml MEM vitamins x100, 2.7ml L-glutamine x100, 6.0ml 0.5M glucose, 13ml water (sterile)

phosphate-free medium : 40.5ml BSS x1, 6.7ml ES x10, 1.6ml $NaHCO_3$, 0.5ml penicillin/streptomycin, 0.5ml new-born calf serum, 5.5ml G-MEM growth medium without tryptose phosphate with 1% new-born calf serum

urea lysis buffer : 9.8M urea, 2% NP-40, 1% 2-mercaptoethanol,

Two 25cm^2 flasks were seeded with 10^6 BHK cells which were allowed to grow until approximately 80% confluent in G-MEM growth medium (Methods 5) containing 10% new-born calf serum but without tryptose phosphate. They were then infected with 3 x 10^7pfu per flask (20pfu/cell) in G-MEM growth medium (Methods 5) containing 1% new-born calf serum but without tryptose phosphate for up to 24h. One hour before the period desired for adding [^{32}P]orthophosphate, this medium was replaced with 5ml phosphate-free medium. [^{32}P]orthophosphate (0.3-1mCi/flask) was added to the cells and the incubation continued for a further 3-12h.

a) Preparation of extracts from infected cells

The cells were harvested by pouring off the medium and scraping the cells into PBS (Methods 8.2). The cells were sedimented by centrifugation in a microfuge for 5min at 13000rpm and washed three

times with PBS. They were then resuspended in urea lysis buffer which brought about lysis of the cells. The lysate was then centrifuged again for 30sec in a microfuge to sediment the nuclear material. The resulting supernatant was then mixed with sample loading buffer (Methods 4.2) and boiled for 3min. This could then be subjected to polyacrylamide gel electrophoresis (Methods 4.2a).

b) Preparation of virions from the medium of infected cells

The medium was poured from the cells and subjected to centrifugation at 2000rpm for 10min to sediment contaminating cellular material. The supernatant was then subjected to centrifugation in a Ti50 rotor at 40000rpm for 2.5h to precipitate the virions. The precipitate was then dissolved in sample loading buffer (Methods 4.2) and boiled for 3min. This could then be subjected to polyacrylamide gel electrophoresis (Methods 4.2a).

9.3 Preparation of autoradiographs

Gels containing radioactive material were stained for protein with Coomassie Blue (Methods 4.2a) and then dried onto filter paper using a gel drier. The dried gel was then exposed to Fuji X-ray film for upto one week before developing the film. Rainbow protein molecular weight markers were used. After the gel had been dried, the positions of the molecular weight markers were marked on the gel with radioactive ink so that their position could be seen on the autoradiograph.

10. Preparation of β-galactosidase fusion-protein for injection into rabbits for the preparation of specific anti-sera

10.1 Expression of β-galactosidase fusion-protein in *E. coli*

40ml L-broth + ampicillin (Methods 1) were inoculated with 2ml overnight culture of *E. coli* transformed with the relevant plasmid (Results A.1) and incubated at 30°C for 5h, before inducing the expression of the fusion-protein by increasing the temperature to 42°C for 2h.

10.2 Preparation of *E. coli* protein extract

The cells were harvested by centrifuging for 10min at 10000rpm, and resuspended in 4ml 0.3M NaCl, 50mM Tris-HCl pH7.5, 1mM EDTA. 0.4ml 40mg/ml H20 lysozyme was added and incubated for 10min on ice before adding 40µl 10% v/v NP-40 in H_2O for a further 10min. This was then centrifuged in a microfuge for 10min and the precipitate resuspended in 4ml 5% w/v SDS, 1% v/v 2-mercaptoethanol in H_2O. It was found necessary to boil this for 5-10min in order to dissolve all the protein.

10.3 Partial purification of the fusion-protein by Sephacryl S300 chromatography

A Sephacryl S300 column (50x1cm) was equilibrated with running buffer (5% SDS, 0.1M NaCl, 10mM Tris-HCl pH7.5) and run at a flow rate of 20ml/h at room temperature. 1ml samples of *E. coli* extract were loaded onto the column and 50 fractions of 1ml were collected.

Fractions were analysed for protein by measuring the A_{280}. Those corresponding to the first peak were then subjected to polyacrylamide gel electrophoresis in order to determine the purity of the fusion-protein. Fractions having sufficient purity (Results A.2) were pooled and dialysed against 3l 10mM Tris-HCl pH7 twice, in order to remove as much of the SDS as possible. The protein was then precipitated with acetone (Methods 4.2c) and redissolved in 0.5ml 10mM Tris-HCl pH7.

10.4 Injection of fusion-protein into rabbits

1st injection: 1mg fusion-protein in a volume of 0.5ml was mixed with an equal volume of Freund's complete adjuvant by sonication, and injected subcutaneously at 4 sites.

2nd injection: after 5 weeks, a further 1mg of the fusion-protein was similarly injected after mixing with an equal volume of Freund's incomplete adjuvant.

Further injections were carried out as for the second injection at 2-3 monthly intervals.

11. Preparation of Anti-sera

11.1 Bleeding of rabbits

Rabbits were bled 10 days after each injection, except for the first one, and also at approximately monthly intervals. 20ml of blood was collected each time from the ear vein.

11.2 Preparation of anti-serum

20ml blood was left to clot for 2h at 4°C in a glass container. The serum was collected from round the clot and centrifuged for 15min at 10000rpm. The supernatant was collected and added to 6g DEAE-cellulose (Whatman DE-52) which had been equilibrated overnight in phosphate buffer (10mM KH_2PO_4/K_2HPO_4 pH7.5). The supernatant was left for 30min on ice with occasional stirring before removing the DEAE-cellulose by filtration. The filtrate was then diluted 1:1 with phosphate buffer before adding 25.8g/100ml solid $(NH_4)_2SO_4$, slowly with continual stirring for 15min, while on ice. The precipitate was sedimented by centrifugation for 15min at 10000rpm at 4°C. The pellet was redissolved in 2ml phosphate buffer and dialysed against two changes of phosphate buffer. The anti-serum was stored as 100µl aliquots at -20°C.

11.3 Affinity-purification of anti-serum by elution from nitrocellulose filters

An extract containing the antigen against which the anti-serum was to be purified, was subjected to electrophoresis and transferred to a nitrocellulose filter (Methods 12). The nitrocellulose filter

was blocked and incubated with the anti-serum as for an immunoblot (Methods 12). Strips were cut from either end of the nitrocellulose filter and incubated with a second antibody and the appropriate reaction solution in order to locate the antigen and hence the position of the desired antibody on the filter. A narrow strip corresponding to this region of the filter was cut out. The antibody was eluted by incubation for 2min in 1ml 0.2M glycine pH2.8, 0.2% gelatin. The filter was then removed and stored in PBS (Methods 8.2) for further use, while 1M Tris base was added to the eluted antibody to give a pH of 7.0. This antibody could then be used in immunoblots.

12. Immunoblotting

transfer buffer: 25mM Tris, 192mM glycine, 20% methanol, 0.025% SDS

blocking buffer: 20mM Tris-HCl pH7.2, 0.15M NaCl, 0.5% Tween 20

solution containing 1st antibody:

20ml blocking buffer, 1ml heat-inactivated horse serum, 40-200µl anti-serum (approximately 20-100 fold dilution of serum)

solution containing 2nd antibody

peroxidase-coupled:

20mM Tris-HCl pH7.2, 0.15M NaCl, 3% bovine serum albumin, 10µl/10ml horse radish peroxidase-coupled donkey anti-rabbit immunoglobulin G

alkaline phosphatase-coupled:

20mM Tris-HCl pH7.2, 0.15M NaCl, 3% bovine serum albumin, 3µl/10ml alkaline phosphatase-coupled goat anti-rabbit immunoglobulin G

reaction solution

peroxidase: 10mg 4-chloro-1-napthol dissolved in 3ml cold methanol (-20°C) + 15ml blocking buffer without Tween 20

alkaline phosphatase: 10ml 0.1M Tris-HCl pH9.5, 0.1M NaCl, 5mM $MgCl_2$, plus 66µl 50mg/ml Nitro Blue Tetrazolium in 70% dimethylformamide plus 33µl 50mg/ml 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide

Protein samples were subjected to polyacrylamide gel electrophoresis (Methods 4.2a). Amersham pre-stained Rainbow molecular weight markers were used as molecular weight standards.

The gel was 'blotted' onto a nitrocellulose filter as follows: the gel was placed in a sandwich between one sheet of 3MM filter paper and a nitrocellulose filter. A further piece of 3MM filter paper was placed upon the nitrocellulose filter. This sandwich was placed in an electrophoretic transfer tank with transfer buffer. The transfer was performed overnight at 60mA or for 6h at 350mA. After the transfer had been performed, the pre-stained molecular weight markers could be seen on the nitrocellulose filter. However, they were found to fade considerably during the course of the subsequent incubations of the filter. Therefore, to avoid losing the positions of the markers,

these were marked with a biro at this stage before commencing the incubations.

The nitrocellulose filter was incubated with approximately 50ml blocking buffer for 1-2h and then with the first antibody for 5-24h at room temperature with gentle shaking. In many cases it was necessary to cut the filters at this stage, either to allow different parts of the filters to be incubated with different anti-sera, or to minimise the volume of the first anti-serum required for the incubation. Filters were subsequently stuck together for photographing, but the cut-lines remain visible as fine lines in the photographs. The first antibody was poured off and the nitrocellulose filter washed twice with 50ml blocking buffer (10min each wash) and once with water. The nitrocellulose filter was incubated for a further 5-24h with the second antibody. After washing as before, the nitrocellulose filter was put in the appropriate reaction solution. The reactions were stopped by washing the filters with H_2O.

The first antibody was reused for many blots until the reaction became significantly weaker. The second antibody was not reused.

Chapter 3

RESULTS

A

Studies with antibodies

It was decided to use an immunochemical approach to determine the relationship between the putative protein kinase gene, *pk*, and PRV-PK, and to investigate the distribution of the product of this gene. The first step was to raise anti-sera against the protein products of the US3 gene of HSV-1 and the *pk* gene of PRV. In order to do this, fragments of both of these genes were expressed as fusion-proteins in *E. coli* and the resulting fusion-proteins were injected into rabbits to raise anti-sera which could cross-react with the products of these viral genes.

A. 1 Construction of recombinants for the expression of fusion-proteins

Constructs were made in the expression vector, pUEX-1 (fig 3. 1a). This is one of a series of vectors, (pUEX-1, 2 and 3 : Materials 6) which allows a fragment of DNA to be expressed in *E. coli* as a fusion-protein with β-galactosidase (Bressan & Stanley, 1987). The fragment to be expressed is cloned into one of five unique restriction sites located at the 3' end of the coding region of the *lac z* gene. The resulting protein thus consists of 110kDa of β-galactosidase, to the C-terminus of which, the foreign protein is fused. The three vectors of this series differ in the precise positions of the unique

restriction sites, so that by choosing the correct vector, the fragment of DNA can be expressed in the correct reading frame, if this is known.

The recombinant, pUEX-P, was constructed in order to express part of the *pk* gene of PRV as a fusion-protein with β-galactosidase. From knowledge of the nucleotide sequence and predicted reading frame of the *pk* gene, it was decided to clone a 0.8kb *Pst* I fragment of this gene, cut from the plasmid, pPRVB 10 (Materials 6 : fig 3.1b), into the *Pst* I site of pUEX-1. This fragment includes most of the coding region of the *pk* gene (amino acids 49-334), and also the translation termination codon.

The orientations of the resulting clones were distinguished by restriction digestion with *Bam* HI. From the restriction maps (fig 3.1b) it can be seen that the desired orientation should yield fragments of 0.8kb and 6.7kb, and the other orientation should produce fragments of 0.03kb and 7.5kb. Figure 3.2a lane 1 shows that the clone selected yielded fragments of 0.8kb and 6.7kb, indicating that it had the correct orientation. This clone was designated pUEX-P.

The recombinant, pUEX-S, was constructed similarly in order to express part of the US3 gene of HSV-1. From knowledge of the nucleotide sequence and predicted reading frame of the HSV-1 US3 gene, it was decided to clone a 0.8kb *Sal* I fragment, cut from the plasmid pRB 425 (Materials 6 : fig 3.1c), into the *Sal* I site of pUEX-1. This should encode a fusion-protein of 140kDa which includes most of the kinase domain of the US3 gene product (amino acids 97-363). Since this fragment does not include a translation termination codon, termination of translation of the fusion-protein should occur at one

of the termination codons present at the C-terminus of *lac z* in the vector.

The 0.8kb *Sal* I fragment was cloned into pUEX-1 and two products, which proved to be the two different orientations, were obtained. Restriction digestion with *Bam* HI was used to distinguish between the two orientations. The restriction map (fig 3.1c) indicates that the desired orientation should yield fragments of 0.8kb and 6.7kb, while the other orientation should produce fragments of 0.03kb and 7.5kb. Figure 3.2b lane 2 shows the clone which yielded fragments of 0.8kb and 6.7kb, indicating that it has the desired orientation. This clone was designated pUEX-S.

A.2 Expression and partial purification of fusion-proteins

The pUEX vectors express the fusion-protein from the *cro* promoter of the bacterial phage lambda. This promoter is repressed by the *λcI* repressor. The vector contains the gene encoding a temperature-sensitive mutant of this repressor, *cI 857*, which is still able to repress expression at the permissive temperature, 30°C, but not at the non-permissive temperature, 42°C.

Transformed *E.coli* containing the recombinant constructs were grown as described (Methods 10.1). A time course of expression of the fusion-protein showed that the maximum amount was produced in 2h, and longer incubation periods did not increase the amount of fusion-protein produced (fig 3.3).

The fusion-protein was produced as an insoluble aggregate. In order to solubilise this material for analysis by polyacrylamide gel

electrophoresis, it was found necessary to boil the material in 5% SDS, 2% 2-mercaptoethanol for 5-10 minutes. Use was made of the insolubility of the fusion-protein in the first step of a purification, by lysing the cells to release the soluble cytosolic proteins before solubilisation of the fusion-protein (Methods 10.2). The extract produced by the solubilisation of the aggregated material remaining after lysis of the cells was found to contain fewer proteins than the total extract (fig 3.4).

It is of interest to note that the fusion-protein expressed from pUEX-P consisted of one major species, whereas pUEX-S produced a fusion-protein with four major species with molecular weights of 160000, 140000, 125000 and 120000 as estimated from polyacrylamide gel electrophoresis (fig 3.6). This was probably because termination of translation was more efficient in pUEX-P where it would be expected to have occurred at the termination codon of the *pk* gene. In pUEX-S, termination of translation at the artificial termination codon in the vector was probably less efficient, giving rise to multiple species.

Analysis of *E.coli* extracts by polyacrylamide gel electrophoresis showed that the fusion-protein was the major high molecular weight protein present in the extract. This suggested that partial purification could be achieved using gel filtration. Extracts were therefore subjected to chromatography on Sephacryl S300 (Methods 10.3). The eluted protein was detected by measuring the A_{280}. Aliquots from the column fractions were subjected to polyacrylamide gel electrophoresis. The fusion-protein eluted in the first fractions which contained protein (fig 3.5 & 3.6). The material from the first one or two fractions of four such columns was pooled (fig 3.7a) and

used for injection into a rabbit (Methods 10.4). Similar material was used for subsequent injections.

A.3 Initial characterisation of anti-sera

The initial bleeds from each rabbit were tested in immunoblots (Methods 12) against extracts containing the partially purified fusion-proteins, in order to determine if antibodies had been raised against the material which had been injected into the rabbits. Figure 3.7b shows immunoblots of anti-serum P (raised against the PRV fusion-protein) and anti-serum S (raised against the HSV fusion-protein) against pooled fractions from both Sephacryl S300 columns which contained the fusion-proteins (fig 3.7a lanes 2 & 3). Pre-immune serum from both rabbits was also tested against the fusion-proteins. No cross-reaction could be detected in the immunoblots with either pre-immune serum. However, anti-sera P and S were both found to react with a number of proteins in the partially purified fractions containing the fusion-proteins. These included several proteins with a high molecular weight (100000 to 160000) which could correspond to the fusion-proteins. The cross-reaction of anti-serum P with the HSV fusion-protein is probably due to antibodies against the β-galactosidase portion of the protein. This would also account for the cross-reaction of anti-serum S with the PRV fusion-protein. The anti-sera also reacted with other bacterial proteins present in the extracts containing the partially purified fusion-proteins.

Both anti-sera were then tested in immunoblots against post-ribosomal extracts (Methods 8.2a) from uninfected BHK cells, BHK cells

infected with HSV-1 and BHK cells infected with PRV. Figure 3.8a shows an immunoblot of anti-serum P against post-ribosomal supernatants from BHK cells infected with PRV (lane 2) and uninfected BHK cells (lane 4). Although, in each extract, the anti-serum reacted with several proteins (a major cross-reacting species having a molecular weight of 34000 and a minor species with a molecular weight of 52000), there were two major bands, corresponding to molecular weights of 38000 and 42000, which were specific to the extract from infected cells, although in many preparations, of these two bands, only the 38000 molecular weight band was seen (eg. fig 3.11b lanes S). This suggested that at least one of these proteins corresponds to the viral product of the *pk* gene of PRV. There were also a number of significantly fainter bands, detected only in the extract from infected cells, corresponding to lower molecular weight proteins. It was unclear if these were viral proteins or virally-induced cellular proteins, but they appeared to be either less abundant or the anti-serum exhibited a lower affinity for them, which suggested that they did not correspond to products of the *pk* gene.

Similarly, anti-serum S, although reacting with a number of proteins, recognised two proteins of 68kDa and 69kDa which were only present in extracts from cells infected with HSV-1. The reaction was rather weak in post-ribosomal supernatants from infected cells (result not shown), but was stronger in fractions from the first stage of purification of HSV-PK (DEAE-cellulose column fractions : fig 3.9 lane 2). Previous work (Frame *et al.*, 1987) has identified HSV-PK as a 68kDa protein. However, it seems likely that both the 68kDa and 69kDa proteins detected in the immunoblot with anti-serum S

correspond to the protein kinase which is the product of the US3 gene of HSV-1.

In an immunoblot in which the same amount of protein (as in fig 3.9 lane 2) from the DEAE-cellulose stage of purification of HSV-PK was transferred to nitrocellulose and incubated with anti-serum P, no reaction was observed (fig 3.8b lane 2). Similarly, when the same amount of a post-ribosomal extract from cells infected with PRV as in figure 3.8a lane 2, was transferred to nitrocellulose and incubated with anti-serum S, only a single band of approximately 34kDa (fig 3.9 lane 4), which was also present in an extract from uninfected cells (fig 3.9 lane 3) was detected. This suggests that the protein products of these two genes encoding protein kinases are not sufficiently closely related for either anti-serum to recognise both proteins.

B

The purification of PRV-PK and the determination of the relationship between this enzyme and the product of the *pk* gene of PRV.

In order to determine the relationship between the product of the *pk* gene of PRV, recognised by anti-serum P, and PRV-PK, the protein kinase which is induced in cells infected with PRV (Introduction C), the latter was purified to homogeneity, and immunoblotting was carried out using anti-serum P on fractions from all stages of the purification. PRV-PK was purified to homogeneity according to the

method of Purves *et al.* (1987a) (Methods 8.2). This involved preparing a post-ribosomal supernatant from infected cells and subjecting this to chromatography on four columns successively: DEAE-cellulose, TSK phenyl-5PW, threonine-Sepharose and protamine-agarose. Figure 3.10 shows the analysis of aliquots from each step of the purification by polyacrylamide gel electrophoresis. The silver-stained gel of the peak fractions from the protamine-agarose column shows that the protein has been purified to homogeneity.

Protein was eluted from the DEAE-cellulose column by applying a gradient of 0 - 0.4M KCl (Methods 8.2bii). Assaying the fractions with protamine, revealed two major peaks of protamine kinase activity (fig 3.11a). From previous work (Katan *et al.*, 1985) the first peak of activity was identified as protein kinase C from the concentration of KCl at which it eluted (0.05M). Similarly, the second peak of activity was identified as PRV-PK from its elution at 0.2M KCl. In immunoblots (fig 3.11b), using anti-serum P, fractions 40-44, which corresponded to the peak of the PRV-PK activity, were found to give a single positive reaction against a protein with the same molecular weight (38000) as the major protein recognised by this anti-serum uniquely in extracts from cells infected with PRV (fig 3.8a lane 2). The intensity of the reaction corresponded to the amount of activity detected in the fractions. Fractions 20-24 also gave a positive reaction in immunoblots with this anti-serum (fig 3.11b). The molecular weight of the protein detected in these fractions was 34000, corresponding to that of the major cellular protein against which anti-serum P cross-reacted in extracts from uninfected cells (fig 3.8a lane 4). The different chromatographic properties of the

34kDa and 38kDa proteins show that they are clearly distinct.

The fractions corresponding to the PRV-PK peak from six DEAE-cellulose columns were concentrated and loaded onto a TSK phenyl-5PW HPLC column, washed and the protein kinase activity eluted with a salt gradient of 1.0 - 0.0 M ammonium sulphate (Methods 8.2c). Four peaks of protamine kinase activity were detected (fig 3.12 : corresponding to fractions 36-46, 48-54, 60-66 & 68-74), in contrast to the single peak eluting at 0M ammonium sulphate obtained in previous purifications (Purves *et al.*, 1987a).

Fractions 37-44, 49-54 and 62-72 corresponding to all four apparent peaks of protein kinase activity were pooled and loaded onto the threonine-Sepharose column, although immunoblots with anti-serum P subsequently detected only the 38kDa protein in fractions 42-56 (fig 3.12). Protein kinase activity was eluted with a salt gradient of 0 - 1.0M KCl (Methods 8.2d) as a single peak (fig 3.13) which did not coincide with the elution of most of the protein from the column. Immunoblotting of fractions from the major peaks of protein and protein kinase activity eluted from the column, showed that anti-serum P reacted with a protein of 38kDa only in the fractions corresponding to the peak of protein kinase activity (fig 3.13). The intensity of the reaction on the immunoblot was related to the amount of protein kinase activity present in the fractions.

Fractions 55-62 from the threonine-Sepharose column were pooled and loaded onto a protamine-agarose column. After washing, the protein kinase activity was eluted with a salt gradient of 0 - 1.0M KCl (Methods 8.2e) as two overlapping peaks, with the peak eluting at the higher salt concentration, corresponding to the

position of elution observed in previous work (Purves *et al.*, 1987b: fig 3.14). Analysis of the protein in the peak fractions by polyacrylamide gel electrophoresis and visualisation by silver staining showed that both peaks contained a single protein of 38kDa, corresponding to PRV-PK (fig 3.10 lane 6a & 6b). In immunoblots, anti-serum P gave a positive reaction against a protein of 38kDa in fractions from both peaks (fractions 30, 38 & 40 : fig 3.14), but the reaction was much more intense for the fractions corresponding to the second peak. It is unclear why the final preparation of PRV-PK in this purification should have eluted as two peaks, and this question is considered further in the Discussion.

From the foregoing, it can be concluded that anti-serum P specifically recognises the 38kDa protein kinase induced by infection with PRV (PRV-PK). Hence PRV-PK is encoded by the *pk* gene of PRV which corresponds to the US3 gene of HSV-1.

C

Location of PRV-PK and HSV-PK within virions

Although previous work (Katan *et al.*, 1985) had indicated that PRV-PK is present in the soluble fraction from infected cells, it was intended to use anti-serum P in immunoflorescence studies to determine the location of PRV-PK more precisely in intact cells. However, since the anti-serum was found to cross-react with cellular proteins (Results A.3), it was first necessary to affinity-purify the anti-serum against PRV-PK (Methods 11.3). Material from the

TSK phenyl-5PW stage of the purification of the protein kinase was transferred to a nitrocellulose filter (Methods 12). Anti-serum P had been shown (fig 3.12) to react with a single protein of 38kDa in this material. After incubating the nitrocellulose filter with anti-serum P, the position of the 38kDa protein was located and a strip corresponding to this part of the nitrocellulose filter was cut out. Antibodies were eluted from this (Methods 11.3) and used in an immunoblot against post-ribosomal extracts and material from the DEAE-cellulose stage of the purification of the protein kinase. These antibodies were found to react with the 38kDa protein, but also with the 34kDa cellular protein (fig 3.15a). This suggests that these two proteins are antigenically related and are reacting with the same antibodies in the anti-serum. This was confirmed in a similar manner by affinity-purifying antibodies against the 34kDa protein. The material transferred to a nitrocellulose filter in this case was from fractions of the DEAE-cellulose column where the 34kDa protein was found to elute (fig 3.11b) and which did not contain the 38kDa protein. Antibodies eluted from the 34kDa band on the immunoblot (fig 3.15b lane 2) also reacted with the 38kDa protein (fig 3.15b lane 1). Thus, anti-serum P, even after affinity-purification, cross-reacted with a cellular protein and hence was unsuitable for immunoflorescence studies.

Nevertheless, this anti-serum was suitable for investigating the location of PRV-PK within PRV virions, using immunoblotting. Mature PRV virions were isolated from the cellular growth medium after infection for 20-24h (Methods 6.2 & 7.1). Figure 3.16a lane 1a shows the analysis of the resulting material by polyacrylamide gel

electrophoresis. The detection of proteins of molecular weight greater than 200kDa indicated the presence of tegument proteins and suggested that the virions were mature. The immunoblot using anti-serum P (fig 3.16a lane 5) against this material gave a single positive reaction against a protein of 38kDa. This indicated that PRV-PK was present in mature PRV virions.

Having shown that PRV-PK is present within PRV virions, studies were carried out to investigate the location of the enzyme within the virions more precisely. Nucleocapsids (ie. immature virions consisting of only the inner two layers of the virion) were isolated (Methods 7.1) from the nuclei of infected cells, where they accumulate before acquiring the envelope by budding through the nuclear membrane. In immunoblots with anti-serum P (fig 3.16a lane 4), this material also gave a single positive reaction against a protein of 38kDa, suggesting that PRV-PK is tightly associated with the nucleocapsid, although the possibility remains that the nucleocapsid preparation may have been contaminated by PRV-PK present in the cytosol of the infected cells.

Similar experiments were carried out to investigate the location of HSV-PK within HSV-1 virions. Mature virions were isolated from cellular growth medium (as for PRV, Methods 7.1), after infection for 20-24h with HSV-1 (as for PRV, Methods 6.2). Nucleocapsids were also isolated from the nuclei of these cells (as for PRV, Methods 7.1). Immunoblots against both nucleocapsids and mature virions using anti-serum S yielded two bands of 68kDa and 69kDa, similar to those in immunoblots against fractions from the DEAE-cellulose stage of purification of HSV-PK (fig 3.17). Thus this protein kinase is also

present within the virion and is probably tightly associated with the nucleocapsid.

The location of PRV-PK within PRV virions was further investigated by the treatment of virions with either 1% NP-40 (Methods 7.2), which was used by Lemaster *et al.* (1980) to solubilise virion envelope proteins, or 10% NP-40, 6M NaCl (Methods 7.2) which was used by Stevely *et al.* (1985) to solubilise protein kinase activity from PRV virions. In an experiment using both methods, 35% of the protamine kinase activity within PRV virions was solubilised. Immunoblots were performed using equivalent amounts (ie. derived from the same number of virions) of the solubilised material and the material which remained insoluble from both methods of extraction (fig 3.16b). The difference in intensity of the reaction with anti-serum P for the solubilised and insoluble material showed that PRV-PK is not efficiently solubilised under either of these conditions, suggesting that the protein kinase solubilised by these treatments was protein kinase C. The fact that neither of these methods efficiently solubilised PRV-PK supports the hypothesis that PRV-PK is tightly associated with the nucleocapsid of the virion.

D

The phosphorylation of viral structural proteins of PRV *in vitro* and *in vivo*

Having established that PRV-PK is present within PRV virions, experiments were performed to see if PRV-PK might have a role in the phosphorylation of virion proteins during infection.

First, the virion proteins phosphorylated *in vivo* were examined by incubating the cells with [^{32}P]orthophosphate during infection with PRV. Extracts were prepared from cells 15h after infection in the presence of [^{32}P]orthophosphate from 12-15h (Methods 9.2a). An extract was also prepared from mock-infected cells which had similarly been incubated with [^{32}P]orthophosphate for 3h. Figure 3.18a shows an autoradiograph of these extracts. Extracts from mock-infected cells (fig 3.18a lanes 4 & 5) contained many more phosphoproteins, suggesting that viral infection inhibits the phosphorylations of cellular proteins. However, the extracts from infected cells contained five phosphoproteins of 210kDa, 112kDa, 43kDa and two of approximately 52kDa, which appeared to be induced by infection (fig 3.18a lane 2 & 3).

In order to investigate whether these virally-induced phosphoproteins were viral structural proteins, a crude preparation of virions was prepared from the growth medium of cells incubated with [^{32}P]orthophosphate from 4-24h after infection (Methods 9.2b). A longer labelling period was used in this experiment in order to allow the virion proteins labelled in the cytosol to be incorporated into

virions and released into the medium. As a control, a similar preparation was made from mock-infected cells. Figure 3.18b lane 1 & 2 shows that four major phosphoproteins of 112kDa, 32kDa and two of approximately 52kDa were detected in this virion preparation. A phosphoprotein of approximately 52kDa was also detected in the preparation from uninfected cells (fig 3.18c lane 2), indicating that one of the phosphoproteins of this molecular weight may be a cellular protein. However the detection of phosphoproteins of 112kDa and 52kDa in this preparation suggests that at least these two of the five phosphoproteins detected in infected cells are viral structural proteins.

The possible role of PRV-PK in the phosphorylation of these viral structural proteins was then investigated by studying the ability of this enzyme to phosphorylate virion proteins *in vitro*. Virions in which the endogenous protein kinase activity had been inactivated by heat, were incubated with purified PRV-PK in the presence of $\gamma[^{32}P]ATP$ (Methods 9.1b). Under these conditions, four major phosphoproteins were observed with molecular weights of 112000, 56000, 38000 and 32000 (fig 3.19 lane 6). The 38kDa phosphoprotein was identified (from fig 3.19 lane 5, which corresponds to the incubation of purified PRV-PK with $\gamma[^{32}P]ATP$ in the absence of virions) as being PRV-PK, having undergone autophosphorylation. However, the identity of the 32kDa and 56kDa proteins is unknown, although it is possible that the 32kDa protein corresponds to the phosphoprotein of this size detected in virions which were labelled *in vivo* (fig 3.18b lane 1). The 112kDa protein, which was also phosphorylated *in vivo*, appears to be the major substrate for PRV-PK *in vitro*. This is one of the major

proteins of PRV virions (fig 3.16a lane 1a) and has been shown here (fig 3.19 lane 2) and in previous work (Stevely *et al.*, 1985) to be phosphorylated by the cellular protein kinase, casein kinase II. It was also found to be a substrate for cAMP-dependent protein kinase (fig 3.19 lane 4). However, studies on the substrate specificities of these protein kinases using peptides as substrates (Purves *et al.*, 1986a; Marin *et al.*, 1986) suggest that it is unlikely that they all phosphorylate the same site(s) on the 112kDa protein.

It was also of interest to examine the phosphorylation of virion proteins by the endogenous protein kinases *in situ* by incubating mature virions or nucleocapsids with $\gamma[^{32}P]$ATP (Methods 9.1a). In nucleocapsids, the major phosphoprotein had a molecular weight of 58000 (fig 3.20 lane 5). Since the phosphorylation of this protein was not detected when heat-inactivated nucleocapsids were incubated with exogenous PRV-PK (fig 3.20 lane 3), it is suggested that this protein is phosphorylated by another protein kinase. The other phosphoproteins of 112kDa, 38kDa and 32kDa may correspond to those phosphorylated by PRV-PK in heat-inactivated mature virions (fig 3.19 lane 6), and hence be substrates for PRV-PK. In mature virions, one major phosphoprotein of 112kDa and three other highly phosphorylated proteins of 56kDa, 38kDa and 32kDa were detected along with several minor phosphoproteins (fig 3.19 lane 8a & b). This pattern of phosphorylation appeared to be similar to that obtained by the incubation of exogenous PRV-PK with heat-inactivated virions (fig 3.19 lane 6), suggesting that PRV-PK may be responsible for these phosphorylations. However, previous work (Stevely *et al.*, 1985) showed that the PRV virion contains at least four protein kinase

activities, including casein kinase II, which was also able to phosphorylate the 112kDa protein.

In order to determine whether PRV-PK as well as casein kinase II contributed to the endogenous phosphorylation of this protein, the effect of the protein kinase inhibitors, heparin and polyarginine, were investigated. The efficiency and specificity of inhibition was initially tested on crude preparations of PRV-PK and casein kinase II. PRV-PK was inhibited 12% by 1μg/ml heparin and 85% by 0.5mg/ml polyarginine, whereas casein kinase II was inhibited 82% by 1μg/ml heparin and 45% by 0.5mg/ml polyarginine. However, although heparin is an effective inhibitor of casein kinase II and polyarginine is an effective inhibitor of PRV-PK, when used at these concentrations, neither inhibitor was found to inhibit the phosphorylation of PRV virion proteins by the endogenous protein kinases *in vitro* (fig 3.21a lane 3, fig 3.21b lane 2). This lack of inhibition may result from the inaccessibility of the protein kinases within the virions to the inhibitors. Alternatively it may be one of the other endogenous protein kinases which is responsible for the observed phosphorylations. The effect of 0.5M KCl on endogenous phosphorylation was also investigated. It was found in a preliminary experiment that PRV-PK is not inhibited at 0.5M KCl, although this concentration of KCl inhibited casein kinase II by 91%. When 0.5M KCl was included in the reaction mixture, it was found to significantly inhibit the phosphorylation of all the proteins observed to be phosphorylated by the endogenous protein kinase activities (fig 3.21a lane 2), thus implying that PRV-PK does not play a major role in these reactions. From these results it would appear that, *in*

vitro, the phosphorylation of the 112kDa protein is catalysed by another enzyme, probably casein kinase II, rather than PRV-PK. However, it cannot be excluded that *in vivo*, PRV-PK might contribute to the phosphorylation of this protein , perhaps during assembly.

E

Attempt to express the US3 gene of HSV-1 in eukaryotic cells.

It was intended to express the US3 gene of HSV-1, in a eukaryotic cell line, with the initial aim of investigating possible physiological substrates for this enzyme, in particular, whether there are any cellular substrates. A comparison of the pattern of phosphorylation in cells with and without the enzyme should allow the identification of a possible cellular substrate. A second aim was to investigate whether the expression or over-expression of this protein kinase gene could cause malignant transformation of the cells, as had been observed for protein kinase C (Housey, *et al.*, 1988; Persons *et al.*, 1988) and oncogenic tyrosine kinases, such as *src* (Johnson *et al.*, 1985), or cause any other changes in the phenotype of the cells. In addition, the over-expression of the gene could provide a plentiful supply of active protein kinase for various studies of the enzyme.

The vector Homer 5 (Spandidos & Anderson, 1987) was chosen for this work. This vector is designed to express a eukaryotic gene from its own promoter, but expression is enhanced by the presence of the enhancer sequence from SV40 which is located beside the unique

Hind III site into which the gene is to be cloned (fig 3.23a). The orientation of the gene relative to the SV40 enhancer is thought to be unimportant (Spandidos & Anderson, 1987). The vector also contains the ampicillin resistance gene for selection of transformants during cloning in *E.coli*, and the geneticin resistance gene to allow the selection of eukaryotic transformants.

Since one of the aims of the work was to investigate whether the expression of this gene could cause malignant transformation, the cell line chosen for transformation, the rat fibroblast cell line, 208F, was one known to have a low frequency of spontaneous malignant transformation (Quade, 1979).

E.1 Construction of the plasmids, transformation of recombinants into 208F cells and selection of transformants.

The fragment of the US3 gene chosen was one of 2.8kb, flanked by *Kpn* I and *Sph* I sites (see fig 3.22b). This includes the promoter for the US3 gene and the information for the complete mRNA transcript. Since the transcripts for US3 and US4 are co-terminal, the entire coding region for the US4 gene would also be present in a construct including this fragment. As a control, it was decided to make a construct from a 1.3kb fragment flanked by *Bam* HI and *Sph* I sites, which contained only the region coding for the US4 gene (fig 3.22b). Although the orientation of the gene(s) in Homer 5 was not expected to affect the expression in the transformants, it was decided to clone both fragments into the *Hind* III site of the vector in both orientations.

In order to make these constructs, both fragments were cut from the plasmid pRB 425 (Materials 6) and first cloned into the appropriate sites of the plasmid vector pIC 20H (fig 3.22a & b) (Materials 6). The pIC-K/S construct was confirmed by restriction digestion with *Xho* I which yielded the expected fragments of 0.9kb and 4.6kb. The pIC-B/S construct was confirmed by restriction digestion with *Bgl* I to yield fragments of 1.0kb and 3.4kb. Having obtained these clones, pIC-K/S and pIC-B/S were digested with *Hind* III and the relevant fragment in each case was isolated from a low melting point agarose gel, and cloned into the *Hind* III site of Homer 5 (fig 3.23b & c). These constructs were confirmed and the orientation of their inserts determined by single restriction digests with *Eco*RI and *Bam* HI (fig 3.24). The expected sizes of the restriction fragments were as follows:

recombinants	***Bam* HI digests**	***Hind* III digests**	***Eco*RI digests**
H5-Ka	1.9, 9.5	2.8, 8.7	
H5-Kb	1.7, 9.7	2.8, 8.7	
H5-Ba	0.35, 9.6	1.3, 8.7	0.35, 0.54, 1.9, 6.8
H5-Bb	1.7, 8.3	1.3, 8.7	0.54, 1.7, 1.9, 5.5

Each of the four plasmids H5-Ka, H5-Kb, H5-Ba and H5-Bb (fig 3.23a & b), were transformed into 208F cells by calcium phosphate precipitation (Spandidos & Wilkie, 1984b). After 3 days, 300µg/ml geneticin was added to the medium to allow selection of transformed cells. After a further 3 days, two geneticin-resistant colonies were

picked from each transformation reaction for further characterisation. The resulting cell lines were named as follows:

expression plasmid	cell line
H5-Ka	Ka-1 and Ka-2
H5-Kb	Kb-1 and Kb-2
H5-Ba	Ba-1 and Ba-2
H5-Bb	Bb-1 and Bb-2

E.2 Attempts to detect HSV-PK and characterisation of the phenotype of transformant cell lines.

The activity of HSV-PK can be detected in an assay using protamine as a substrate. However, since protamine is also a substrate for protein kinase C, it is necessary to separate the two kinases in order to detect the activity of the viral kinase. This can be achieved by subjecting the post-ribosomal supernatant to chromatography on DEAE-cellulose (Methods 8.2bi). Protein kinase C elutes at a KCl concentration of approximately 0.05M, whereas the viral kinase elutes at approximately 0.2M KCl (Katan *et al.*, 1985). Extracts were prepared from five confluent roller bottles of the transformants Ka-2 and Kb-2, and the parent cell line, 208F (Methods 8.2a). Comparison of the protein kinase activity profiles from chromatography of each extract showed no appreciable differences (fig 3.25). Thus, if there was expression of the protein kinase it

was not sufficient to be detected by this method.

The phenotype of the transformant cell lines was also investigated. Comparison of the appearance of the transformants with the parent cell line 208F (fig 3.26), suggested that these cell lines were not malignantly transformed.

Cell growth curves were performed for the cell lines 208F, Ka-2 and Kb-2 (fig 3.27) (Methods 5.3). These showed that the density of the cells at confluence did not differ significantly between the parent cell line and Kb-2 but was slightly less for Ka-2. This may indicate that Ka-2 cells are less transformed than the parent cell lines.

After nine months in culture, growth curves were performed on the cell lines in the presence and absence of 300μg/ml geneticin (Methods 5.3) in order to determine if expression of geneticin resistance, which had been used to select the transformants, had been retained even though the cells had since been grown under conditions which did not require the expression of this gene. Comparison of the growth curves for Ka-2 and Kb-2 in the presence and absence of 300μg/ml geneticin with those for 208F showed that, although the growth of Ka-2 and Kb-2 was slower in the presence of geneticin, the sensitivity to this antibiotic, was much less than that of the parent cell line which was completely inhibited at this concentration (fig 3.28). Thus Ka-2 and Kb-2 have maintained the expression of the geneticin resistance gene.

The results of this work were inconclusive since over-expression of the viral kinase was not achieved. It has been found in other studies with stable transformants that there can be a large variation (eg. 50-fold) in the extent of expression of foreign genes for which there is no direct selection (Housey *et al.*, 1988). Low levels of expression of the foreign gene may result from deletions occurring within the gene during the process of integration, but this variation may also be due to differences in the number of copies of the gene which are incorporated in the cellular genome, the location at which integration occurs, and the degree of methylation of the gene. Any of these factors may explain the lack of detectable viral protein kinase activity in the four clones selected for resistance to geneticin but which did not appear to express the viral protein kinase.

The chance of obtaining clones over-expressing the viral protein kinase could have been increased by analysing more clones. For practical reasons, this would have required a more efficient method for screening the clones. At the time of this work, detection of the viral protein kinase was dependent on making an extract from the cells, subjecting this to chromatography on an analytical scale, and detecting the protein kinase activity in certain fractions from the column. This necessarily required the cells to be grown on a relatively large scale. However, as a result of other aspects of the work described in this thesis, there is now another approach which might allow screening to be carried out on fewer cells. Anti-serum S (raised against the HSV-1 protein kinase fusion-protein) might be suitable for use in immunoprecipitations or immunoblots to detect HSV-PK in extracts prepared from the transformants. Although

immunoblots were performed on post-ribosomal supernatants, and various fractions from analytical DEAE-cellulose chromatography of extracts (Methods 8.2bi) from the transformants Ka-2 and Kb-2, no positive signal was detected. Since immunoblots using this anti-serum were able to detect HSV-PK in post-ribosomal supernatants and the relevant fractions from analytical DEAE-cellulose chromatography of extracts from cells infected with HSV-1, it would suggest that this approach would allow the detection of HSV-PK, in cells in which it is expressed.

The extent of expression of a fragment of foreign DNA is partly determined by the strength of the promoter from which it is expressed and also possible enhancer elements. In the constructs used in this work, the US3 gene was to be expressed from its own promoter, enhanced by the SV40 enhancer. This enhancer can significantly increase the level of expression of a gene, as is indicated by the work of Spandidos and Wilkie (1984a). Transformants expressing oncogenic Ha-*ras*-1 from its own promoter alone were not morphologically transformed, whereas 25% of the colonies were morphologically transformed when this gene was expressed in Homer 5 in the presence of the SV40 enhancer. However, a further 4-fold enhancement was achieved by expressing the gene in Homer 6 in which the gene is flanked by both the SV40 enhancer and the Moloney murine sarcoma virus long terminal repeat. Such an approach might also increase the expression of the US3 gene. There is also the possibility that over-expression of the viral protein kinase may inhibit cell growth, in which case, expression of the gene from an inducible promoter might be more successful (eg. the mouse metallothionein promoter which can be

induced by zinc; Spandidos & Wilkie, 1984b).

Another approach to increase the yield of high-expression cell lines is to cotransfect with two marker genes and select for cells expressing both of them. Wirth *et al.* (1988) have shown that when cells were cotransfected with the genes conferring resistance to geneticin and puromycin along with the gene for interferon β, and selected for resistance to both antibiotics, the resulting cells secreted 20 times as much interferon β as cells selected for resistance to geneticin alone. Clones expressing a large amount of the foreign protein did not necessarily have a high copy number of the transfected DNA, suggesting that other factors such as the site of integration and the extent of methylation are more important in determining the level of expression.

Thus, there are several approaches which could be used in the future to improve the chances of obtaining efficient expression of the US3 gene from transformed eukaryotic cells.

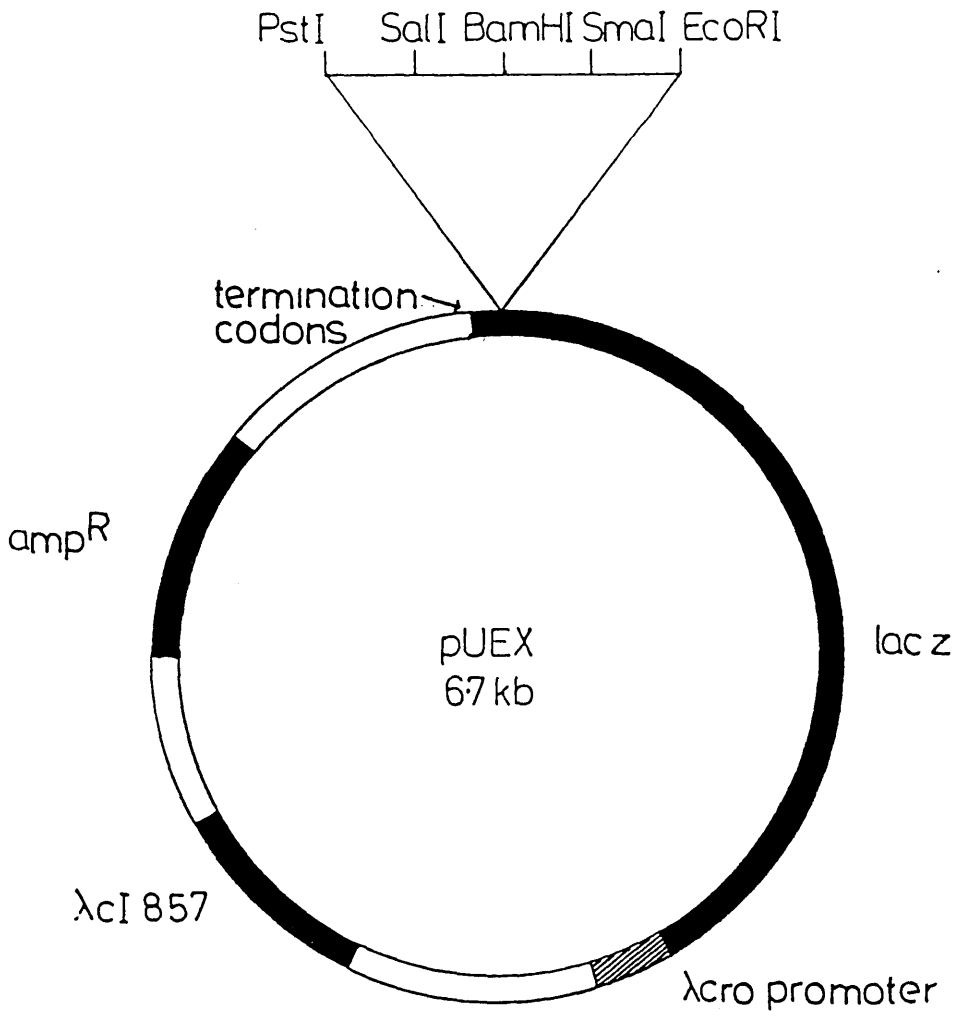


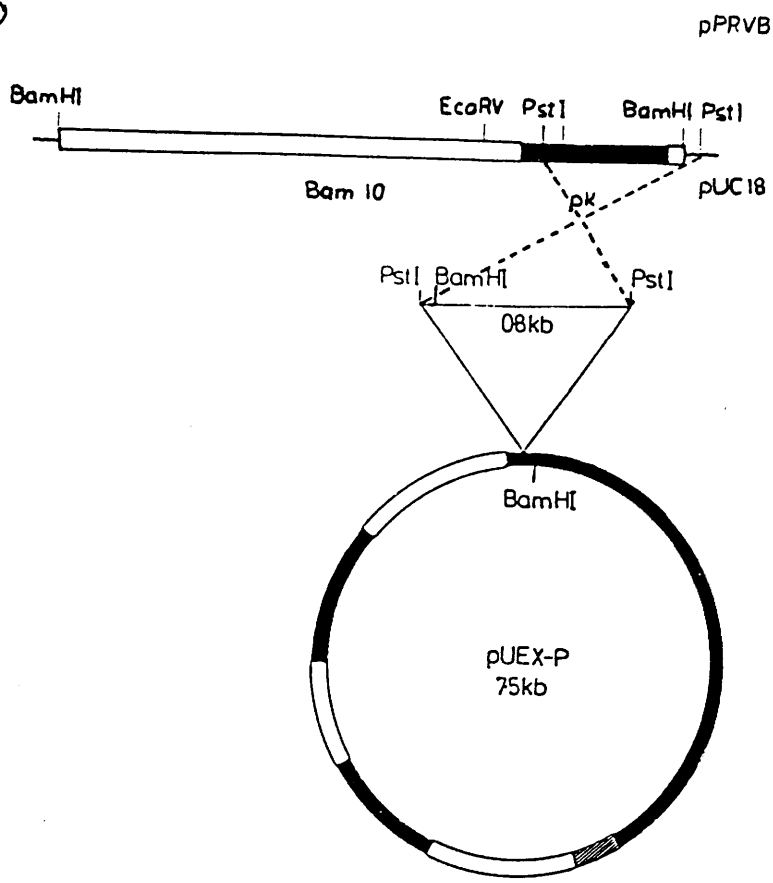
Figure 3.1 Schematic diagrams of the expression vector pUEX and the recombinants pUEX-P and pUEX-S for expression of protein kinase fusion-proteins.

a) pUEX contains three genes, *lac z*, *amp*^{R} and the bacterial phage lambda gene, *cI 857*. The *lac z* gene is expressed from the lambda *cro* promoter, which is sensitive to repression by the lambda repressor, *cI*. *cI 857* is a temperature sensitive mutant which is non-functional at 42°C. The DNA to be expressed, is inserted into the multiple cloning site located at the 3' end of the *lac z* gene. Expression of the fusion-protein in *E. coli* is induced by incubation at 42°C.

b) pUEX-P, the construct for expression of the PRV fusion-protein. A 0.8kb *Pst* I fragment from pPRVB 10 (Materials 6), which contains most of the coding region of the *pk* gene, was cloned into the *Pst* I site of pUEX-1.

c) pUEX-S, the construct for expression of the HSV-1 fusion-protein. A 0.8kb *Sal* I fragment from pRB 425 (Materials 6), which encodes most of the protein kinase catalytic domain of the US3 gene, was cloned into the *Sal* I site of pUEX-1.

b


pPRVB 10
BamHI
EcoRV
PstI
BamHI
PstI
Bam 10
pk
pUC18
PstI
BamHI
PstI
0·8kb
BamHI
pUEX-P
7·5kb

C

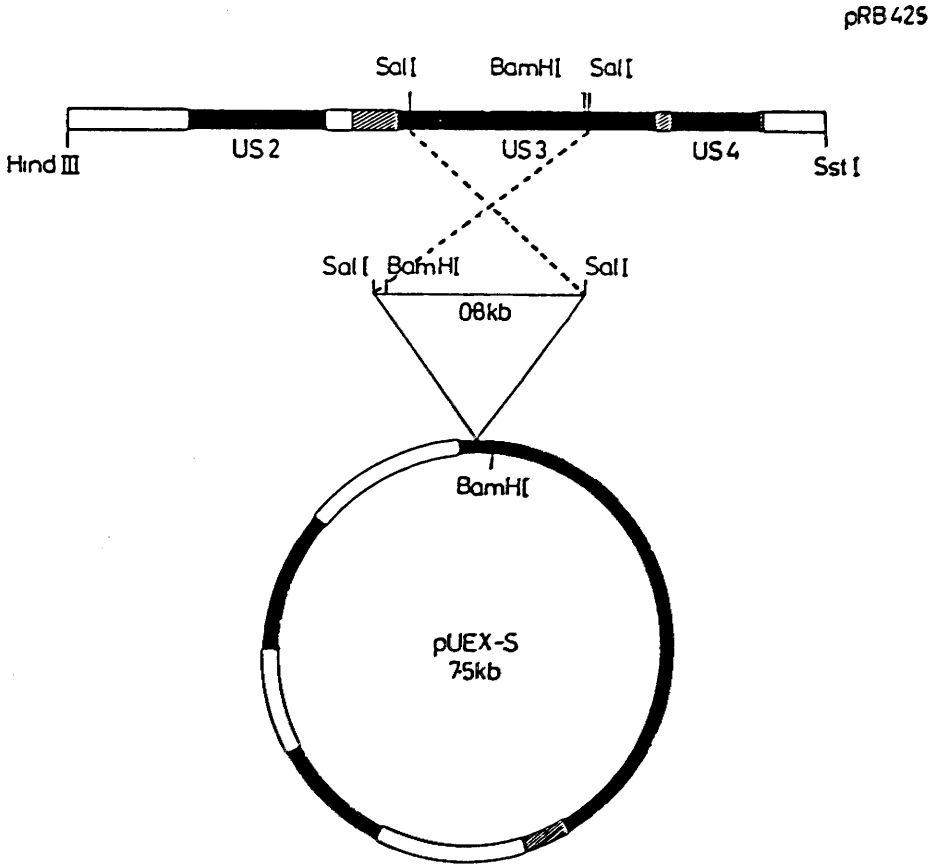

pRB425
SalI
BamHI
SalI
HindIII
US2
US3
US4
SstI
SalI
BamHI
SalI
0·8kb
BamHI
pUEX-S
7·5kb

Figure 3.2 Construction of pUEX-P and pUEX-S: Identification of clones with the insert in the desired orientation by restriction digestion with *Bam* HI.

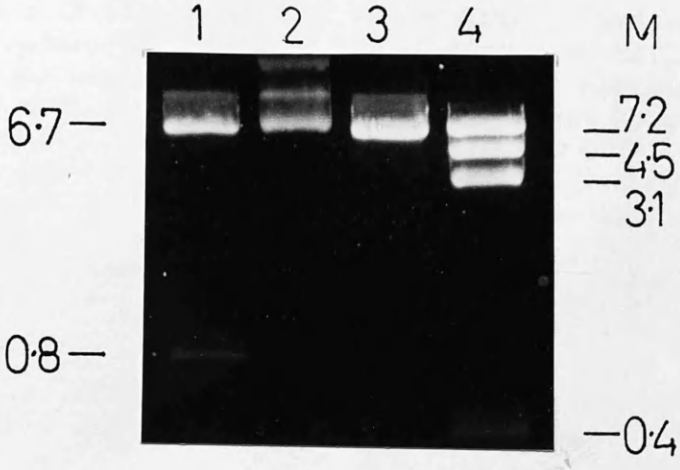
Restriction digestions were carried out (Methods 3.1) and subjected to electrophoresis on a 1% agarose gel (Methods 4.1a).

a) Construction of pUEX-P
   1) Restriction digestion of clone having the desired orientation
   2) Uncut DNA from clone having the correct orientation
   3) Restriction digestion of pUEX-1
   4) DNA size markers : 7.2kb, 4.5kb, 3.1kb, 0.4kb

b) Construction of pUEX-S
   1) Restriction digestion of clone having the undesired orientation
   2) Restriction digestion of clone having the desired orientation
   3) Restriction digestion of pUEX-1
   4) DNA size markers : 7.2kb, 4.5kb, 3.1kb, 0.4kb

Numbers to the right of figures indicate the sizes of DNA markers in kb. Numbers to the left of figures indicate the sizes of DNA fragments in kb.

a



b

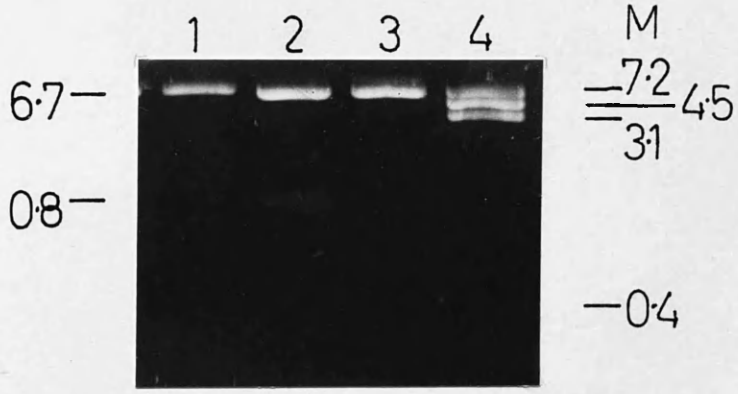


Figure 3.3 Time course of expression of β-galactosidase and the HSV-1 fusion-protein in *E. coli*.

E. coli transformed with pUEX-1 (a & b) or pUEX-S (c & d) (Methods 1.2) were grown for 5h at 30°C. Expression of β-galactosidase (b) and the HSV-1 fusion-protein (d) was then induced by increasing the temperature of incubation to 42°C (Methods 10.1). In a and c, the cells were maintained at 30°C. At 0h, 1h, 2h, 3h and 4h after induction, 1ml aliquots were collected and used to prepare total cell extracts. The cells were sedimented by centrifugation and the precipitates were dissolved in sample loading buffer (Methods 4.2) These were subjected to polyacrylamide gel electrophoresis and the gel was stained for protein with Coomassie Blue (Methods 4.2a).

M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 96kDa, 69kDa, 46kDa (Materials 2).
F indicates the induced fusion-protein.
β indicates induced β-galactosidase.

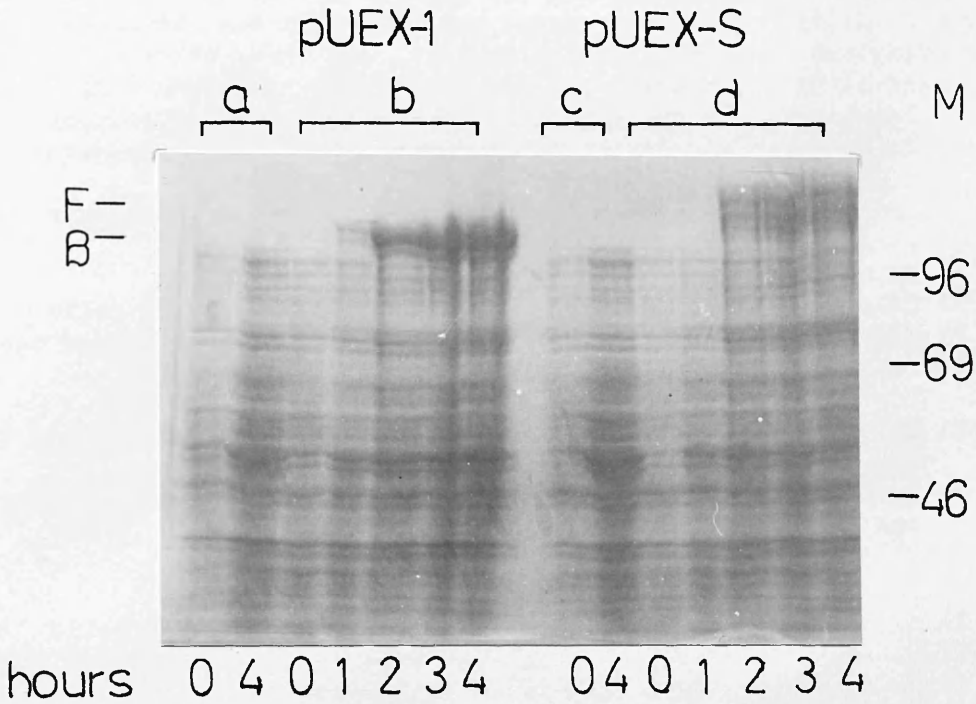

pUEX-1
pUEX-S
a
b
c
d
M
F
B
-96
-69
-46
hours
0 4 0 1 2 3 4
0 4 0 1 2 3 4

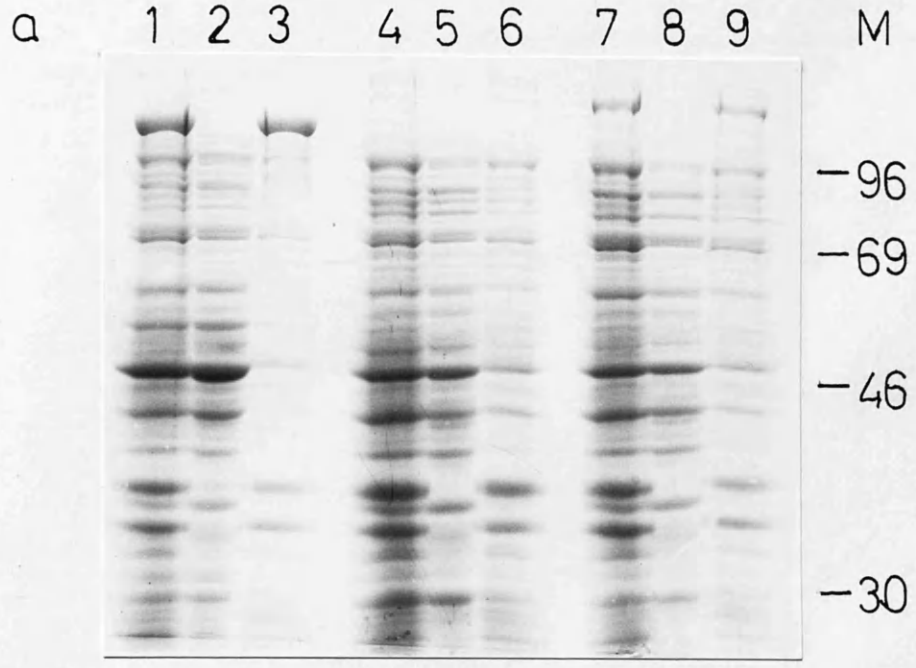
Figure 3.4 Extraction of fusion-proteins from *E. coli*.

Extracts were prepared from *E. coli* either by resuspending the sedimented cells in sample loading buffer (Methods 4.2) to give a total cell extract, or lysing the cells with lysosyme and NP-40 (Methods 10.2) and sedimenting the insoluble material by centrifugation. This supernatant was collected and the precipitate was dissolved in sample loading buffer. All three extracts were analysed by polyacrylamide gel electrophoresis (Methods 4.2a).

a 1) Total cell extract from cells expressing β-galactosidase
2) Extract containing soluble material from cells expressing β-galactosidase
3) Extract containing precipitated material from cells expressing β-galactosidase
4) Total cell extract from uninduced cells
5) Extract containing soluble material from uninduced cells
6) Extract containing precipitated material from uninduced cells
7) Total cell extract from cells expressing the PRV fusion-protein
8) Extract containing soluble material from cells expressing the PRV fusion-protein
9) Extract containing precipitated material from cells expressing the PRV fusion-protein

b 1) Total cell extract from uninduced cells
2) Extract containing soluble material from uninduced cells
3) Extract containing precipitated material from uninduced cells
4) Total cell extract from cells expressing the HSV-1 fusion-protein
5) Extract containing soluble material from cells expressing the HSV-1 fusion-protein
6) Extract containing precipitated material from cells expressing the HSV-1 fusion-protein

M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 96kDa, 69kDa, 46kDa, 30kDa. (Materials 2)


a
1 2 3 4 5 6 7 8 9 M
96
69
46
30

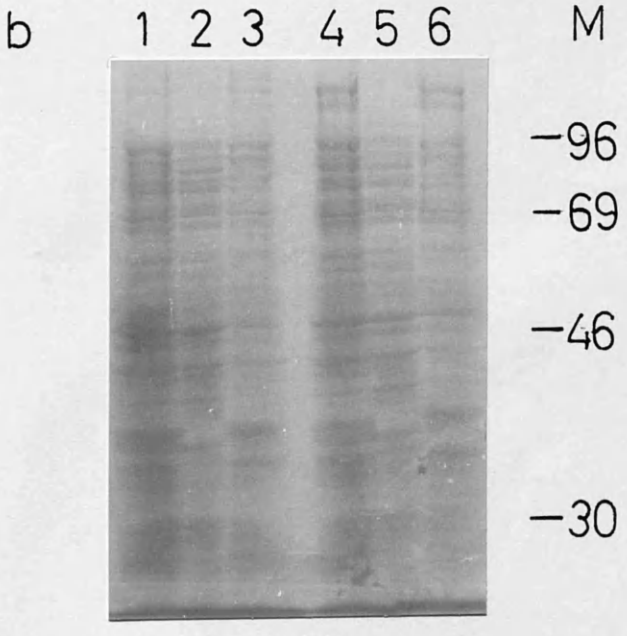
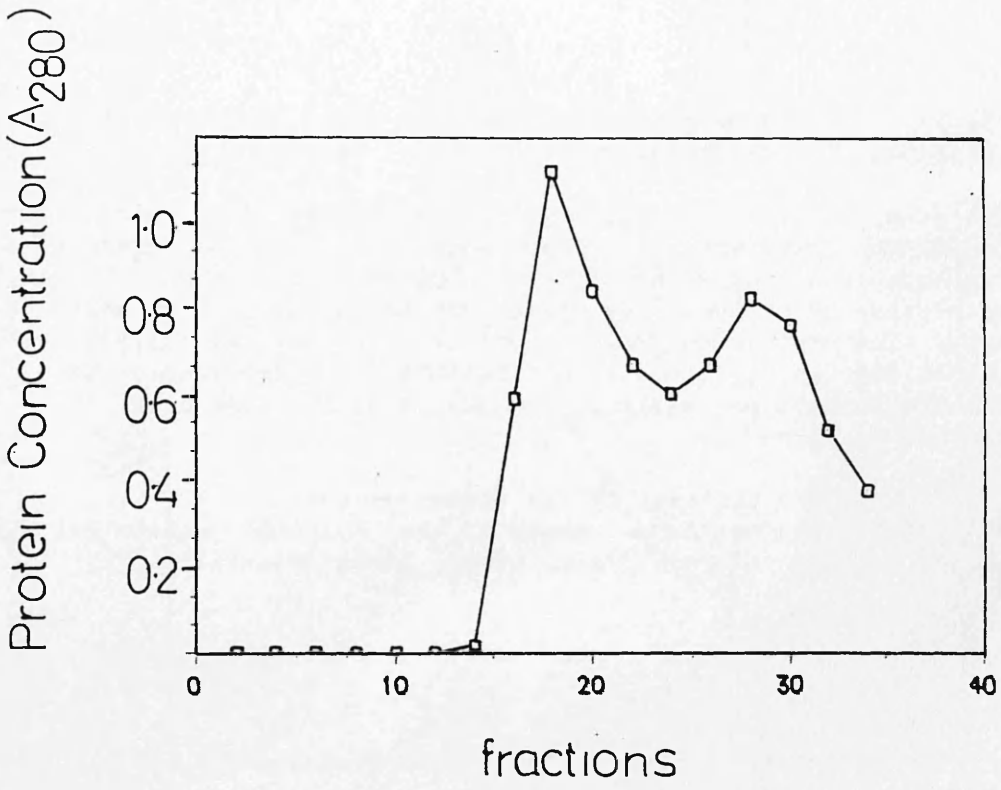

b
1 2 3 4 5 6 M
96
69
46
30

Figure 3.5 Partial purification of the PRV fusion-protein by chromatography on Sephacryl S300.

Extracts from cells expressing the fusion-protein (prepared according to Methods 10.2) were subjected to chromatography on Sephacryl S300 (Methods 10.3) and 40 fractions of 1ml were collected. The position of elution of protein from the column was determined by measuring the A_{280}. The protein composition of the fractions was analysed by subjecting 30µl aliquots from fractions to polyacrylamide gel electrophoresis and staining the gel with Coomassie Blue (Methods 4.2a).

F indicates the position of the fusion-protein.


Protein Concentration (A_{280})
1·0
0·8
0·6
0·4
0·2
0
10
20
30
40
fractions

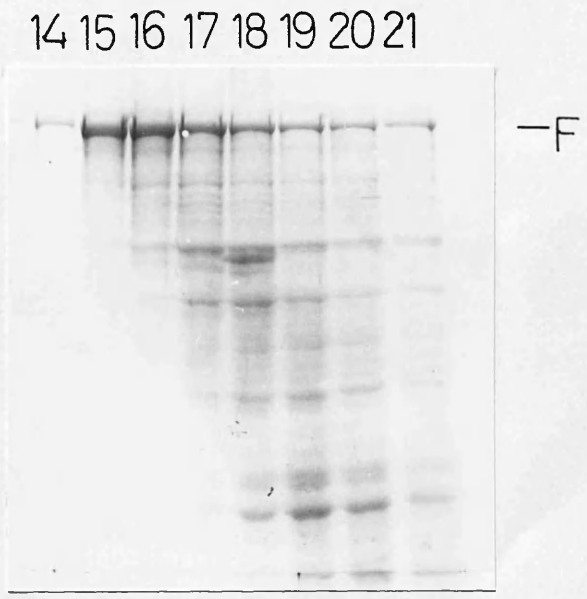

14 15 16 17 18 19 20 21
F

Figure 3.6 Partial purification of the HSV-1 fusion-protein by chromatography on Sephacryl S300.

Extracts from cells expressing the fusion-protein (prepared according to Methods 10.2) were subjected to chromatography on Sephacryl S300 (Methods 10.3) and 40 fractions of 1ml were collected. The position of elution of protein from the column was determined by measuring the A_{280}. The protein composition of the fractions was analysed by subjecting 30µl aliquots from fractions to polyacrylamide gel electrophoresis and staining the gel with Coomassie Blue (Methods 4.2a).

F indicates the position of the fusion-protein.
M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 96kDa, 69kDa, 46kDa (Materials 2)

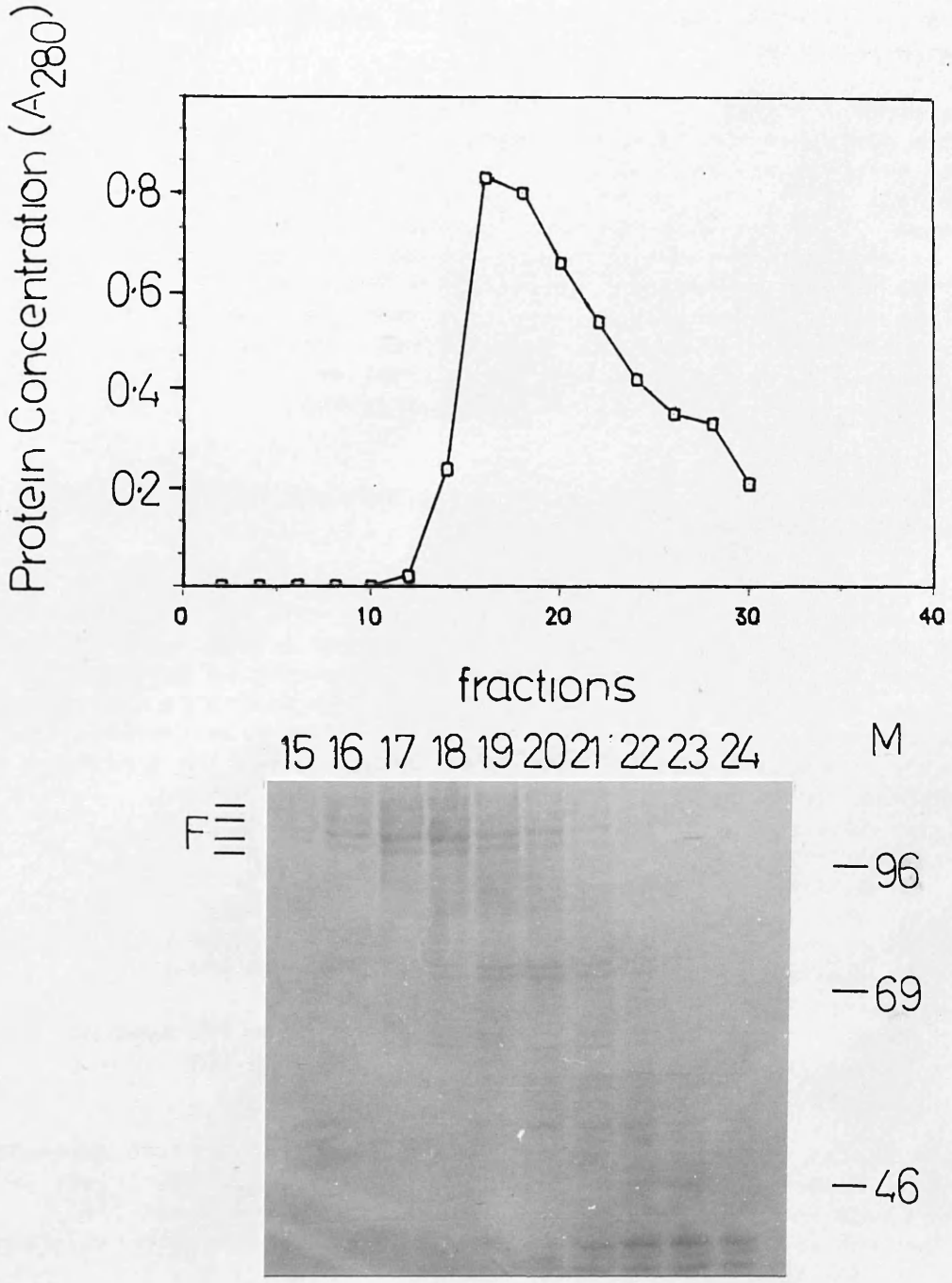

Protein Concentration (A_{280})
0·8
0·6
0·4
0·2
0
10
20
30
40
fractions
15 16 17 18 19 20 21 22 23 24
M
F III
96
69
46

Figure 3.7
a) Polyacrylamide gel electrophoresis of partially purified fusion-proteins.

The first fraction (from the Sephacryl S300 column; Methods 10.3) which contained the fusion-protein, was pooled from four columns. This material was used to inject into rabbits to raise anti-sera (Methods 10.4). The second fractions from the columns were also pooled. This material was used in immunoblots to test the anti-sera.

1) PRV fusion-protein: pooled second fraction
2) PRV fusion-protein: pooled first fraction
3) HSV-1 fusion-protein: pooled second fraction
4) HSV-1 fusion-protein: pooled first fraction
5) β-galactosidase: pooled first fraction
6) β-galactosidase: pooled second fraction

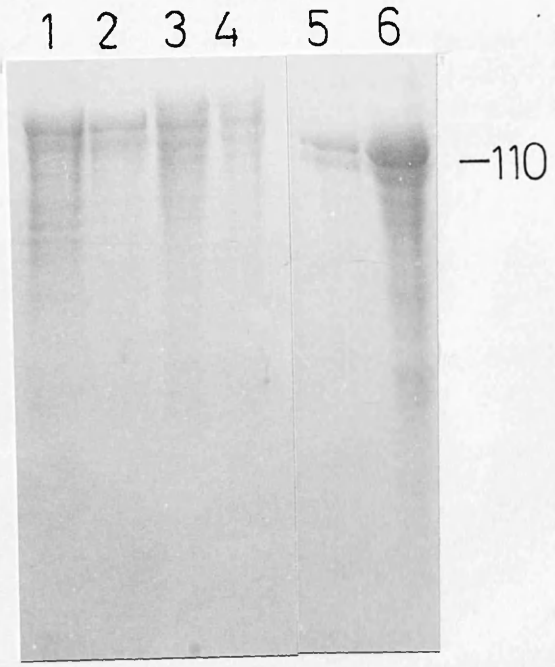
b) Characterisation of anti-sera and pre-immune sera with respect to the fusion-proteins.

Anti-sera and pre-immune sera were prepared according to Methods 11.2. The following dilutions were used for the anti-sera and pre-immune sera: anti-serum P was diluted 1:2000, anti-serum S was diluted 1:400, pre-immune serum corresponding to anti-serum P was diluted 1:400 and the pre-immune serum corresponding to anti-serum S was diluted 1:100. These were used in an immunoblot (Methods 12) against fractions from the S300 columns containing the partially purified fusion proteins (fig 3.7a lane 2 & 3)

1) anti-serum P against the PRV fusion-protein
2) anti-serum P against the HSV-1 fusion-protein
3) anti-serum S against the PRV fusion-protein
4) anti-serum S against the HSV-1 fusion-protein
5) pre-immune serum from rabbit S against the PRV fusion-protein
6) pre-immune serum from rabbit S against the HSV-1 fusion-protein
7) pre-immune serum from rabbit P against the PRV fusion-protein
8) pre-immune serum from rabbit P against the HSV-1 fusion-protein

M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 200kDa, 96kDa, 69kDa, 46kDa (Materials 2). The lines between lanes 2 & 3, 4 & 5, 6 & 7, and between the molecular weight markers, 46 and 69 are cuts in the nitrocellulose filter (Methods 12).

a



b

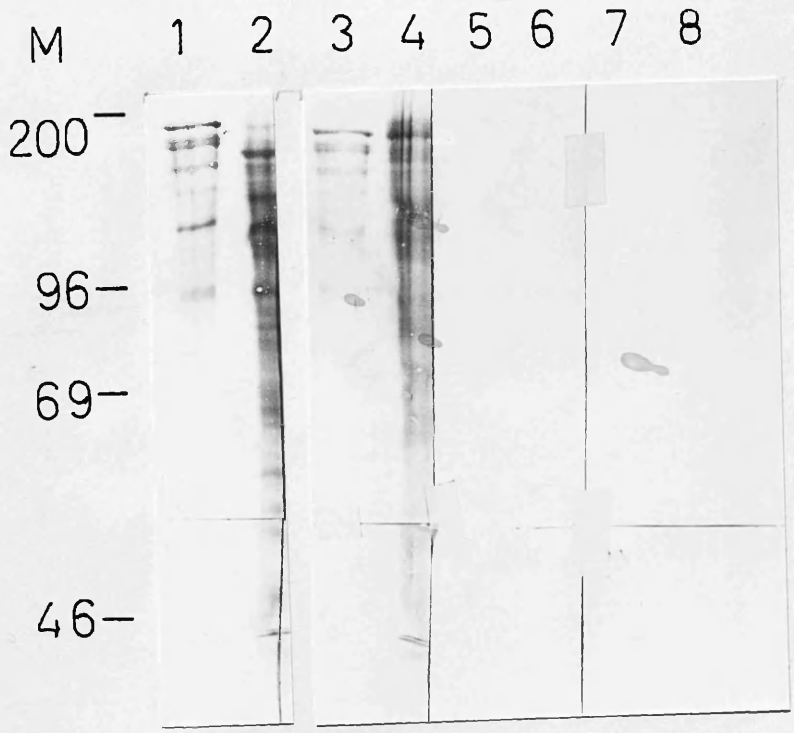


Figure 3.8 Activity of anti-serum P towards extracts from uninfected cells and cells infected with PRV and HSV-1.

Anti-serum P (prepared according to Methods 11.2) was used at a dilution of 1:400 in an immunoblot (Methods 12) against the following protein extracts:

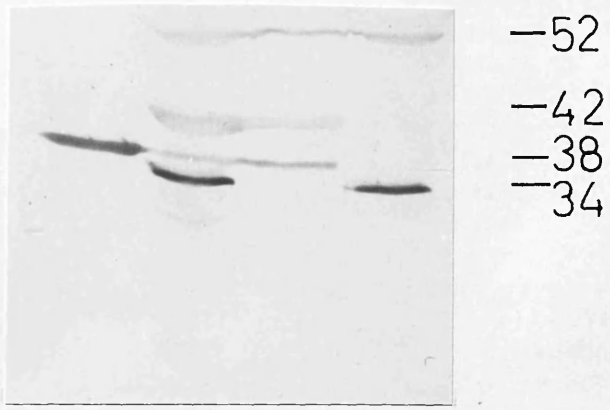
a 1-3) stages from purification of PRV-PK (Methods 8.2):
1) TSK phenyl-5PW
2) post-ribosomal supernatant
3) DEAE-cellulose
4) post-ribosomal supernatant from uninfected cells (Methods 8.2a)

Numbers to the right indicate the molecular masses of the bands in kDa.

b 1) post-ribosomal supernatant from uninfected cells (Methods 8.2a)
2) material from DEAE-cellulose stage of the purification of HSV-1 PK (as for PRV-PK, Methods 8.2b)
3) material from TSK phenyl-5PW stage of the purification of PRV-PK (Methods 8.2c)

Lane 3 also contains 'Rainbow' molecular weight markers (Materials 2). The position of these were marked with a biro before incubation with the first anti-serum and had molecular masses of 69kDa, 46kDa and 30kDa as indicated (numbers to the right). Numbers to the left indicate the molecular masses of the bands in kDa.

a



b

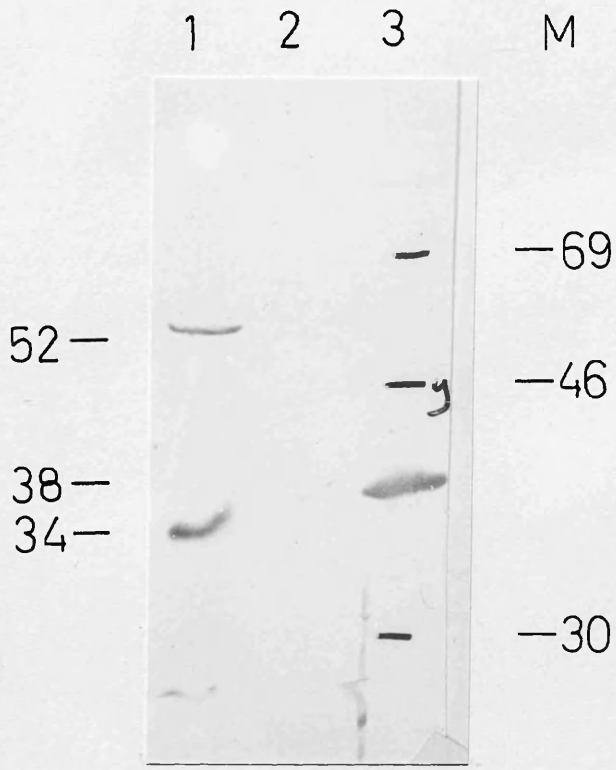


Figure 3.9 Activity of anti-serum S towards extracts from uninfected cells and cells infected with PRV or HSV-1.

Anti-serum S (prepared according to Methods 11.2) was used at a dilution of 1:100 in an immunoblot (Methods 12) against the following protein extracts:

1) partially purified HSV-1 fusion-protein (fig 3.7a lane 3)
2) material from DEAE-cellulose stage of HSV-1 protein kinase purification (as for PRV-PK, Methods 8.2b)
3) post-ribosomal supernatant from uninfected cells (Methods 8.2a)
4) post-ribosomal supernatant from cells infected with PRV (Methods 8.2a)

Numbers to the left indicate the molecular masses of the bands in kDa.
M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 96kDa, 69kDa, 46kDa, 30kDa (Materials 2).

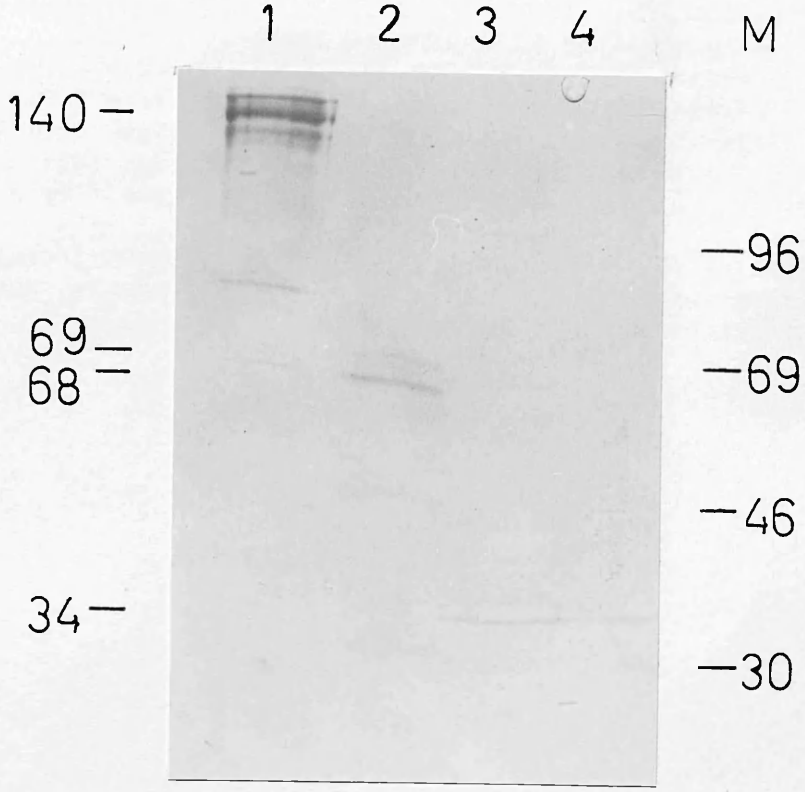
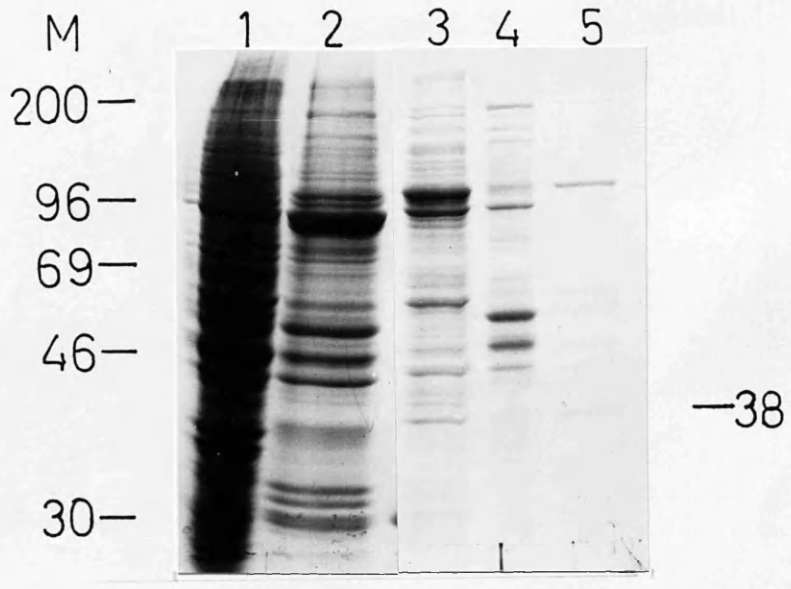

1
2
3
4
M
140
69
68
34
96
69
46
30

Figure 3.10 Purification of PRV-PK.

Fractions from the stages of the purification were analysed by polyacrylamide gel electrophoresis (Methods 4.2a) and stained with Coomassie Blue (1-5) or by silver staining (lane 6a & 6b; Methods 4.2b).

1) post-ribosomal supernatant (Methods 8.2a)
2) pooled fractions from DEAE-cellulose column after concentration using Aquacide II (Methods 8.2bii)
3) fraction 46 from TSK phenyl-5PW column (fig 3.12)
4) fraction 70 from TSK phenyl-5PW column (fig 3.12)
5) fraction 60 from threonine-Sepharose column (fig 3.13)
6a) fraction 30 from protamine-agarose column (fig 3.14)
6b) fraction 37 from protamine-agarose column (fig 3.14)

M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 200kDa, 96kDa, 69kDa, 46kDa, 30kDa (Materials 2).


M
1
2
3
4
5
200
96
69
46
30
38

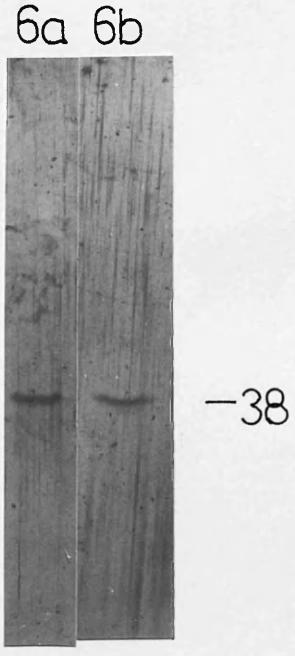

6a
6b
38

Figure 3.11 Purification of PRV-PK: DEAE-cellulose column.

a) Protein was eluted from this column using a salt gradient of 0 - 0.4M KCl (Methods 8.2bii). 60 fractions of 7ml were collected and assayed for protein kinase activity (Methods 8.1).

—□— indicates protein kinase activity (cpm $x10^{-3}$)
- - - - indicates KCl concentration (M).

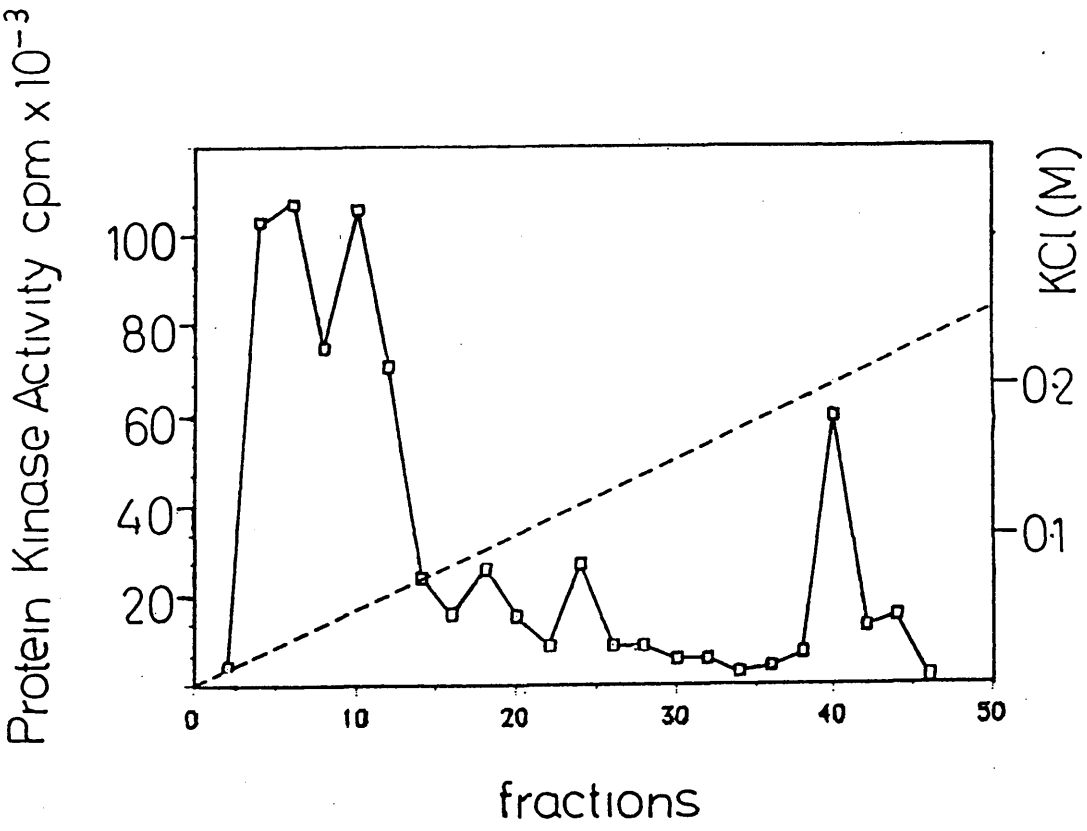
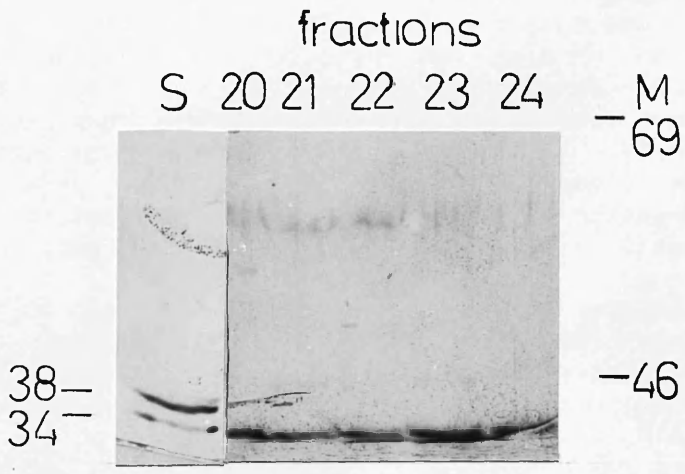

Protein Kinase Activity cpm x 10^{-3}
100
80
60
40
20
0
10
20
30
40
50
fractions
KCl (M)
0·2
0·1

Figure 3.11 Purification of PRV-PK: DEAE-cellulose column.

b) Immunoblots (Methods 12) were performed on the material from various fractions after precipitation of the protein with acetone (Methods 4.2c). Anti-serum P (prepared according to Methods 11.2) was used at a dilution of 1:400.

M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 69kDa, 46kDa, 30kDa (Materials 2).
S indicates the lane which contained material from the post-ribosomal supernatant from cells infected with PRV (Methods 8.2a)
Numbers to the left indicate the molecular masses of the bands in kDa.
The line within lane M corresponds to where the nitrocellulose filter was cut (Methods 12).


fractions
S 20 21 22 23 24
M
69
46
38
34

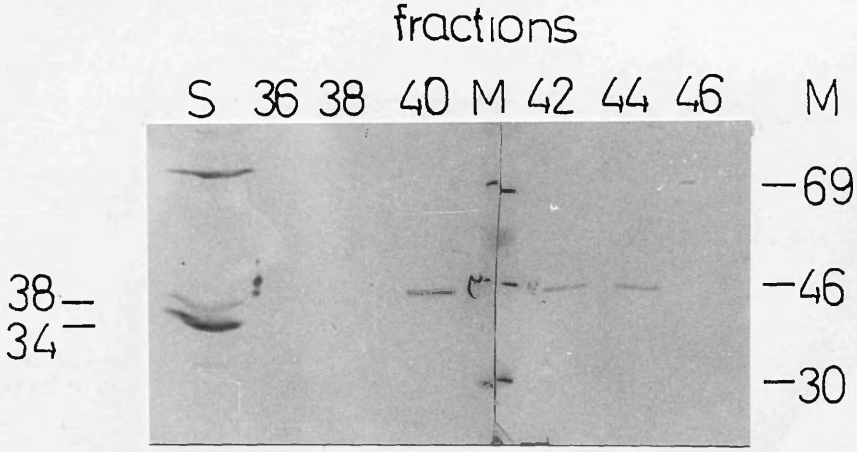
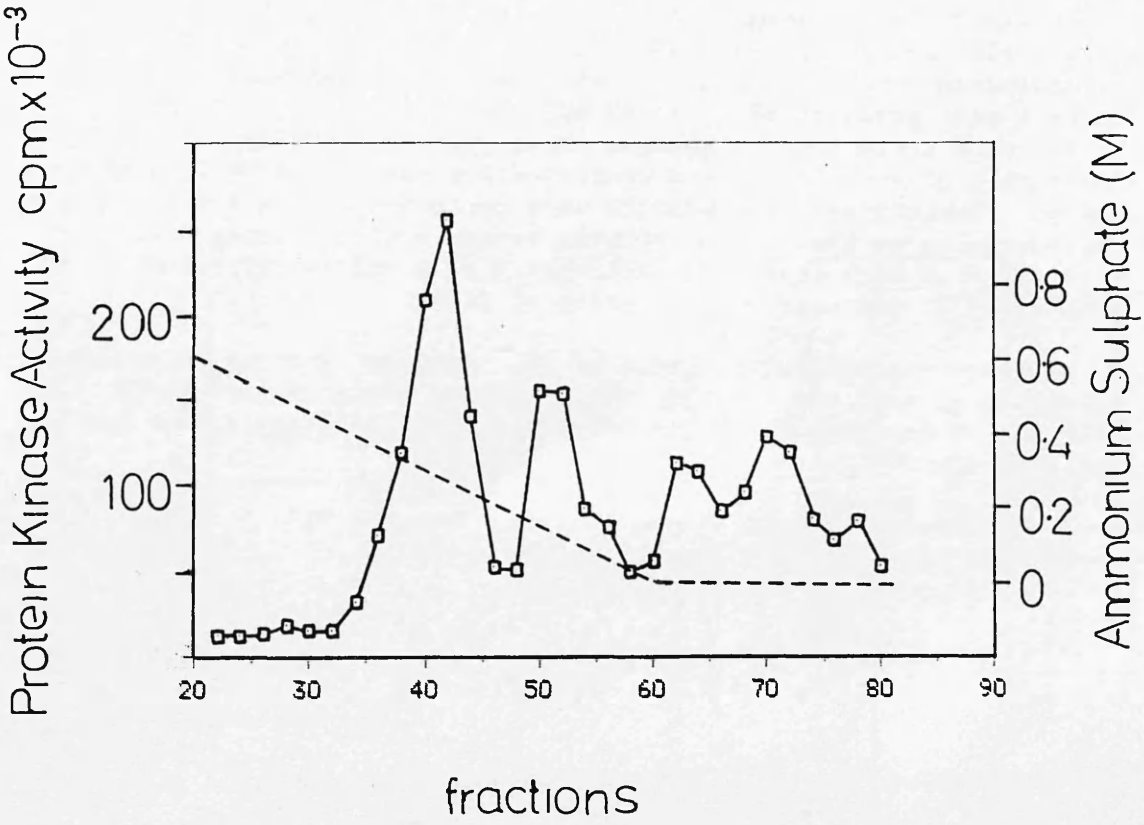

fractions
S 36 38 40 M 42 44 46
M
69
46
30
38
34

Figure 3.12 Purification of PRV-PK: TSK phenyl-5PW column.

Fractions corresponding to the peak of PRV-PK from six DEAE-cellulose columns were pooled and subjected to chromatography on a TSK phenyl-5PW column. Protein was eluted from this column using a salt gradient of 1.0 - 0M ammonium sulphate (Methods 8.2c). 80 fractions of 1ml were collected and assayed for protein kinase activity (Methods 8.1). Immunoblots (Methods 12) were performed on the fractions shown to contain protein kinase activity. 50µl aliquots of each fraction were used. Anti-serum P (prepared according to Methods 11.2) was used at a dilution of 1:400

M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 69kDa, 46kDa, 30kDa (Materials 2).
Number to the right indicates the molecular mass of the band in kDa.
The line between fractions 46 & 48 is where the nitrocellulose filter was cut (Methods 12).

—□— indicates protein kinase activity (cpm $x10^{-3}$)
- - - - indicates ammonium sulphate concentration (M).


Protein Kinase Activity cpm x10⁻³
200
100
20
30
40
50
60
70
80
90
fractions
0·8
0·6
0·4
0·2
0
Ammonium Sulphate (M)

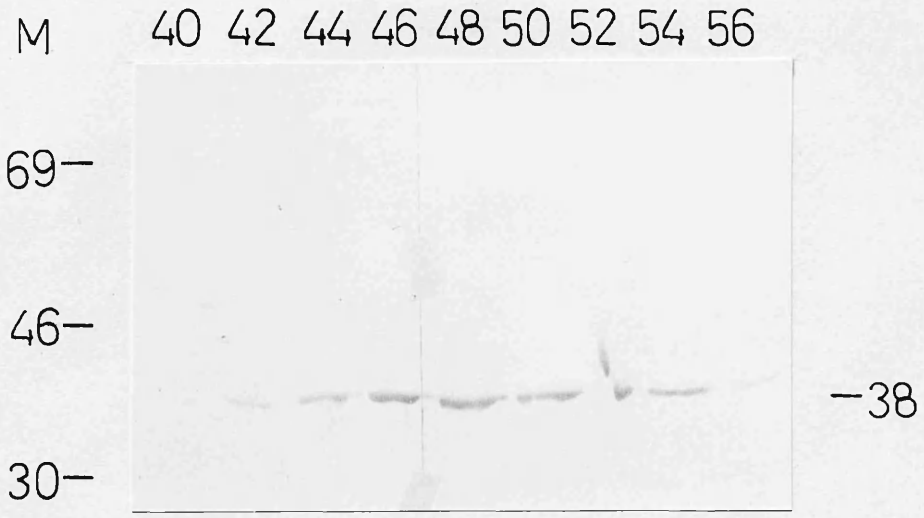
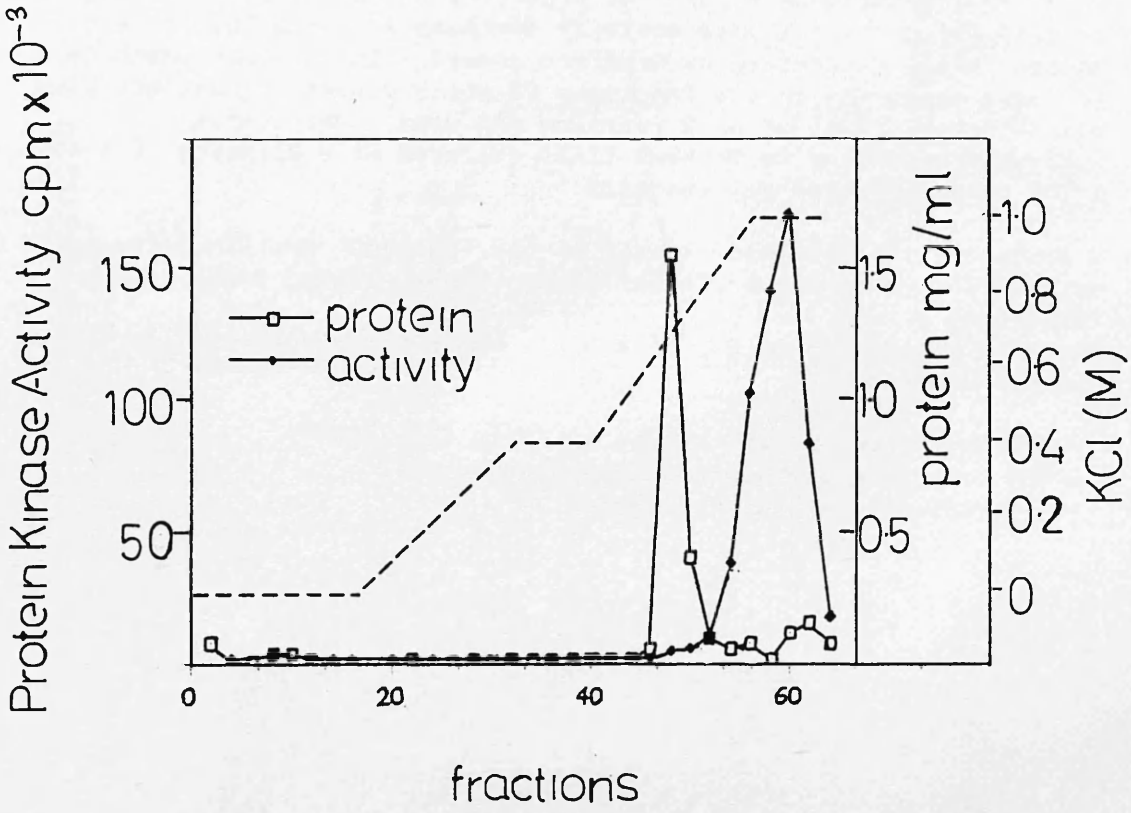

M
40 42 44 46 48 50 52 54 56
69
46
30
38

Figure 3.13 Purification of PRV-PK: Threonine-Sepharose column.

Fractions 37-44, 49-54 and 62-72 from the TSK phenyl-5PW column (fig 3.12) were pooled and subjected to chromatography on a threonine-Sepharose column. Protein was eluted from this column using a salt gradient of 0 - 1.0M KCl (Methods 8.2d) . 70 fractions of 2ml were collected and assayed for protein kinase activity (Methods 8.1) and the protein concentration determined by Bradford assays. Immunoblots (Methods 12) were performed on the fractions corresponding to the peak of protein kinase activity using 50µl aliquots from each fraction. Anti-serum P (prepared according to Methods 11.2) was used at a dilution of 1:400.

M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 96kDa, 69kDa, 46kDa, 30kDa (Materials 2). The line within fraction 59 is where the nitrocellulose filter was cut (Methods 12).

—◆— indicates protein kinase activity (cpm x10^{-3})
—□— indicates protein concentration (mg/ml)
- - - - indicates KCl concentration (M).


Protein Kinase Activity cpm x 10⁻³
150
100
50
protein
activity
1·5
1·0
0·5
protein mg/ml
1·0
0·8
0·6
0·4
0·2
0
KCl (M)
0
20
40
60
fractions

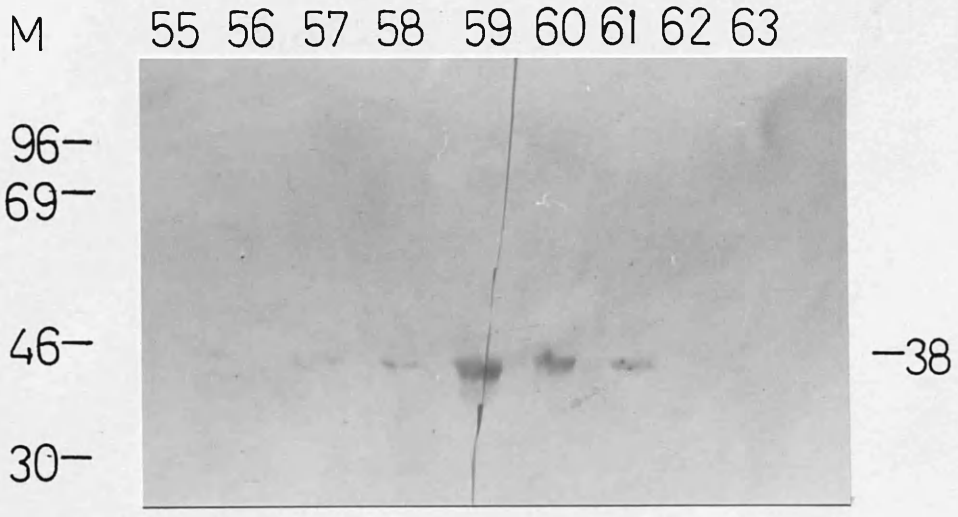
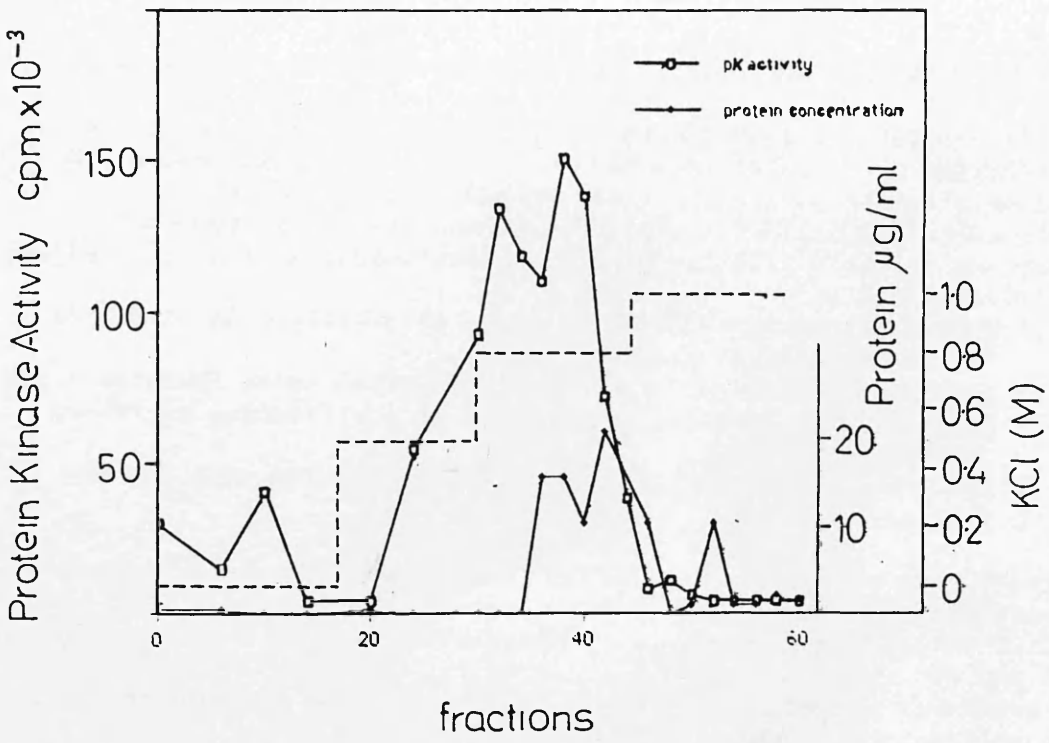

M
55 56 57 58 59 60 61 62 63
96
69
46
30
38

Figure 3.14 Purification of PRV-PK: Protamine-agarose column.

Fractions 55-62 from the threonine-Sepharose column (fig 3.13) were pooled and subjected to chromatography on a protamine-agarose column. Protein was eluted from this column using a salt step gradient of 0 - 1.0M KCl (Methods 8.2e). 60 fractions of 1ml were collected and assayed for protein kinase activity (Methods 8.1) and the protein concentration determined by Bradford assays. Immunoblots (Methods 12) were performed on the fractions in which protein kinase activity was detected. 50µl of each fraction was used. Anti-serum P (prepared according to Methods 11.2) was used at a dilution of 1:400. A 15% polyacrylamide gel was used.

M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 200kDa, 96kDa, 69kDa, 46kDa, 30kDa, 21kDa (Materials 2).
The line between fractions 36 & 38 is where the nitrocellulose filter was cut (Methods 12).

—□— indicates protein kinase activity (cpm $x10^{-3}$)
—◆— indicates protein concentration (µg/ml)
- - - - indicates KCl concentration (M).


Protein Kinase Activity cpm x10⁻³
pK activity
protein concentration
150
100
50
0
20
40
60
fractions
Protein µg/ml
20
10
KCl (M)
1·0
0·8
0·6
0·4
0·2
0

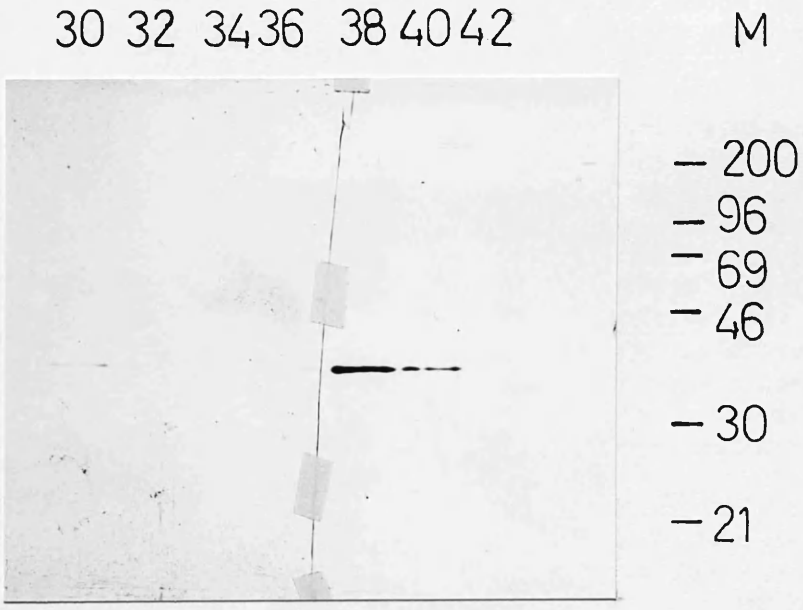

30 32 34 36 38 40 42
M
200
96
69
46
30
21

Figure 3.15 Characterisation of affinity-purified antibodies.

a) Immunoblot using antibodies affinity-purified against PRV-PK. Antibodies were eluted from PRV-PK (material from TSK phenyl-5PW column stage of purification ie. Methods 8.2c) bound to nitrocellulose filters (Methods 11.3) and used in an immunoblot (Methods 12) at a dilution of 1:5 against extracts from infected and uninfected cells:

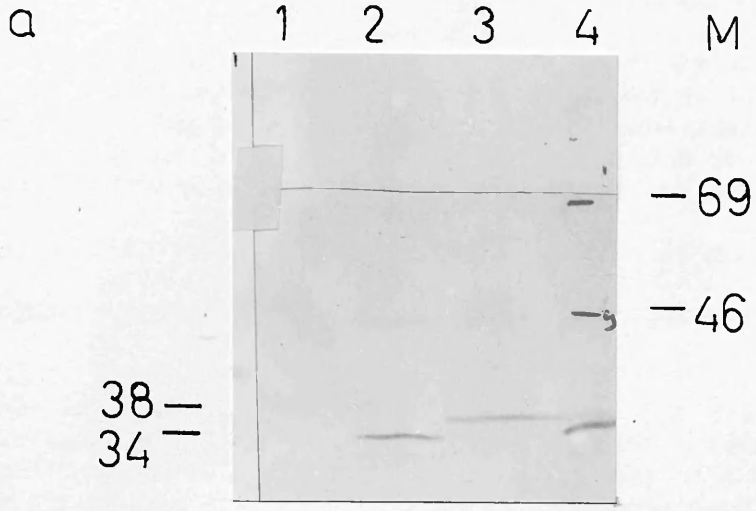
1) material from DEAE-cellulose stage of purification of HSV-PK (as for PRV-PK, Methods 8.2bii)
2) post-ribosomal supernatant from uninfected cells (Methods 8.2a)
3) material from DEAE-cellulose stage of purification of PRV-PK (Methods 8.2bii)
4) post-ribosomal supernatant from cells infected with PRV-PK (Methods 8.2a)

Lane 4 also contained 'Rainbow' protein molecular weight markers, the positions of which were marked in biro before incubation with the first anti-serum (Methods 12). These correspond to the lines at 69 and 46.
M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 69kDa, 46kDa (Materials 2).
Numbers to the left indicate the molecular masses of the bands in kDa.
The line above the 69 marker is where the nitrocellulose filter was cut (Methods 12).

b) Immunoblot using antibodies affinity-purified against the 34kDa cellular protein. Antibodies were eluted from the 34kDa cellular protein (Methods 11.3) and used in an immunoblot at a dilution of 1:4 (Methods 12).

1) Immunoblot of antibodies affinity-purified against the 34kDa cellular protein against material from the TSK phenyl-5PW stage of the purification of PRV-PK.
2) Immunoblot of anti-serum P (prepared according to Methods 11.2) against material which eluted from the DEAE-cellulose column at 0.1M KCl during the purification of PRV-PK (fig 3.11). This shows the 34kDa band from which antibodies were eluted.

Lane 2 also contained 'Rainbow' protein molecular weight markers, the positions of which were marked in biro before incubation with the first anti-serum (Methods 12). These correspond to the lines at 69 46 and 30.
The line in lane 1 below the 38kDa band is where the nitrocellulose filter was cut (Methods 12).
M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 69kDa, 46kDa, 30kDa (Materials 2).


a
1
2
3
4
M
69
46
38
34

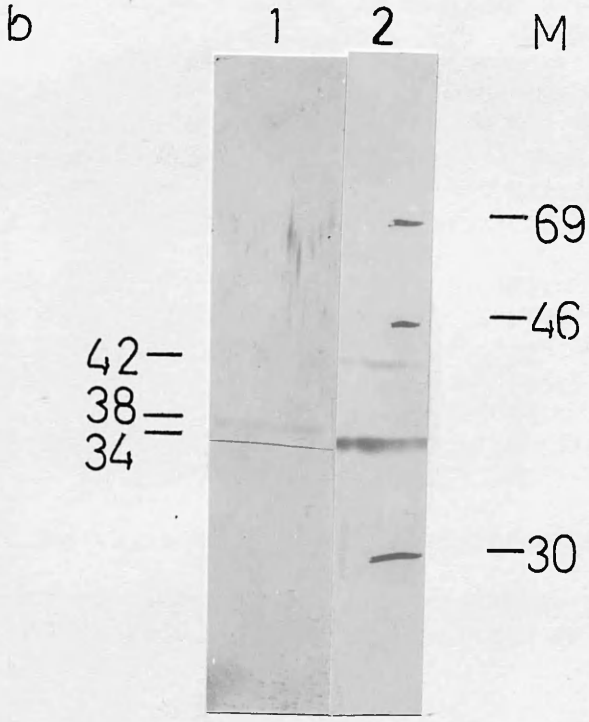

b
1
2
M
69
46
42
38
34
30

Figure 3.16
a) Protein and immunochemical analysis of PRV virions and nucleocapsids.

Virions and nucleocapsids were prepared according to Methods 7.1. 10µl of virions and nucleocapsids were subjected to polyacrylamide gel electrophoresis and stained for protein with Coomassie Blue (Methods 4.2a)

lane 1a : virions prepared from the growth medium of infected cells
lane 1b : virions prepared from the cytosol of infected cells
lane 2 : nucleocapsids prepared from the nuclei of infected cells

Anti-serum P (prepared according to Methods 11.2) was used in an immunoblot (Methods 12) at a dilution of 1:400 against virions (from 3 roller bottles of infected cells) and nucleocapsids (prepared according to Methods 7.1), and an extract from infected cells (the concentrated material from the DEAE-cellulose column equivalent to that produced from 0.03 roller bottles of infected cells : Methods 8.2bii)

lane 3 : DEAE-cellulose material from infected cells
lane 4 : PRV nucleocapsids
lane 5 : PRV virions prepared from the growth medium of infected cells

b) Immunochemical analysis of extracts of PRV virions after treatment with NP-40.

10µl of PRV virions (prepared according to Methods 7.1) were treated with 1% NP-40 or 10% NP-40, 0.6M NaCl (Methods 7.2). Anti-serum P (prepared according to Methods 11.2) was used in an immunoblot (Methods 12) against the solubilised material and the precipitate resulting from both these extraction procedures at a dilution of 1:400

lane 1 : precipitate after extraction with 1% NP-40
lane 2 : soluble material from extraction with 1% NP-40
lane 3 : precipitate after extraction with 10% NP-40, 0.6M NaCl
lane 4 : soluble material from extraction with 10% NP-40, 0.6M NaCl
lane 5 : DEAE-cellulose material from infected cells (Methods 8.2bii)

The line within lane 3 is where the nitrocellulose filter was cut (Methods 12).
M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 200kDa, 96kDa, 69kDa, 46kDa, 30kDa (Materials 2).

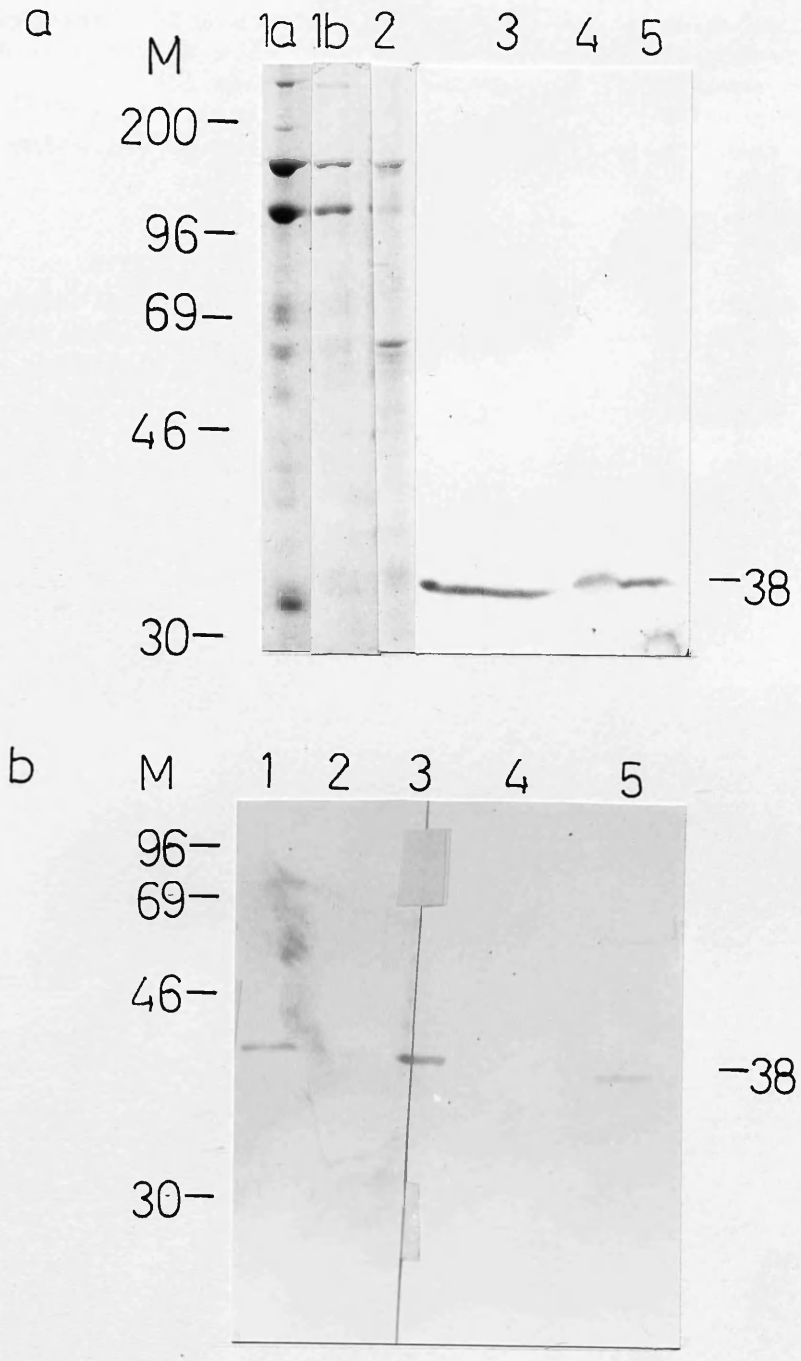

a
M
1a
1b
2
3
4
5
200
96
69
46
30
38
b
M
1
2
3
4
5
96
69
46
30
38

Figure 3.17 Immunochemical analysis of HSV-1 virions using anti-serum S.

Mature HSV-1 virions were prepared from the medium from infected cells (as for PRV, Methods 7.1). Nucleocapsids were prepared from the nuclei of infected cells (as for PRV, Methods 7.1). Anti-serum S (prepared according to Methods 11.2) was used at a dilution of 1:100 in immunoblots against these preparations (Methods 12).

1) partially purified HSV-1 fusion-protein (fig 3.7 lane 3)
2) material from the DEAE-cellulose stage of the purification of HSV-1 PK (as for PRV, Methods 8.2bii)
3) HSV-1 nucleocapsids
4) HSV-1 mature virions

M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 96kDa, 69kDa, 46kDa, 30kDa (Materials 2). Numbers to the left indicate the molecular masses of the bands in kDa.

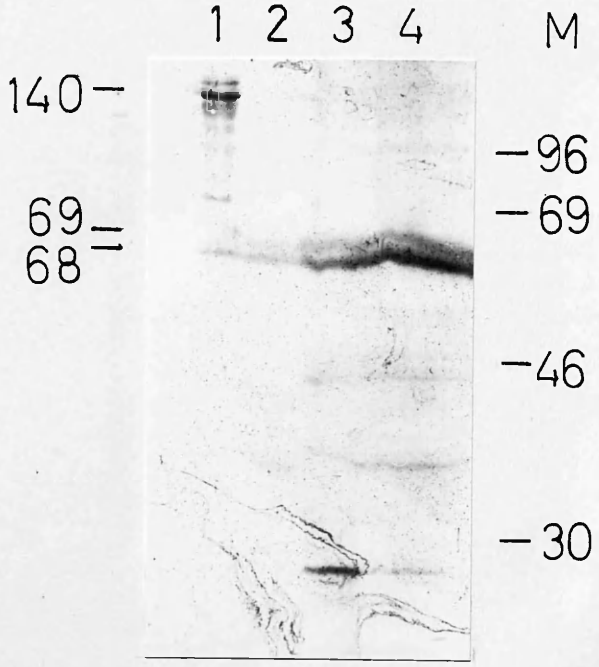

1 2 3 4
M
140
69
68
96
69
46
30

Figure 3.18
a) The proteins phosphorylated in extracts prepared from cells incubated with [^{32}P]orthophosphate during infection with PRV or during mock-infection.

Two 25cm^2 flasks were infected and 0.3mCi/flask [^{32}P]orthophosphate was added 12h post-infection. (Methods 9.2). The cells were harvested 3h later (Methods 9.2a) and an extract produced (200µl). Similarly, an extract was made from two 25cm^2 flasks of mock-infected cells after labelling for 3h. These were subjected to polyacrylamide gel electrophoresis (Methods 4.2a) and an autoradiograph produced (Methods 9.3).

lane 1: 1µl purified virions incubated with γ[^{32}P]ATP (ie. phosphorylation by endogenous protein kinases *in vitro*. Methods 9.1a)
lane 2: 10µl of extract from infected cells
lane 3: 30µl of extract from infected cells
lane 4: 10µl of extract from mock-infected cells
lane 5: 30µl of extract from mock-infected cells

b & c) The phosphorylation of the viral structural proteins of PRV *in vivo*.

Two 25cm^2 flasks were infected and 1mCi/flask [^{32}P]orthophosphate added 3h post-infection (Methods 9.2). The virions were harvested 20h after infection (Methods 9.2b) and an extract produced (200µl). An extract was similarly prepared from two 25cm^2 flasks of cells mock-infected and incubated with 1mCi/flask [^{32}P]orthophosphate for 17h. These were subjected to polyacrylamide gel electrophoresis (Methods 4.2a) and an autoradiograph produced (Methods 9.3).

b) lane 1: 30µl virion extract
lane 2: 20µl virion extract
lane 3: 1µl purified virions incubated with γ[^{32}P]ATP (ie. phosphorylation by endogenous protein kinases *in vitro*. Methods 9.1a)

c) lane 1: 30µl virion extract
lane 2: 30µl extract prepared from mock-infected cells

M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 200kDa, 96kDa, 69kDa, 46kDa, 30kDa (Materials 2).
Numbers to the right of a and c, and to the left of b indicate the molecular masses of the bands in kDa.

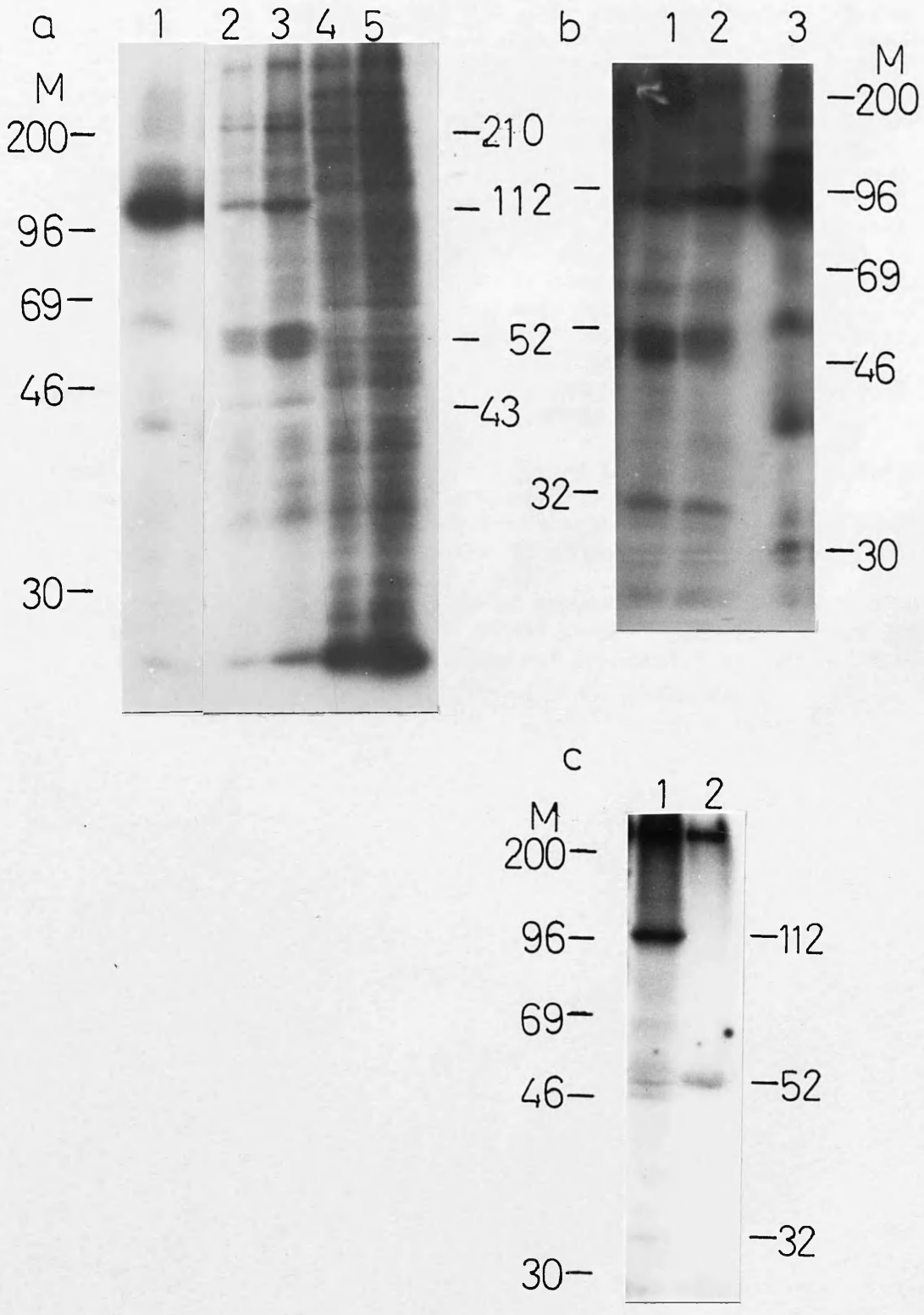

a
1 2 3 4 5
M
200
96
69
46
30
210
112
52
43
b
1 2 3
M
200
96
69
46
32
30
c
1 2
M
200
96
69
46
30
112
52
32

Figure 3.19 The phosphorylation of structural proteins of heat-inactivated PRV virions by PRV-PK, cAMP-dependent protein kinase and casein kinase II.

1µl of virions (prepared according to Methods 7.1) were heat-inactivated and incubated with γ[^{32}P]ATP (Methods 9.1b) in the presence or absence of exogenously added protein kinases and subjected to electrophoresis on a 15% polyacrylamide gel (Methods 4.2a). The protein kinases were also incubated with γ[^{32}P]ATP under the same conditions, but in the absence of the heat-inactivated virions, and subjected to electrophoresis. An autoradiograph was produced (Methods 9.3).

lane 1: crude preparation of casein kinase II incubated with γ[^{32}P]ATP without heat-inactivated virions
lane 2: crude preparation of casein kinase II incubated with γ[^{32}P]ATP and heat-inactivated virions
lane 3: pure cAMP-dependent protein kinase incubated with γ[^{32}P]ATP without heat-inactivated virions
lane 4: pure cAMP-dependent protein kinase incubated with γ[^{32}P]ATP and heat-inactivated virions
lane 5: highly purified PRV-PK (from threonine-Sepharose column) incubated with γ[^{32}P]ATP without heat-inactivated virions
lane 6: highly purified PRV-PK (from threonine-Sepharose column) incubated with γ[^{32}P]ATP and heat-inactivated virions
lane 7: heat-inactivated virions incubated with γ[^{32}P]ATP
lane 8a & b: virions incubated with γ[^{32}P]ATP (Methods 9.1a)

M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 96kDa, 69kDa, 46kDa, 30kDa (Materials 2). Numbers to the right indicate the molecular masses of the bands in kDa.

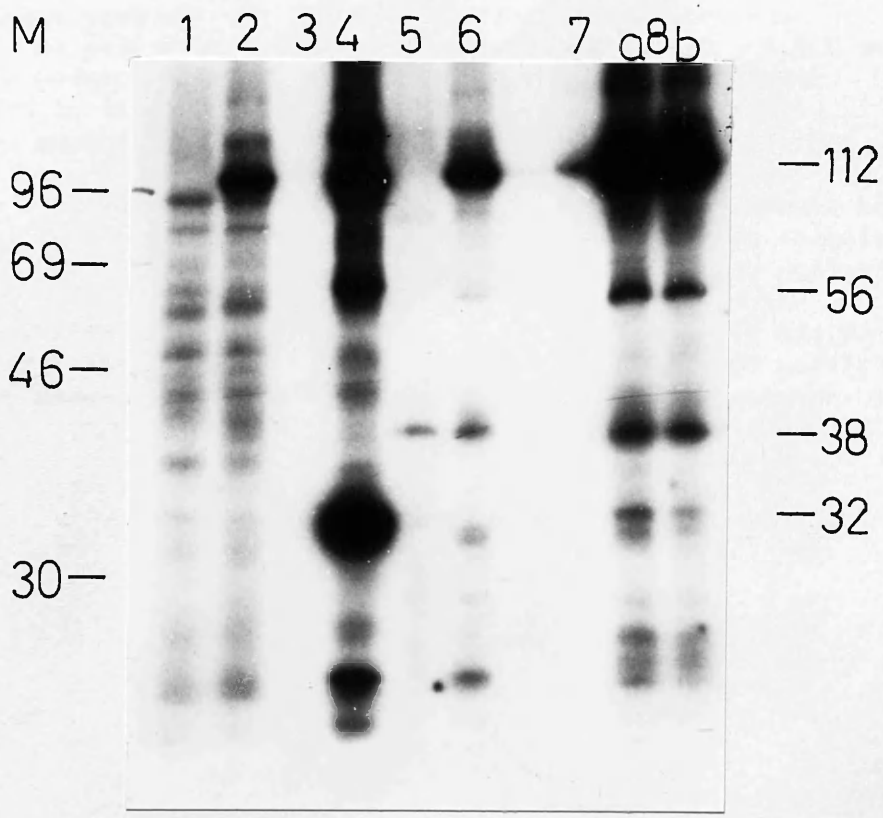

M
1
2
3
4
5
6
7
a
8
b
96
69
46
30
112
56
38
32

Figure 3.20 The phosphorylation of nucleocapsids by endogenous and exogenous protein kinases *in vitro*.

lane 1 : phosphorylation of PRV virions by endogenous protein kinases (Methods 9.1a)

lanes 2 & 5 : 5µl of nucleocapsids (prepared according to Methods 7.1) were incubated with γ[^{32}P]ATP in the presence of 0.5M (2) or 50mM (5) KCl (Methods 9.1a)

lanes 3 & 4 : 5µl of nucleocapsids (prepared according to Methods 7.1) were heat-inactivated and incubated with γ[^{32}P]ATP in the presence (3) or absence (4) of PRV-PK (highly purified PRV-PK from threonine-Sepharose column) (Methods 9.1b).

After the incubations, the reaction mixture was subjected to polyacrylamide gel electrophoresis (Methods 4.2a) and an autoradiograph produced (Methods 9.3).

Numbers to the left show molecular masses (in kDa) of proteins phosphorylated by endogenous protein kinases as calculated from fig 3.19. Numbers to the right indicate the molecular masses deduced for the phosphoproteins in the nucleocapsids.

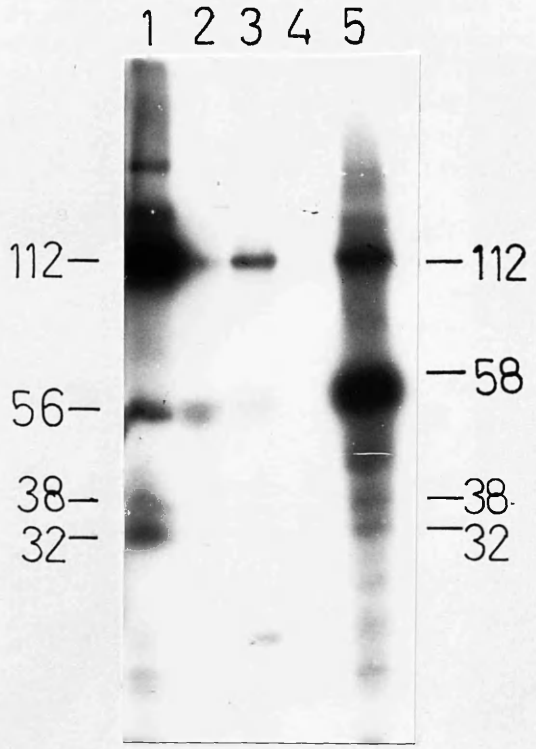

1 2 3 4 5
112—
56—
38—
32—
—112
—58
—38
—32

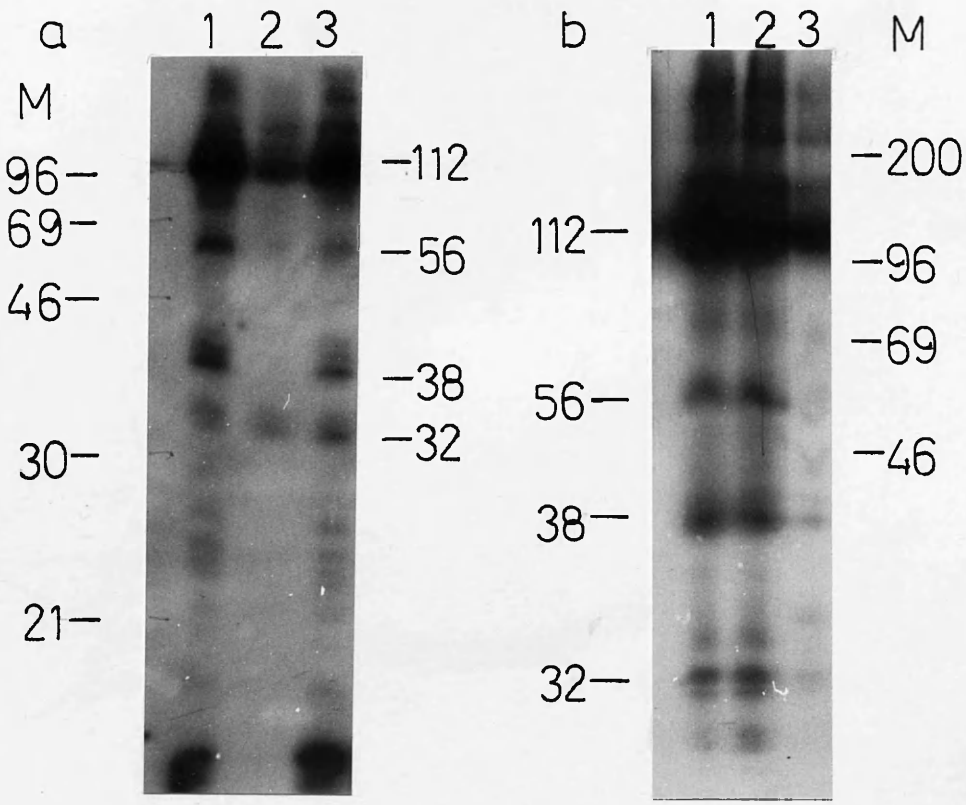
Figure 3.21 The effect of heparin, polyarginine and 0.5M KCl on the phosphorylation of PRV virions by the endogenous protein kinase activity *in vitro*.

1µl of virions (prepared according to Methods 7.1) were incubated with γ[^{32}P]ATP (Methods 9.1a) in the presence or absence of 0.5M KCl, 1µg/ml heparin, or 0.5mg/ml polyarginine and subjected to polyacrylamide gel electrophoresis (Methods 4.2a) on a 15% gel (a) or 10% gel (b). Autoradiographs were produced (Methods 9.3)

a) lane 1 : endogenous phosphorylation in absence of inhibitors
   lane 2 : endogenous phosphorylation in presence of 0.5M KCl
   lane 3 : endogenous phosphorylation in presence of 1µg/ml heparin

b) lane 1 : endogenous phosphorylation in absence of inhibitors
   lane 2 : endogenous phosphorylation in presence of 0.5mg/ml polyarginine
   lane 3 : endogenous phosphorylation in presence of 0.5M KCl

M indicates the molecular masses of the 'Rainbow' protein molecular weight markers in kDa : 200kDa, 96kDa, 69kDa, 46kDa, 30kDa, 21kDa (Materials 2).
Numbers to the right of a and left of b indicate the molecular masses of the bands in kDa.


a
1 2 3
M
96
69
46
30
21
112
56
38
32
b
1 2 3 M
112
56
38
32
200
96
69
46

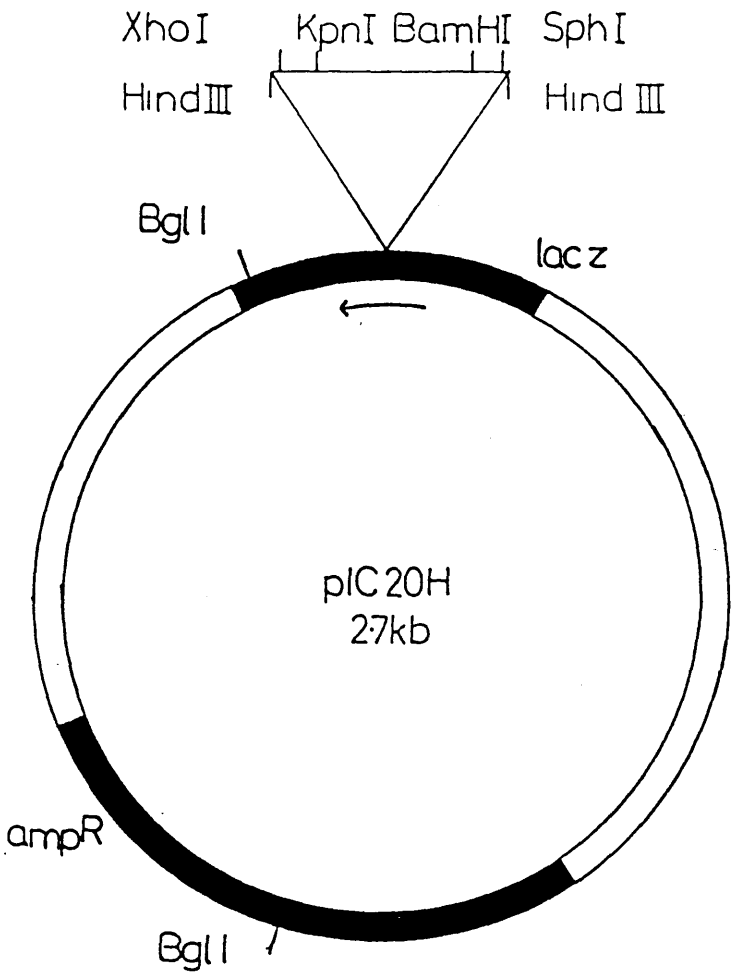
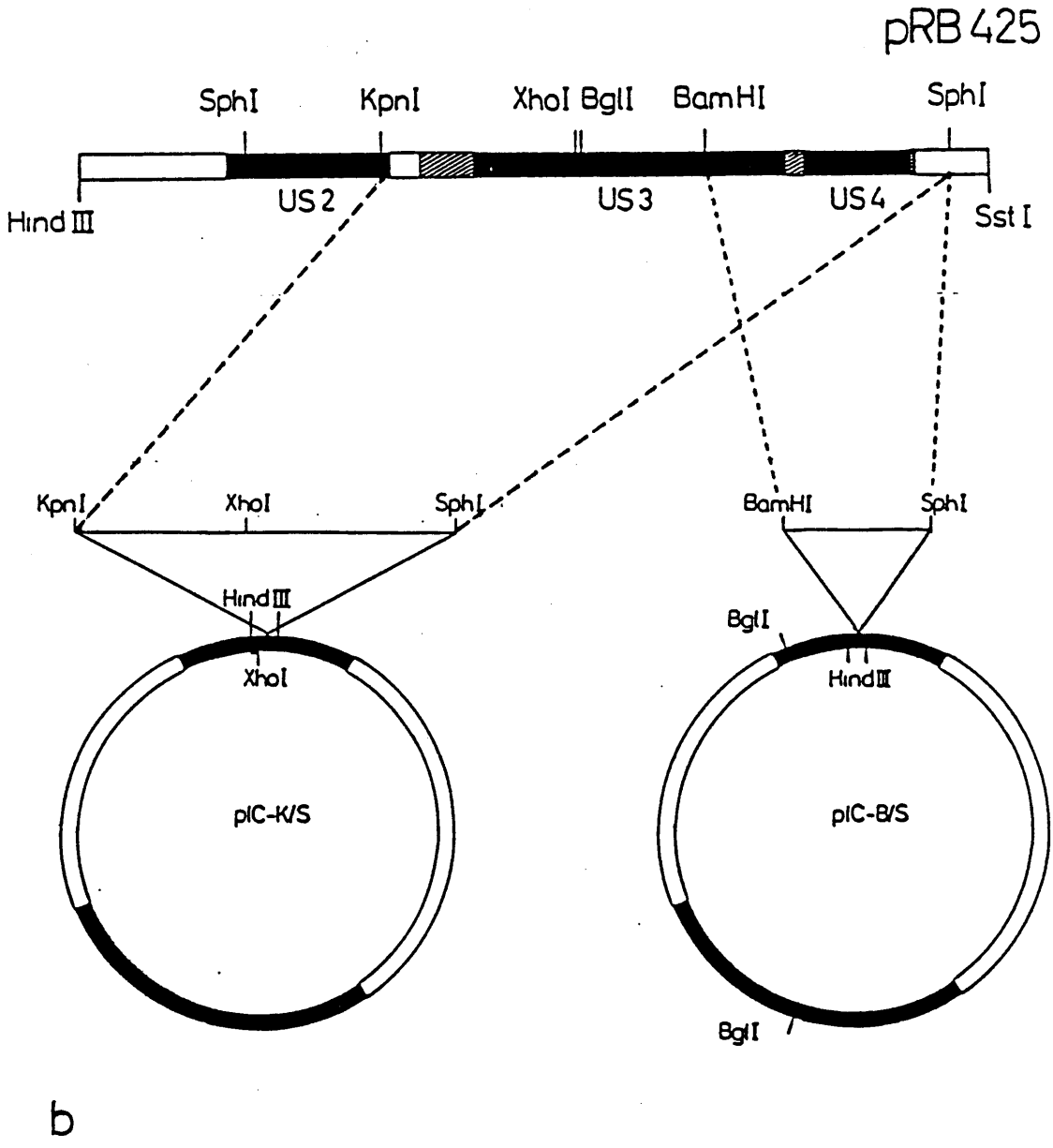


Figure 3.22 Schematic diagrams of pIC 20H and the recombinants pIC-K/S and pIC-B/S.

a) pIC 20H is a vector which allows a restriction fragment which has been produced by restriction digestion with one or two of a number of restriction endonucleases, including *Kpn* I, *Bam* HI and *Sph* I, to be converted to a *Hind* III fragment. The vector has a sequence which contains unique sites for a number of restriction endonucleases. These sites are flanked by two sites for *Hind* III. This sequence is located within *lac z*, thus allowing recombinants to be identified by colour selection using IPTG and X-gal.

b)
The construction of pIC-K/S. The 2.8kb *Kpn* I / *Sph* I fragment from pRB 425 (Materials 6) was inserted into the multiple cloning site of pIC 20H. This fragment includes the promoter and the information for the complete mRNA transcript of US3 and US4.
The construction of pIC-B/S. The 1.3kb *Bam* HI /*Sph* I fragment from pRB 425 was inserted into the multiple cloning site of pIC 20H. This fragment contains the coding region of US4.


pRB 425
SphI
KpnI
XhoI BglI
BamHI
SphI
HindIII
US2
US3
US4
SstI
KpnI
XhoI
SphI
HindIII
XhoI
pIC-K/S
BamHI
SphI
BglI
HindIII
pIC-B/S
BglI

b

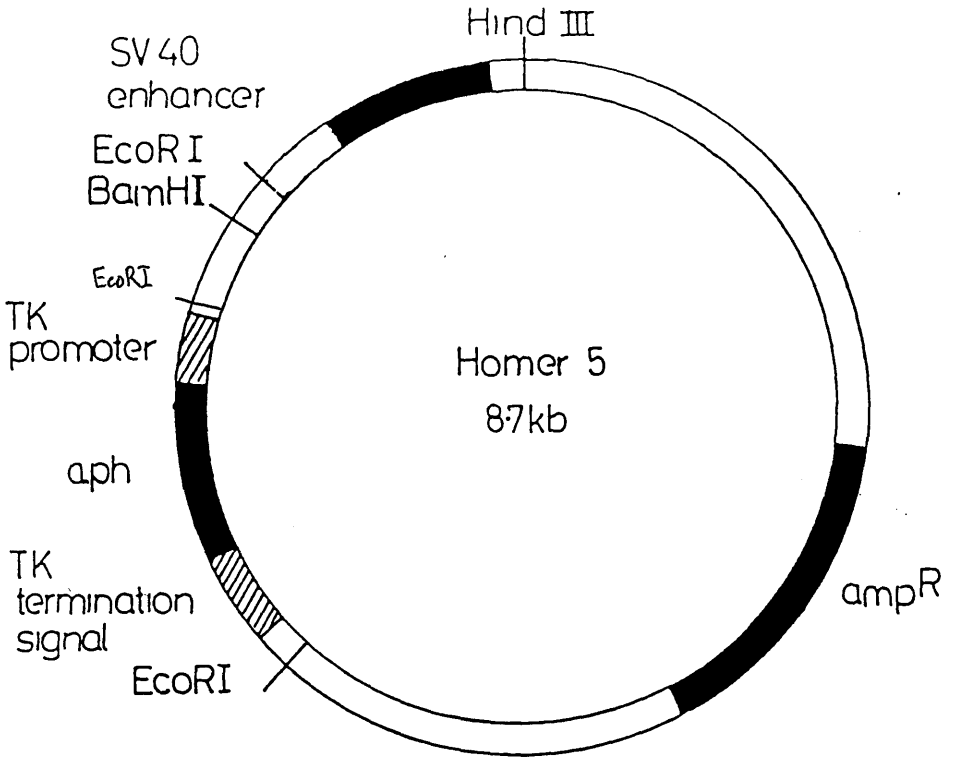
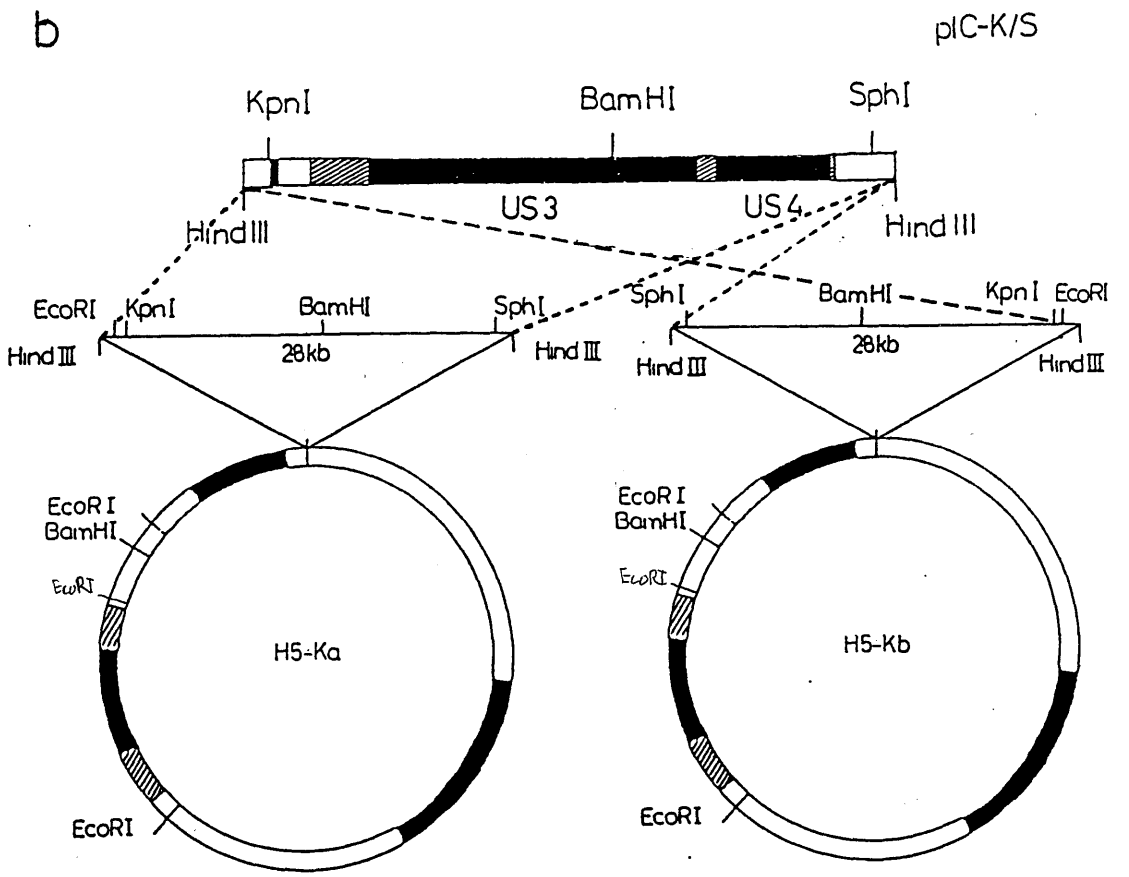


Figure 3.23 Schematic diagrams of Homer 5 and the recombinants H5-Ka, H5-Kb, H5-Ba and H5-Bb.

a) Homer 5 contains two genes, *amp*^{R} and *aph* which confer resistance to ampicillin and geneticin respectively. It also contains the SV40 enhancer located near the unique *Hin*d III site, which is the cloning site for the gene to be expressed. Expression of *aph*, which is a bacterial gene, is made possible by the promoter and termination signal sequences from the thymidine kinase gene (TK) of HSV-1 which flank the gene.

b) The construction of H5-Ka and H5-Kb. The 2.8kb *Hin*d III fragment from pIC-K/S was cloned into the *Hin*d III site of Homer 5. The orientation of the insert was determined by restriction digestion with *Bam* HI.

c) The construction of H5-Ba and H5-Bb. The 1.3kb *Hin*d III fragment from pIC-B/S was cloned into the *Hin*d III site of Homer 5. The orientation of the insert was determined by restriction digestion with *Bam* HI and *Eco*RI.


b
pIC-K/S
KpnI
BamHI
SphI
US3
US4
HindIII
HindIII
EcoRI
KpnI
BamHI
SphI
HindIII
28kb
HindIII
SphI
BamHI
KpnI
EcoRI
HindIII
28kb
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EcoRI
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BamHI
EcoRI
H5-Kb
EcoRI

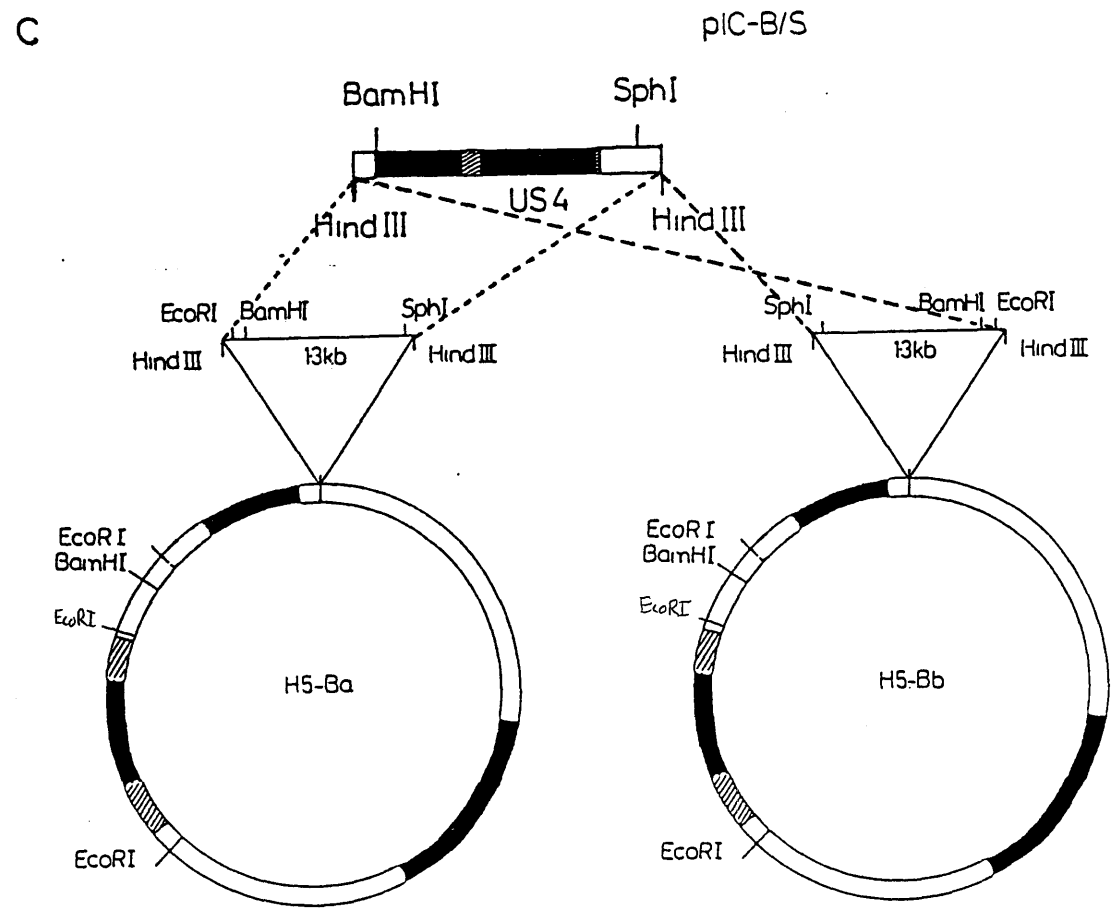

C
pIC-B/S
BamHI
SphI
US4
HindIII
HindIII
EcoRI
BamHI
SphI
HindIII
13kb
HindIII
SphI
BamHI
EcoRI
HindIII
13kb
HindIII
EcoR I
BamHI
EcoRI
H5-Ba
EcoRI
EcoR I
BamHI
EcoRI
H5-Bb
EcoRI

Figure 3.24 Construction of H5-Ka, H5-Kb, H5-Ba, and H5-Bb: Confirmation of identity of clones and determination of orientation by restriction digestion.

Restriction digestions were carried out according to Methods 3.1 and subjected to electrophoresis on a 1% agarose gel (Methods 4.1a)

lane M : DNA size markers: 5.9kb, 4.3kb, 3.3kb & 1.7kb
lane 1 : restriction digestion of H5-Kb with *Bam* HI
lane 2 : restriction digestion of H5-Kb with *Hin*d III
lane 3 : restriction digestion of H5-Ka with *Bam* HI
lane 4 : restriction digestion of H5-Ka with *Hin*d III
lane 5 : restriction digestion of H5-Bb with *Eco*RI
lane 6 : restriction digestion of H5-Bb with *Bam* HI
lane 7 : restriction digestion of H5-Bb with *Hin*d III
lane 8 : restriction digestion of H5-Ba with *Eco*RI
lane 9 : restriction digestion of H5-Ba with *Bam* HI
lane 10: restriction digestion of H5-Ba with *Hin*d III

M indicates the sizes of the DNA size markers in kb.
Numbers to the right indicate the sizes of the bands in kb.

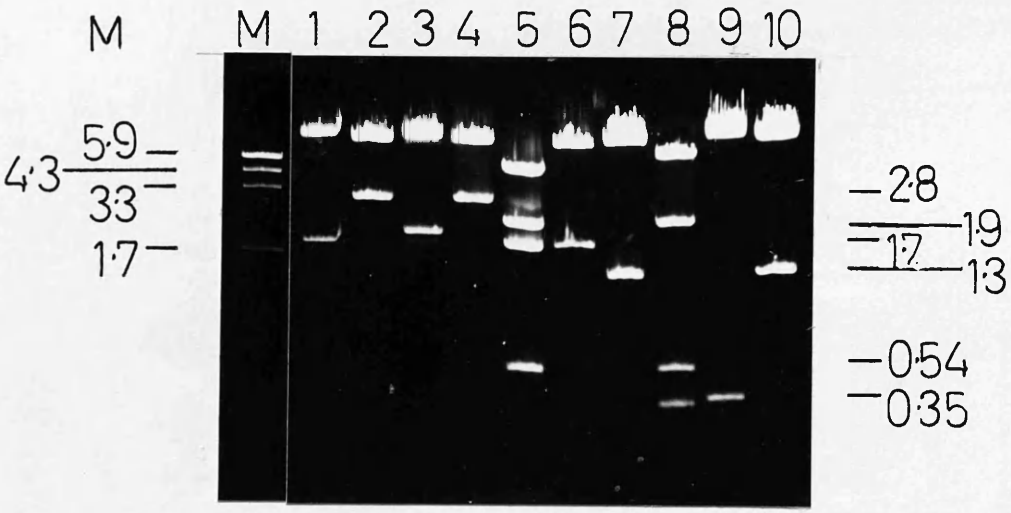

M
M 1 2 3 4 5 6 7 8 9 10
4·3
5·9
3·3
1·7
2·8
1·9
1·7
1·3
0·54
0·35

Figure 3.25 Analysis of protein kinase activity present in the cell lines 208F, Ka-2 and Kb-2.

Post-ribosomal supernatants (Methods 8.2a) prepared from five roller bottles of each cell line were subjected to chromatography on DEAE-cellulose (Methods 8.2bi). Protein was eluted from the column using a salt gradient of 0 - 0.4M KCl. Protein kinase activity was detected by assaying with protamine as the substrate (Methods 8.1).

—□— indicates protein kinase activity (cpm $x10^{-3}$)
- - - - indicates KCl concentration (M)

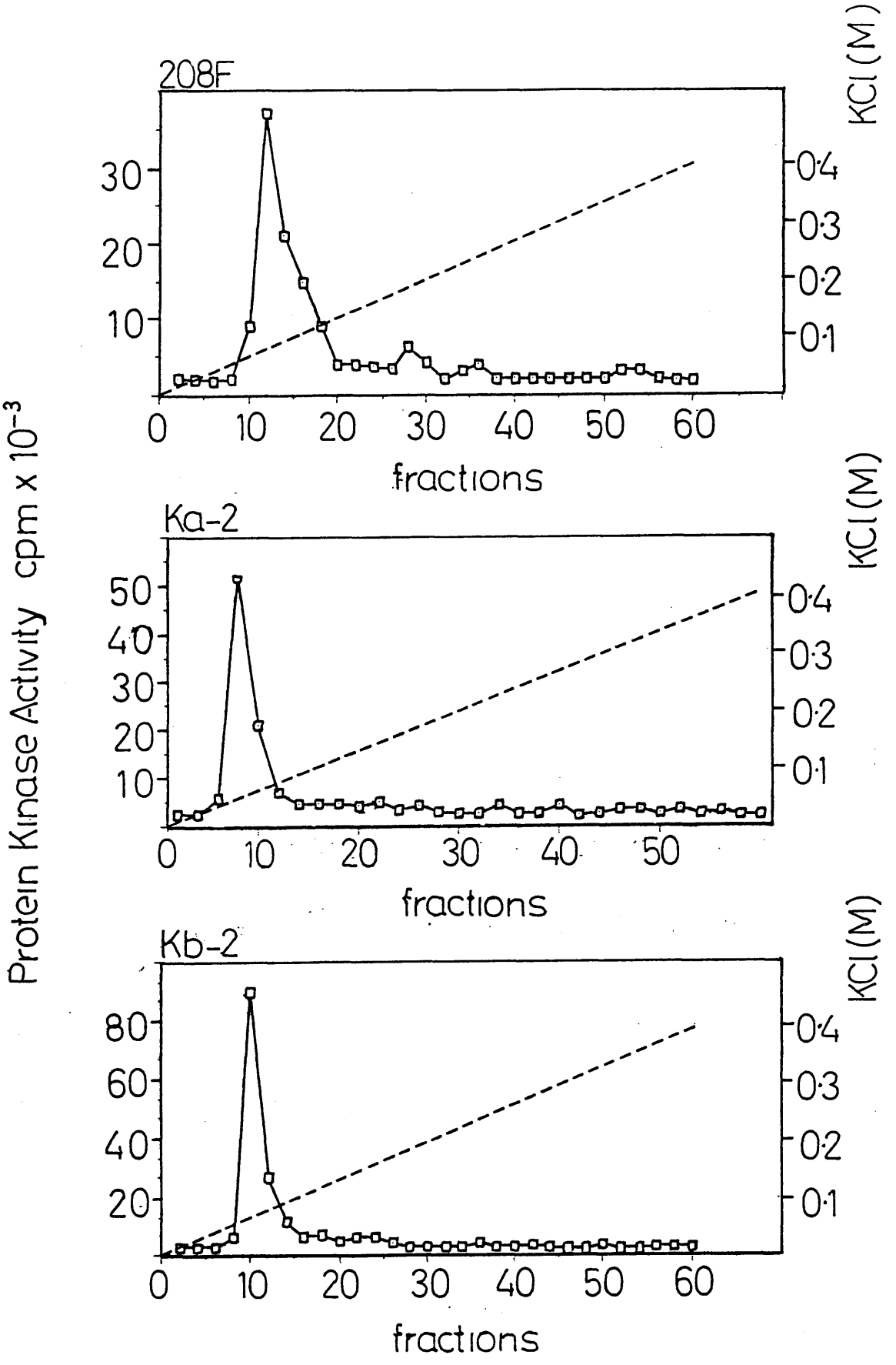
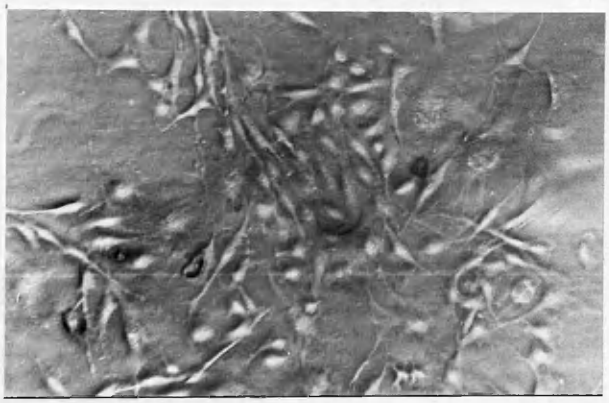

208F
Ka-2
Kb-2
Protein Kinase Activity cpm x 10^{-3}
KCl (M)
fractions
0
10
20
30
40
50
60
0·1
0·2
0·3
0·4

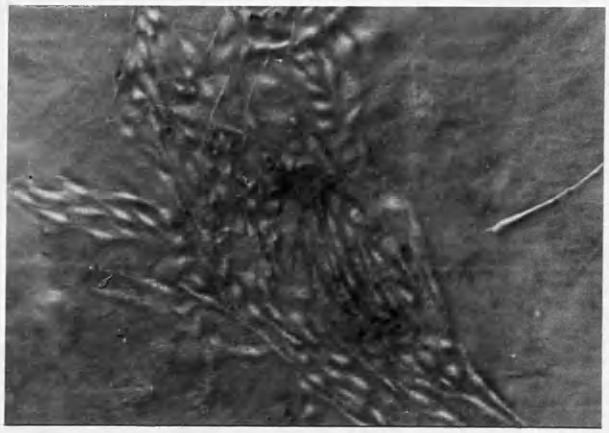
Figure 3.26 Light microscopic appearance of the cell line used for transformation with Homer constructs and resulting geneticin resistant clones.

The cell line 208F was transformed with the constructs H5-Ka and H5-Kb by calcium phosphate precipitation. Transformants (Ka and Kb) were selected for by culturing in the presence of 300µg/ml geneticin.

208F



Ka



Kb

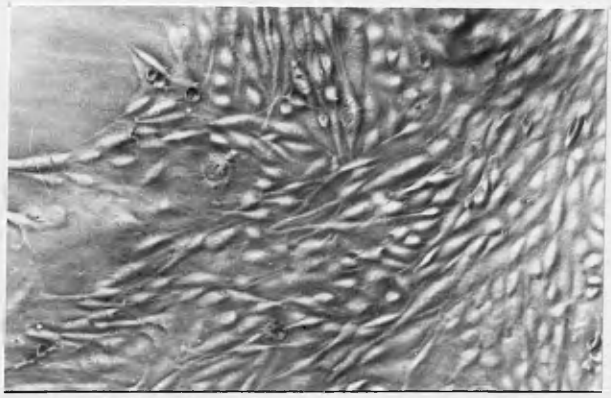


Figure 3.27 Cell growth curves showing the rates of growth and saturation density of the cell lines 208F, Ka-2 and Kb-2.

Cells were grown in 60mm dishes (Methods 5.3), harvested by trypsinisation and counted using a Coulter counter. (The errors in the values for each point on the graph were less than 8%.)

—□— indicates the cell line Kb-2
—◆— indicates the cell line 208F
—■— indicates the cell line Ka-2

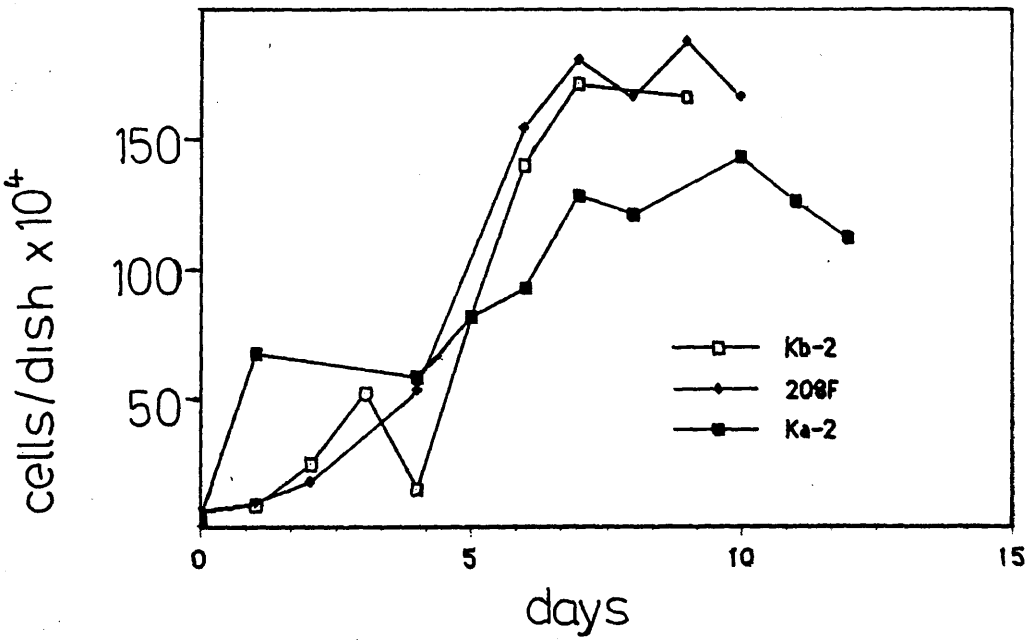

cells/dish x 10⁴
150
100
50
0
5
10
15
days
Kb-2
208F
Ka-2

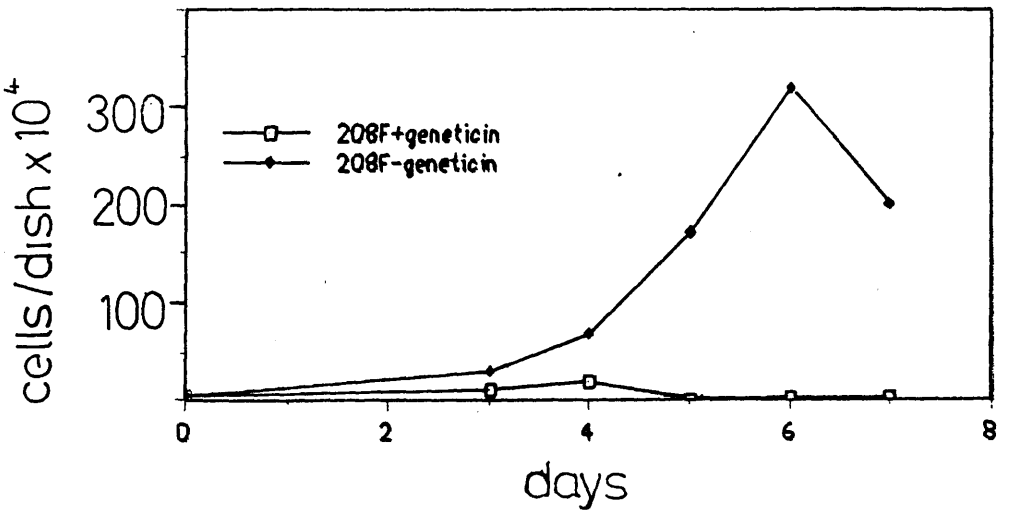
Figure 3.28 Cell growth curves showing the rates of growth of the cell lines 208F, Ka-2 and Kb-2 in the presence and absence of 300μg/ml geneticin.

Cells were grown in 60mm dishes (Methods 5.3), harvested by trypsinisation and counted using a haemocytometer. (The errors in the values for each point on the graph were less than 8%.)

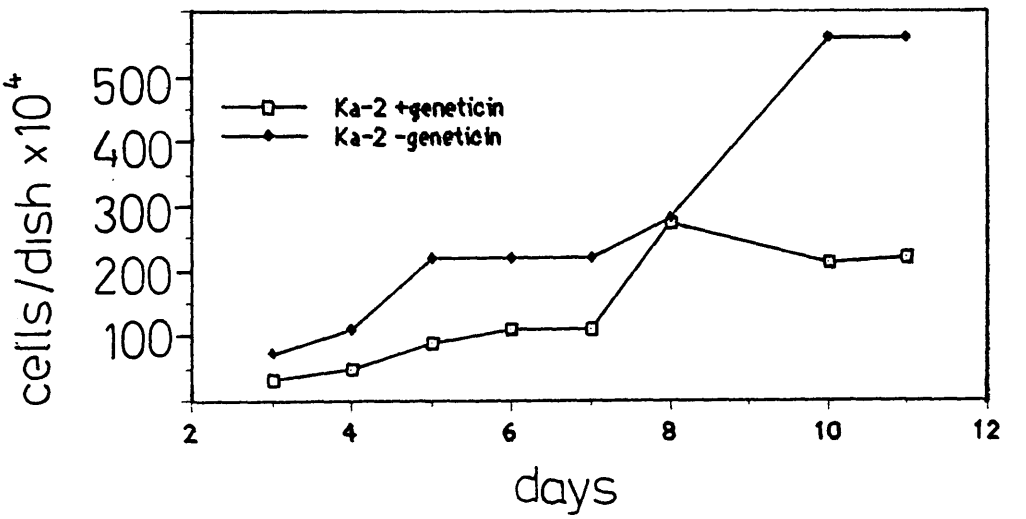
a) 208F
b) Ka-2
c) Kb-2

—□— indicates cells grown in the presence of 300μg/ml geneticin
—◆— indicates cells grown in the absence of geneticin

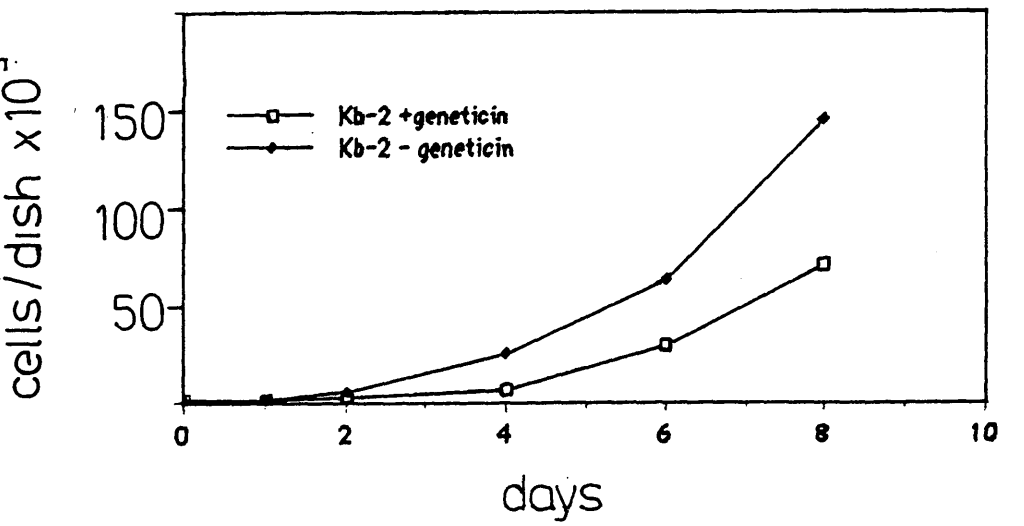
a


cells/dish x 10⁴
300
200
100
208F+geneticin
208F-geneticin
0
2
4
6
8
days

b


cells/dish x10⁴
500
400
300
200
100
Ka-2 +geneticin
Ka-2 -geneticin
2
4
6
8
10
12
days

c


cells/dish x10⁵
150
100
50
Kb-2 +geneticin
Kb-2 - geneticin
0
2
4
6
8
10
days

Chapter 4

DISCUSSION

A. The relationship between the 38kDa protein kinase (PRV-PK) and the PRV *pk* gene.

The main aim of the work described in this thesis was to determine whether the 38kDa protein kinase (PRV-PK) purified from cells infected with PRV was the product of the *pk* gene of PRV, homologous to the US3 gene of HSV-1. The anti-serum raised against a fusion-protein of part of the gene showed immuno-reactivity against PRV-PK during its purification, including homogeneous material from the final stage, when transferred to nitrocellulose (fig 3.14). From this it can be concluded that PRV-PK is encoded by the *pk* gene of PRV and hence is of viral origin.

Previous work on HSV-1 (Frame *et al.*, 1987) also used an immunochemical approach to determine the relationship between the US3 gene and the protein kinase induced in cells infected with HSV-1. This enzyme was purified 100-fold, and the final stage of its purification yielded material containing two species, of 65kDa and 68kDa, of which only the 68kDa protein was phosphorylated when incubated with $\gamma[^{32}P]ATP$. Anti-serum against a synthetic oligopeptide corresponding to the carboxy-terminal eight amino acids of the US3 protein was found to react with the protein of 68kDa in immunoblots of fractions from the final two stages of the purification of HSV-PK. This showed that the 68kDa protein was the

product of the US3 gene and suggested that it contained the HSV-PK activity which underwent autophosphorylation. The possibility remained, however, that the 65kDa protein contained the HSV-PK activity and that the viral 68kDa protein was a substrate for it. In contrast, in this study, the purity of PRV-PK eliminates any possibility that the anti-serum is reacting with a protein other than the protein kinase.

The demonstration that PRV-PK is the product of the *pk* gene of PRV is of added importance in the light of recent papers by Smith & Smith (1989) and Chee *et al.* (1989) concerning another putative protein kinase gene in several different herpes viruses, namely HSV-1, VZV, EBV and HCMV. This gene, which corresponds to UL13 in HSV-1, was identified using the sequence motifs diagnostic of the catalytic domain of protein kinases (Introduction B.2). It is much more highly divergent from known cellular protein kinases than are the US3 gene and its homologues, but the presence of these motifs is quite suggestive that it could encode a protein kinase. The conservation of this gene among herpesviruses suggests that the genome of PRV may also contain a similar gene. However, this work identifies PRV-PK as being the product of the protein kinase gene which is related to that of the HSV-1 US3 gene rather than to that related to the UL13 gene.

One minor point that deserves comment is the fact that the behaviour of PRV-PK during purification differed in certain respects from that reported by Purves *et al.* (1987a). The final protamine-agarose column was found to yield two peaks of activity (fig 3.14). The major peak eluted at the position in the salt

gradient previously observed (Purves *et al.*, 1987a), but another peak eluted at a slightly lower ionic strength. Analysis of fractions containing protein kinase activity by polyacrylamide gel electrophoresis and visualising with silver stain showed that a single species of 38kDa was present in fractions from both peaks of activity (fig 3.10 lane 6a & 6b). Multiple peaks were also observed in the material eluted from the TSK phenyl-5PW column (fig 3.12), but the first of these may have been protein kinase M, a proteolytic product of protein kinase C which elutes at a higher salt concentration than the latter on DEAE-cellulose and may not have been completely resolved from PRV-PK on that column. Although the coelution of an inhibitor with PRV-PK in some fractions may account for some of the apparent peaks, they may also result from the presence of multiple forms of PRV-PK, differing in their chromatographic properties. This may also account for the two peaks observed in the material eluted from the protamine-agarose column. The fact that the only protein detected in both peaks from the protamine-agarose column had a molecular weight of 38kDa makes it unlikely that the differences result from proteolysis. Autophosphorylation, which the enzyme is known to undergo (Purves *et al.*, 1987a), is another possible explanation for the phenomenon, but as this question was peripheral to the main aim of the work, it was not further investigated.

Although the anti-serum reacted with PRV-PK through all stages of purification of the enzyme, it also showed one major cross-reaction with a cellular protein of 34kDa. This protein was present in post-ribosomal supernatants from infected and uninfected cells

(fig 3.8), but was resolved from PRV-PK by chromatography on DEAE-cellulose (fig 3.11). Affinity-purification of the anti-serum, by binding to PRV-PK immobilised on a nitrocellulose filter followed by elution, gave anti-serum which still cross-reacted with this 34kDa protein, indicating that the latter must be antigenically related to PRV-PK (fig 3.15a). Consistent with this result was the observation that anti-serum eluted from the 34kDa protein immobilised on nitrocellulose, cross-reacted with PRV-PK (fig 3.15b).

One possible explanation for this cross-reaction is that the 34kDa protein is a cellular homologue of the viral protein kinase. Since the 34kDa protein was not fully resolved from protein kinase C on DEAE-cellulose (fig 3.11a), it was not possible to determine if this protein did show protein kinase activity. However it seems unlikely that a cellular homologue of PRV-PK would be sufficiently homologous for immuno-reaction, since the anti-serum to PRV-PK did not cross-react with the homologous protein kinase of HSV-1 (fig 3.8b). Nor did anti-serum aganist HSV-PK cross-react with PRV-PK (fig 3.9). It seems more likely that the 34kDa protein is not a protein kinase but fortuitously has common epitopes.

B. Occurence and implications of the presence of PRV-PK within the virions.

It was intended to use the anti-serum which recognised PRV-PK for immunofloresence studies to determine the location of this enzyme within infected cells. This might have shed some light on the physiological substrate of the viral protein kinase, and, hence, its function. However the fact that the anti-serum cross-reacted with a cellular protein, even after affinity-purification, meant that the anti-serum was unsuitable for such studies. Nevertheless, it was possible to use the anti-serum in immunoblotting to investigate a different aspect of the distribution of the viral protein kinase, namely its occurence within the virion.

The anti-serum was found to react with a single protein of 38kDa in purified PRV virions (fig 3.16a). This indicated that PRV-PK is present within the virions. This result is unlikely to arise from contamination of the purified virions with proteins present in the infected cells for several reasons. Firstly, the virions were derived from the medium of infected cells rather than from the cytosol of infected cells, which reduces such contamination. Secondly, the 34kDa cellular protein present in cellular extracts was not detected in the immunoblot. This result is not inconsistent with previous studies (Stevely *et al.*, 1985) in which the protein kinase activities of PRV virions were investigated. A protein kinase activity was observed that could be PRV-PK on the basis of its preference for protamine as a substrate and its chromatographic

properties on DEAE-cellulose. However, further characterisation of the enzyme was not carried out and thus its identity must still be considered ambiguous.

What is the significance of the location of the protein kinase in the virion? Although PRV-PK is present within PRV virions, it can be estimated from the immunoblot (fig 3.16a) that there is at least a 10^2 times as much PRV-PK in the cytoplasm of infected cells as in the virions, even assuming that the yield of virions in the preparation was only 10%. Stevely *et al.* (1985) identified at least four protein kinase activities within PRV virions. The properties of the two activities which showed a preference for casein as a substrate suggested that they corresponded to the cellular protein kinases, casein kinase I and II. Of the two activities showing a preference for protamine, one was suggested to be protein kinase C, while the other enzyme could have been PRV-PK. Assuming this identification to be correct, the amount of PRV-PK present within the virion is approximately the same as that of these cellular protein kinases which are assumed to become fortuitously incorporated into the virion during the process of assembly. The fact that there appears to be no specific accumulation of PRV-PK as compared to the cellular protein kinases in PRV virions, along with the much greater quantity of PRV-PK in the cytosol of infected cells suggests that the major functional role(s) of this protein kinase are carried out within infected cells rather than within the virion.

However, *in vitro*, PRV-PK is able to phosphorylate one of the major virion structural proteins of molecular weight 112,000 (fig

3.19 lane 6). *In vivo*, this is the major phosphoprotein in virions (fig 3.18b) and can also be detected in extracts made from cells infected with PRV in the presence of [^{32}P]orthophosphate (fig 3.18a). This indicates that the 112kDa protein is a substrate for PRV-PK. In previous studies, Stevely *et al.*, (1985) found that casein kinase II was able to phosphorylate this viral protein, (a result confirmed here, fig 3.19 lane 2) and they concluded that this cellular protein kinase was responsible for the phosphorylation of the major phosphoprotein of PRV virions. However, it now appears that the 112kDa protein can be phosphorylated by two distinct protein kinases. This probably occurs at different sites, since these protein kinases differ in their substrate specificities (Purves *et al.*, 1986a; Marin *et al.*, 1986). Experimental analysis and comparison of the sites phosphorylated *in vivo* and *in vitro* are needed to determine the relative contributions of these two enzymes to the phosphorylation of this protein, the function of which still remains to be investigated.

It can thus be concluded that although the location of PRV-PK within the virion may be important for the phosphorylation of the major phosphoprotein of PRV virions, the abundance of the enzyme within the cytosol of infected cells suggests that the major role of PRV-PK is played within infected cells, involving the phosphorylation of substrate(s), possibly of cellular origin, which are yet to be identified.

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