THE PROTEIN KINASES INDUCED IN CELLS INFECTED WITH HERPESVIRUSES

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"If conquests useful to humanity touch your heart; if you stand amazed before the surprising effects of electric telegraphy, the daguerreotype, anaesthesia and so many other admirable discoveries; if you are jealous of the part your country can claim in the further flowering of these wonders - take an interest, I urge upon you, in those holy dwellings to which the expressive name of Laboratories is given. Ask that they be multiplied and adorned. They are the temples of the future, of wealth and well-being. It is there that humanity grows bigger, strengthens and betters itself. It learns there to read in the works of nature, works of progress and universal harmony, whereas its own works are too often those of barbarity, fanaticism and destruction."

L. Pasteur

(Inscription on the walls of the Institut du Radium, Pavillon Curie, Paris, France).

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ABBREVIATIONS AND DEFINITIONS

The abbreviations recommended by the Biochemical Journal in its instructions to Authors [Biochem J (1981) 193, 4-27] have been used throughout the thesis with the following additions:

BHK cells	Baby hamster kidney fibroblasts
c.p.m.	counts per minute
DATD	Diallytartardiamide
EBV	Epstein-Barr virus
FPLC	Fast protein liquid chromatography
Hepes	[4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid]
HSV-1 (G or F)	Herpes simplex virus type-1 Strain 17 Glasgow or Strain F
HSV-2	Herpes simplex virus type-2
HSV-1 PK	Herpes simplex type-1 protein kinase
HPLC	High performance liquid chromatography
HCMV	Human cytomegalovirus
kbp	kilobase pair
Mr	apparent relative molecular mass
m.u.	map units
PBS	Phosphate-buffered saline
p.f.u.	plaque-forming unit
PRV	Pseudorabies virus
PRV PK	Pseudorabies protein kinase
rpm	revolutions per min
SDS	Sodium dodecyl sulphate
TEMED	NNN'N'-tetramethylethylene-diamine
TK	Thymidine kinase
US3	The third open reading frame of the unique short (U_S) region of the HSV-1 genome
VZV	Varicella zoster virus
Vero cells	African green monkey kidney cells
ViPK	Virus-induced protein kinase

DEFINITION OF UNITS

$1A_{260}$ unit is the quantity of material contained in 1ml of solution which has an absorbance of 1 at 260 nm, when measured in a cell with 1 cm light path.

1 unit of protein kinase activity is defined as the amount of enzyme that catalyses the incorporation of 1 nmol phosphate per min into protein substrate under standard assay conditions.

SUMMARY

The subject of this thesis is a protein kinase that had previously been detected in cellular cytoplasmic extracts of cells infected with the α-herpesvirus, pseudorabies (PRV). The object of the work undertaken here was to purify the enzyme to allow its proper characterisation, and to determine its genetic origin.

The enzyme induced by infection of hamster fibroblasts with PRV was purified to apparent homogeneity by a combination of DEAE-cellulose, high-performance hydrophobic, threonine-sepharose, protamine-agarose and high-performance gel-exclusion chromatography. This purification resulted in an enzyme with a specific activity in excess of 1,000 nmol units/mg, a figure comparable with other homogeneous protein kinases. Polyacrylamide gel electrophoresis of the purified enzyme under denaturing conditions revealed a single stained band at a position of migration corresponding to an apparent molecular weight of 38,000. Polyacrylamide gel electrophoresis of the purified enzyme after incubation with [γ^{32}P]ATP in the absence of added substrate resulted in incorporation of ^{32}P into this protein band, consistent with the 38,000 molecular weight protein being a protein kinase with a capacity for autophosphorylation. The isoelectric point of the phosphorylated form of the enzyme was approximately 4.9. The molecular weight of the native enzyme was determined by gel-exclusion chromatography and found to be approximately 70,000. It is therefore likely that the protein kinase is homodimeric.

When hamster fibroblasts or Vero cells were infected with Herpes simplex type-1 (HSV-1) a new protein kinase activity was also detected. The protein kinases from cells infected with HSV-1 and PRV had similar

catalytic properties. Both catalysed the transfer of phosphate from ATP (but not GTP) to basic (but not acidic) proteins, protamine being the preferred substrate in vitro, and they appear to be independent of known regulatory molecules. These protein kinases were also active at 1M KCl, a particularly striking characteristic.

The protein kinase activity from cells infected with HSV-1 was partially purified using the methods used for the enzyme from cells infected with PRV. The final purified preparation contained two major species of apparent molecular weights 68,000 and 61,000 when analysed by polyacrylamide gel electrophoresis under denaturing conditions. In addition, when incubated with [γ^{32}P]ATP, the purified enzyme preparation phosphorylated a protein species of molecular weight 68,000 and isoelectric point 5.6. It is probable that this represents autophosphorylation of the HSV-1 protein kinase, which may be one of the two major species observed in the purified preparation. The molecular weight of the native enzyme was investigated by gel-exclusion chromatography and was found to be in the range of 150,000 - 200,000. This suggested that the enzyme may be a homodimer like the PRV protein kinase.

Synthetic peptides were used to investigate the substrate-specificity of the protein kinases. The enzyme from cells infected with PRV had similar substrate preferences to the enzyme from cells infected with HSV-1, but these were distinct from those of other known protein kinases. Both could catalyse the phosphorylation of both seryl and threonyl residues in peptides containing several arginyl residues on the amino terminal side of the target residue. At least two basic N-terminal residues were required and the best substrates contained four to six such residues. Other amino acids on both the N-terminal and C-terminal sides of the phosphate acceptor could exert a modulating influence on substrates for the viral kinases. There were indications that hydrophobic amino acids immediately adjacent to

the target residue on its N-terminal side caused a decrease in phosphorylation of the target residue by the viral kinases. The amino acid, proline, exerted a marked effect when adjacent to the target residue, depending on whether it was N or C-terminal. If it was positioned on the N-terminal side, phosphorylation was enhanced, but if present on the C-terminal side, phosphorylation of the synthetic peptide substrate was decreased. Acidic residues positioned on the C-terminal side of the target residue appeared capable of negative modulation of the phosphorylation of peptides by the viral kinases. The basic residues, lysine or ornithine, were much inferior to arginine as positive determinants for substrates of the viral protein kinases.

The amino acid sequences of proteins predicted to be viral phosphoproteins in HSV and VZV were examined for conserved phosphorylation sites suitable for the viral protein kinases. The only one that was identified was an immediate-early protein which from other considerations is unlikely to be the physiological substrate for this enzyme. It is therefore possible that the physiological substrate for the viral protein kinase is cellular.

Initial indirect experiments were consistent with the protein kinases being encoded by the viral genome. The enzyme activities obtained from cells infected with each virus differed in their chromatographic properties on anion-exchange and gel-permeation resins. The kinetics of induction of these enzymes were similar to the viral early protein, DNA polymerase. The amount of enzyme detected depended on the multiplicity of infection, and no enzyme was detected in cells infected with virus which had been irradiated with ultra-violet light. When cells were infected with HSV-1 strain tsK, which has a temperature-sensitive mutation in the gene for the immediate-early protein required for transition to early protein synthesis, enzyme was induced at the permissive but not the restrictive temperature.

Direct evidence that the protein kinase detected in cells infected with HSV-1 was encoded by the viral genome was obtained from exploring the relationship of this protein kinase to the protein predicted to be encoded by gene US3, as the latter contained motifs found in known protein kinases. Hamster fibroblasts and Vero cell lines were infected with a mutant of HSV-1 with a genetically engineered deletion in the US3 open reading frame. The protein kinase was not present in cells infected with the deletion mutant but was detected when cells were infected with a recombinant in which the US3 open reading frame had been rescued.

Confirmation that the protein kinase was the product of the US3 gene was obtained using an antiserum to a synthetic oligopeptide corresponding to the carboxy-terminal eight amino acids of the predicted US3 protein. This antiserum reacted weakly with a polypeptide of 68,000 molecular weight in extracts of cells infected with HSV-1, and with increasing intensity with fractions of increasing specific activity from the enzyme purification. The immunoreactive species in the purified preparation corresponded in electrophoretic mobility to the phosphorylated protein species of 68,000 molecular weight obtained by incubation with $[\gamma^{32}P]$ATP in the absence of exogenous substrate.

This demonstration that the α-herpesviruses encode a protein kinase provides the first example of an authentic protein kinase encoded by a eukaryotic virus (the protein kinases of certain acute transforming retroviruses being properly regarded as the products of transduced cellular genes). Although the experiments with the deletion mutant indicate that the protein kinase is not essential for growth of the virus in tissue culture cells, the conservation of this function between human and pig α-herpesviruses implies that it confers a selective advantage for the virus, even though the nature of this is unknown at present.

INTRODUCTION

I PROTEIN KINASES AND PROTEIN PHOSPHORYLATION

Protein phosphorylation is of central importance in the response of cells to various internal and external signals. Many cellular enzymes involved in the control of metabolic pathways can exist in either a phosphorylated or a dephosphorylated form, and their activity is regulated by this reversible phosphorylation. An example of this is the control of phosphorylase kinase activity by cyclic AMP - dependent protein kinase (Cohen, 1985; Krebs, 1985). Proteins other than enzymes are also regulated by phosphorylation. Examples include the phosphorylation of histone H1 in the nucleus, which is thought to control chromosome condensation (Matthews, 1980), and muscle myosin light chain, the degree of phosphorylation of which has been implicated in the control of muscle contraction (England, 1980). More recently it has been shown that the lamins, proteins of the nuclear envelope, are also phosphorylated, and it is thought that this may control the breakdown of the nuclear envelope during mitosis (Fisher, 1987).

The phosphorylation and dephosphorylation reactions are catalysed by two different types of enzymes, and it is the differential modulation of the activity of these enzymes that determines the extent of phosphorylation and hence regulates the function of a particular protein. The enzymes which catalyse the phosphorylation event are the protein kinases, and those responsible for dephosphorylation are the phosphoprotein phosphatases.

There are many more protein kinases than phosphatases known, and this seems to reflect a greater substrate-specificity in the former class of enzyme. Each protein kinase can catalyse the phosphorylation of

either a single protein or a restricted range of different proteins, although sometimes one protein serves as a substrate for more than one kinase. In contrast most phosphoprotein phosphatases have a much wider substrate range, although some more specific ones have been reported (Ingebritsen and Cohen, 1983).

From these considerations it would be expected that the control of the phosphorylation reactions is primarily through the control of protein kinases, and this indeed appears to be the case. Protein kinases catalyse the transfer of phosphoryl groups from ATP, or ATP and GTP, to seryl, threonyl or, in some instances, tyrosyl residues positioned in characteristic recognition sequences within the protein substrate. The protein kinases may be classified in relation to this acceptor amino acid residue as serine/threonine protein kinases or tyrosine protein kinases. A variety of effectors can regulate the activity of different protein kinases. These effectors can be hormones or growth factors (in the case of membrane receptor kinases), second messengers (cyclic AMP, cyclic GMP, diacylglycerol) or small intracellular molecules (e.g. Ca^{2+}, haemin, double-stranded RNA, acetyl CoA). Many protein kinases have the ability to transfer phosphoryl groups from ATP to their own hydroxyl residues in an intramolecular reaction; and this phenomenon, which may be a fundamental general feature of these enzymes, is termed autophosphorylation.

The various different aspects of protein kinases, which are discussed in more detail below, can be used to classify these enzymes; and one such classification is shown in Table I1.

1.1 Regulation

The effectors that regulate protein kinase activity may arise from extracellular signals, either directly *via* first messengers or indirectly *via* second messengers; or they may be intracellular, either small non-enzymic molecules or other protein kinases. Many protein kinases do not appear to be regulated by any effector molecules, although for other protein kinases there may well be effectors, as yet unknown, governing their activity.

Protein kinases regulated by first messengers are the hormone or growth factor receptor kinases. Examples of these are the receptors for insulin (Ullrich *et al.*, 1985), epidermal growth factor (Cohen *et al.*, 1982; Hunter *et al.*, 1984; Staros *et al.*, 1985), platelet-derived growth factor (Cooper *et al.*, 1982, Yarden *et al.*, 1986) and insulin-like growth factor (Ullrich *et al.*, 1986). The binding of the ligand appears to activate the intrinsic tyrosine protein kinase activity of the receptor, which is manifest experimentally as autophosphorylation of the receptor. It is at present uncertain whether the chain of intracellular events elicited by the hormone is a consequence of a conformational change in the receptor resulting from this autophosphorylation, or whether it results from the phosphorylation of an extrinsic substrate, the identity of which is still unknown.

Other protein kinases are regulated by a relatively small number of chemical mediators or second messengers, which can alter the phosphorylation states of many intracellular proteins in concert. Cyclic AMP regulates the activity of the cyclic AMP-dependent protein kinases. These enzymes exist in isoenzymic forms designated as type I and type II (Reimann *et al.*, 1971) consisting of two catalytic

subunits and two regulatory subunits which bind the effector. In the absence of cyclic AMP the regulatory (R) and catalytic (C) subunits have a high affinity for each other, but on binding the nucleotide this affinity is decreased by a factor of 10^4. The holoenzyme (R_2C_2) is inactive because a part of the regulatory subunit directly impedes the active site of the catalytic subunit. It has been proposed that cyclic AMP binds to the R subunit of R_2C_2 forming an intermediate ternary complex: cyclic AMP. R_2C_2. A conformation change then occurs in the R subunits, and they dissociate from the C subunits. [R_2 (cyclic AMP)$_4$ + 2C] (Hoppe, 1985). Cyclic GMP can also regulate protein kinase activity. The cyclic GMP-dependent protein kinase consists of two identical subunits, and upon binding of cyclic GMP activation of the enzyme occurs without physical dissociation of the subunits:

inactive E_2 + 2 cyclic GMP $\rightleftharpoons$ E_2 - cyclic GMP_2 active

Intracellular regulation of protein kinases can be achieved by small non-enzymic effectors. Ca^{2+} ions activate many protein kinases, either alone or in association with calmodulin, a Ca^{2+} - binding protein. Indeed one of the subunits of phosphorylase kinase (Jesse - chan et al., 1982), is itself calmodulin (Cohen et al., 1978). This enzyme can be activated by the binding of Ca^{2+} to its calmodulin subunit (δ), and the subsequent initiation of a change in the conformation of the enzyme. Two types of myosin light-chain kinase (Stull et al., 1985) and a less specific Ca^{2+}/calmodulin-dependent protein kinase, designated as the multifunctional protein kinase by many investigators (McGuiness et al., 1983; 1985; Woodgett et al., 1984), are all activated by Ca^{2+} ions. Another Ca^{2+} ion-dependent

serine protein kinase, but one that does not utilise calmodulin, is protein kinase C, discovered by Nishizuka and co-workers (Ioune et al., 1977; Takai et al., 1977; Nishizuka, 1983). The principal effector controlling the activity of protein kinase C in vivo is thought to be diacylglycerol (Nishizuka, 1984). Phorbol esters have been shown to mimic diacylglycerol in this respect (Castagna et al., 1982).

There are other protein kinases regulated by small molecules, including double-stranded RNA, haemin, and metabolites involved in the pyruvate dehydrogenase reaction. These latter protein kinases are examples of the enzymes with the narrowest substrate specificity.

Protein kinases themselves may act as catalytic effectors for other protein kinases, as exemplified by phosphorylase kinase. This latter enzyme is not only regulated by Ca^{2+} ions but also by phosphorylation - dephosphorylation, the effector kinase responsible for this being the cyclic AMP-dependent protein kinase. Not only phosphorylation by other protein kinases, but also autophosphorylation has been implicated in the regulation of activity of certain protein kinases. An example of this is protein kinase C, where autophosphorylation increases the activity of the enzyme by decreasing (at least with the artificial substrate, histone H1) the Km for its substrate in vitro (Mochly-Rosen and Koshland, 1987). The potential biological importance of autophosphorylation has also been investigated by site-specific mutagenesis of potential autophosphorylation sites in the protein kinase product of the cell-cycle control gene, cdc 2, of

the fission yeast, Schizosaccharomyces pombe (Booher and Beach, 1986). It was reported that mutations within a region which may be the site of phosphorylation resulted in a loss of cdc 2 protein kinase activity. In the case of pp60 v-src, the tyrosyl protein kinase product of the v-src gene of Rous sarcoma virus (Collett and Erikson, 1978), the N-terminal region of this protein may regulate its catalytic activity since autophosphorylation of pp60 v-src in this locale is correlated with elevated protein kinase activity (Collett et al., 1984; Brown and Gordon, 1984). In addition, proteolytic removal of the entire N-terminal half of pp60 src resulted in increased activity (Levinson et al., 1981; Brugge and Darrow, 1984). It is becoming apparent that almost all cellular tyrosine protein kinases are subject to tight negative regulation by their non-catalytic domains, and these enzymes can be converted into transforming proteins by the removal of such negative regulatory elements. Similarly, a point mutation that changes Tyr 527 to Phe in the c-src gene-product can have this consequence (Hunter, 1987).

Finally there is a fairly large group of protein kinases for which no regulatory agents are known. These are termed independent protein kinases, examples of these being listed in Table I1. Included are the so-called casein kinases (casein is certainly not their physiological substrate), which are abundant and ubiquitous enzymes that do not appear to have any regulatory effectors; and the protein kinases encoded by several oncogenes, the effectors for which (for it is reasonable to assume there must be such) are as yet unknown.

1.2 Site-specificity

Of the factors responsible for the specificity of protein kinases, the primary structure around the phosphorylation site of the substrate plays an important role (Nimmo and Cohen, 1977; Pinna et al., 1979; Gould, 1985; Glass et al., 1986; Pinna et al., 1986; Michnoff et al., 1986; Ferrari et al., 1987). Table I2 illustrates this in summarising the results of experiments using synthetic peptides modelled on phosphorylation sites of proteins phosphorylated in vivo or in vitro by the particular protein kinase. Using these peptides it is possible to define the minimum primary structural requirements of the protein kinase for the particular substrate.

The substrate-specificity of cyclic AMP-dependent protein kinase has been particularly well defined: the enzyme requires two basic residues on the N-terminal side of the target residue and a hydrophobic residue immediately adjacent to the target residue is also required (eg RRAS). Serine residues are also preferred to threonine as targets by these enzymes, although phosphorylation of the latter does occur. Protein kinase C will phosphorylate peptides with blocks of basic residues on the C-terminal and N-terminal sides of the target. Other types of protein kinases, for example casein kinases I and II, do not have this preference for basic residues but phosphorylate substrates with large blocks of acidic residues clustered on the N-terminal side of the target, in the case of casein kinase I, or on the C-terminal side for casein kinase II (Marin et al., 1986; Pinna et al., 1986). Although the use of these synthetic peptides has provided information regarding primary

structure determinants of protein kinases, it should be pointed out that the substrate specificity of protein kinases is also determined by secondary, tertiary and quaternary structures of the protein. Examples of this are phosphorylase kinase and glycogen synthase kinase-3, the substrate specificities of which must rather be considered in terms of particular three-dimensional domains (Pinna et al., 1986).

Although some protein kinases (for example cyclic AMP-dependent protein kinase and casein kinase II) have quite broad specificities, it can be seen that they nevertheless are relatively easy to distinguish using artificial protein substrates of different amino acid compositions, or more precisely, using synthetic model peptides (Engström et al., 1984).

Table I2 does not include the most highly specific protein kinases such as those that phosphorylate eIF-2. Although the site phosphorylated in vivo is known in this (Kudlicki et al., 1987) and other similar cases, little work has been done to define which features of the environment of the target are the major determinants of specificity, and indeed, whether it is primary or higher-order structure that is involved.

1.3 Structure

Protein kinases have a wide variety of structures, as can be seen from Table I3: some are monomeric, eg. kinase C and casein kinase I, whereas others are multimeric. The simplest of these latter are homodimeric, for example the cyclic GMP-dependent protein kinases, whereas phosphorylase kinase, which has four different types of subunit all of different apparent molecular weights, is an example of

a more complex quaternary structure. Although protein kinases have a wide range of molecular weights and subunit structures, it is possible to identify within the primary structures of most protein kinases a regulatory domain or subunit, which is responsible for binding the effector, and a second domain (kinase domain) or subunit responsible for the catalysis (Fig I2).

(i) **Conserved domains in protein kinases**

The amino acid sequences of all eukaryotic protein kinases so far examined share a number of highly conserved motifs that are unique to, and diagnostic of, the catalytic domain of this class of enzyme (Barker and Dayhoff, 1982; Takio et al., 1984a). Examples of these motifs are shown in Fig I1.A. Within this overall homology certain subsidiary features even allow one to distinguish those protein kinases that phosphorylate tyrosyl residues from those that phosphorylate seryl and threonyl residues (Hunter and Cooper, 1985). This is clear on examination of the C-terminal domain (Fig I1B): certain amino acids are conserved in both nature and position in these kinases and the serine/threonine protein kinases do not share this homology.

In the context of the explosion in the availability of nucleotide sequence information and, with it, of predicted amino acid sequences, the recognition of the conserved motifs of the catalytic domain has provided an alternative route to the identification of additional members of the protein kinase family. This predictive approach has been exploited

successfully in the study of the oncogenic retroviruses (Sefton and Hunter, 1984; Hunter and Cooper, 1986), circumventing the often laborious protein purification procedures usually required in identification of protein kinases. In yeast several genes identified by mutant phenotypes in growth or progression through the cell cycle have recently been predicted to encode protein kinases on the basis of such sequence comparisons. (Lörincz and Reed, 1984; Patterson et al., 1986; Simon et al., 1986; Teague et al., 1986; Celenza and Carlson, 1986). In the case of CDC 28 (Reed et al., 1985) and SNF 1 (Celenza and Carlson, 1986) this prediction has been confirmed by subsequent characterisation of the gene-product.

(ii) **Divergent domains in protein kinases**

As well as conserved areas in their structure, protein kinases also display divergent structural domains. In some protein kinases it has been clearly demonstrated that the binding site for the effector is on a separate subunit to that containing the catalytic function. This is illustrated by cyclic AMP-dependent protein kinase, with its regulatory subunit, and phosphorylase kinase, with its Ca^{2+} - binding calmodulin subunit. The structure of the domain or subunit involved in binding the effector is highly specific and depends on the nature of the particular effector molecule. For instance protein kinases regulated by cyclic nucleotides, cyclic AMP-dependent protein kinases and cyclic GMP-dependent protein kinases, have characteristic nucleotide binding sites (Fig I2)

which have also been shown to share homology with the otherwise unrelated cyclic AMP binding protein (CAP) of E.Coli (Titani et al., 1984; Takio et al., 1984(a) and (b); Weber et al., 1982).

In other cases the evidence that the divergent domain binds the effector is more indirect. Thus, partial proteolysis of protein kinase C generates a fragment lacking the N-terminal divergent domain but still maintaining catalytic activity. This latter, however, is now constitutive, being independent of the effectors, Ca^{2+} and phospholipid (Parker et al., 1986). Perhaps surprisingly, examination of the primary structure of the C-terminal domain of protein kinase C revealed no similarity to that of the other well characterised Ca^{2+} - binding protein, calmodulin. However a possible similarity to the Ca^{2+} and phospholipid-dependent protein, phospholipase A2, has been pointed out (Maragonore, 1987).

TABLE I.1

CLASSIFICATION OF PROTEIN KINASES

EFFECTORS		KINASE	PHYSIOLOGICAL SUBSTRATE	RESIDUE PHOSPHORYLATED
I EXTRACELLULAR REGULATION	1ST MESSENGER: Insulin	Insulin receptor	?	Tyr
	Epidermal growth factor (EGF)	EGF receptor	?	Tyr
	Platelet-derived growth factor (PDGF)	PDGF receptor	?	Tyr
	Insulin-like growth factor (IGF-1)	IGF-1 receptor	?	Tyr
	2ND MESSENGER: Cyclic AMP (cAMP)	cAMP-dependent protein kinase I/II	Phosphorylase kinase Glycogen synthase and many others	Ser/Thr
	Cyclic GMP (cGMP)	cGMP-dependent protein kinase	? wide range	Ser/Thr
	Diacylglycerol	Protein kinase C	? wide range of substrates <u>in vitro</u>	Ser/Thr

(continued overleaf)

TABLE I.1 (cont.)

EFFECTORS		KINASE	PHYSIOLOGICAL SUBSTRATE	RESIDUE PHOSPHORYLATED
II INTRACELLULAR REGULATION	SMALL MOLECULES:			
	Ca^{2+}(calmodulin)	Phosphorylase kinase	Glycogen phosphorylase	Ser/Thr
		Skeletal muscle myosin light-chain kinase	Skeletal muscle myosin light chain	Ser/Thr
		Smooth muscle myosin light-chain kinase	Smooth muscle myosin light chain	Ser/Thr
		Protein kinase C	Wide range?	Ser/Thr
		Multifunctional Ca^{2+}/ calmodulin-dependent protein kinase	Wide range?	Ser/Thr
		Calmodulin-dependent glycogen synthase kinase	Glycogen synthase (Site 2)	Ser/Thr
	Double standed RNA (dsRNA)	dsRNA-dependent eIF2 kinase 1	Initiation factor eIF2	Ser/Thr
	Haem	Haem-regulated eIF2 kinase 2	Initiation factor eIF2	Ser/Thr
	Acetyl CoA, NADH, Pyruvate, ADP	Pyruvate dehydrogenase kinase	Pyruvate dehydrogenase	Ser/Thr
	Protein kinases cAMP-dependent protein kinase	Phosphorylase kinase	Glycogen phosphorylase	Ser/Thr

(continued overleaf)

TABLE I.1 (cont.)

EFFECTORS		KINASE	PHYSIOLOGICAL SUBSTRATE	RESIDUE PHOSPHORYLATED
III	Unknown or non-existent	Casein kinase I	Wide range?	Ser/Thr
		Casein kinase II	Wide range? including glycogen synthase site 5	Ser/Thr
		Glycogen synthase kinases 3 and 4	Glycogen synthase ?	Ser/Thr
		p60 src	Various proteins ?	Tyr
		Growth-associated protein kinases	Ribosomal protein S6 ?	Ser/Thr

TABLE I.2

THE SUBSTRATE-SPECIFICITY OF PROTEIN KINASES DEFINED BY SYNTHETIC PEPTIDES
(Data from Pinna et al., 1986)

Protein kinases	Examples of suitable peptide substrates	Suggested minimum primary structure requirement
Cyclic AMP-dependent PK	RRA<u>S</u>VA	R(K)RXS(T)XX
Cyclic GMP-dependent PK	RKR<u>S</u>RKE	R....S(T)R
Myosin light chain kinase	KKRPERAT<u>S</u>NVFA	$R(X)_{2-3}$RXXS(T)XXX
Multifunctional calmodulin-dependent protein kinase	PLSRTL<u>S</u>VSS	RXXS
Phosphorylase kinase	KRKQI<u>S</u>VRGL L<u>S</u>YRGYSL	SXR (higher structure required)
H4 Protein kinase (protease activated)	VKRI<u>S</u>GL	KRXS ....
Physiological casein kinase (Mammary gland)		$STXEDS^P$
Multifunctional/casein kinase I	EQLS^{P}TS^{P}EEN<u>S</u>KKTVDM	Acidic Cluster-SXX
Multifunctional/casein kinase II	<u>S</u>EEEEE <u>S</u>AAEEEEEE RREEE<u>T</u>EEE	$S(T)X_{(0-2)}$-$(EDS^P)n$ n = 3

(continued)

TABLE I.2 (CONTINUED)

Protein kinases	Examples of suitable peptide substrates	Suggested minimum primary Structure requirement
Pyruvate dehydrogenase kinase	YHGHSMSDPGVSYR	....SXXD....
Glycogen synthase kinase-3	Inactive on short peptides	Integrity of large domain
Tyrosine protein kinases	KLIEDNEYTAR	EX_nY
	DRVYIHPF	
Protein kinase C	GKGRGLSLSRFSWGA	
	GSRRRRRRY	$(R)_n$...S or T...(R)n
	RRLSSLRA	?

- denotes residues that are phosphorylated.

P denotes phosphoamino acid.

TABLE I.3. STRUCTURAL CHARACTERISTICS OF PROTEIN KINASES

Protein kinase	Monomer molecular weights	Subunit structure	Autophosphorylated subunits
Cyclic AMP-dependent protein kinases Type I and Type II	45,000 R 40,000 C	R_2C_2	C only Type I CR Type II
Cyclic GMP-dependent protein kinase	74-81,000 E	E_2	E
Phosphorylase kinase	α 118-145,000 β 108-128,000 γ 40,000 δ 17,000 (calmodulin)	$\alpha_4\beta_4\gamma_4\delta_4$	α and β
Myosin light-chain kinase (skeletal muscle)	70,000	monomer	+
Multi-functional protein kinase	isoform I 50,000 II 58-60,000	monomer	+
Protein kinase C	77-87,000	monomer	+
eIF-2 kinase	80-95,000	monomer	+
Casein kinase I	32-42,000	monomer	+
Casein kinase II	42-44,000α 38-40,000α' 24-26,000β	$\alpha\alpha'\beta_2$	β
$pp60^{v\text{-}src}/pp60^{c\text{-}src}$	60,000	monomer	+
EGF Receptor (A341 cells)	170,000	monomer	+
Insulin Receptor (adipocyte)	α 130,000 β 95,000 (glycoproteins)	$\alpha_2\beta_2$	$\alpha_2\beta_2$

Fig. I1A.

Highly conserved motifs in the catalytic domain of eukaryotic protein kinases

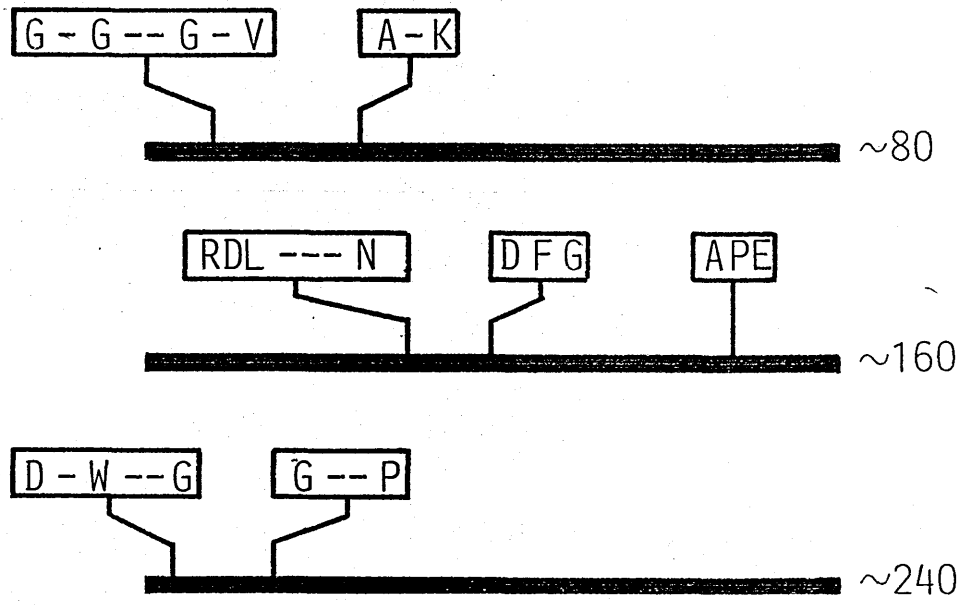


Fig. I.1B. Amino acid residues diagnostic of tyrosine protein kinases within conserved common regions of tyrosine and serine/threonine protein kinases

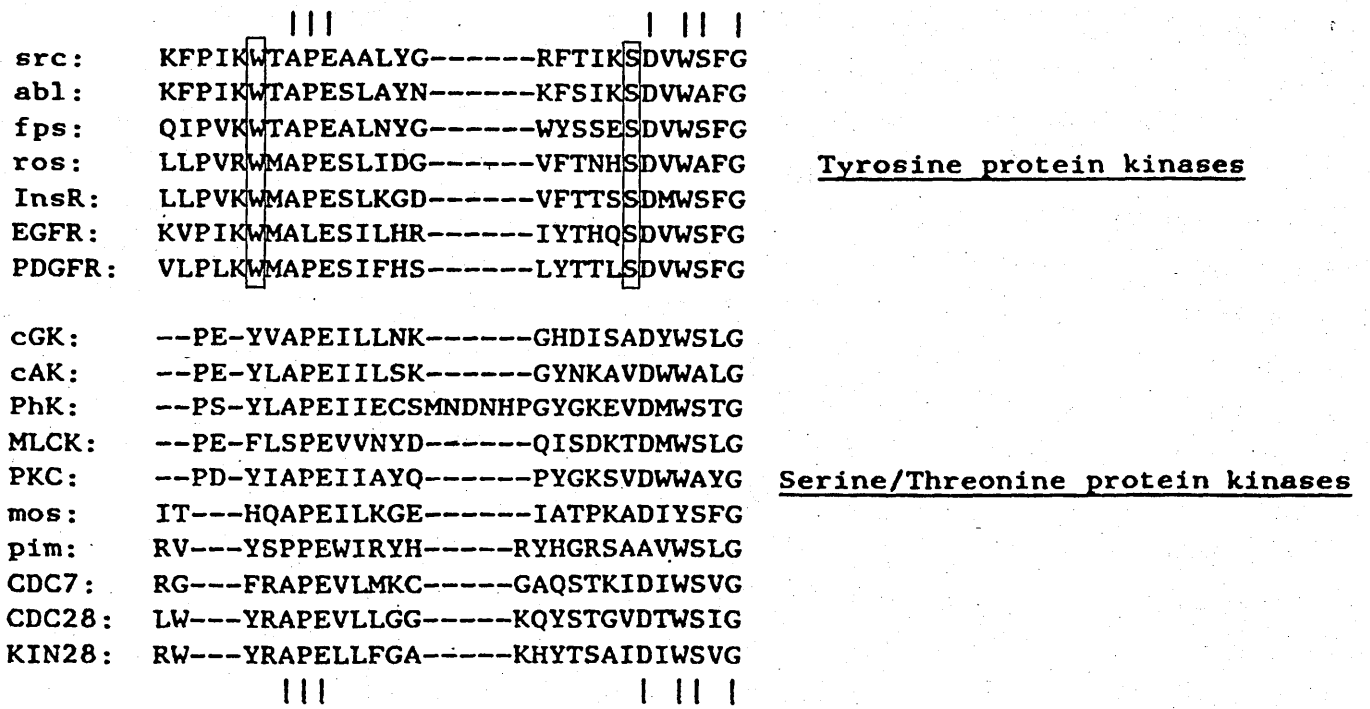
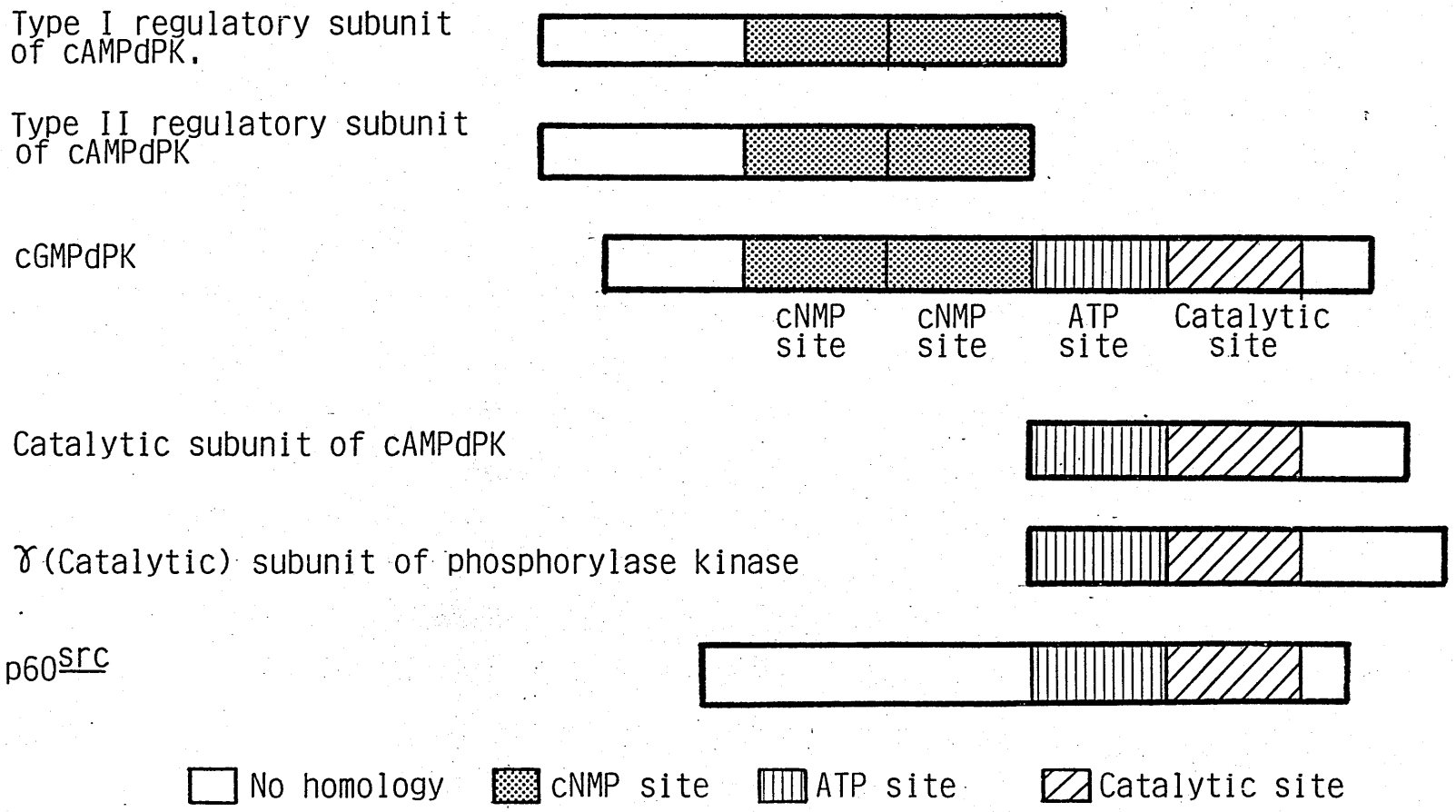


Fig I2. Structural comparison of some protein kinases



II THE GENETIC REPERTOIRE OF HERPESVIRUSES

The particular aspect of protein phosphorylation and protein kinases dealt with in this thesis relates to cells infected with certain α-herpesviruses. This section deals with some of the general characteristics of these viruses. Herpesviruses may be defined as viruses of eukaryotes, having single, large, linear, double-stranded DNA genomes, which are replicated and packaged into icosahedral nucleocapsids within the nuclei of infected cells. These then are enclosed in protein teguments and glycoprotein and lipid envelopes to give their infectious extracellular forms (Roizman, 1982). The participation of essential virus-coded gene products in the replication of virus DNA, the formation of concatameric replicative intermediates and their maturation into progeny genomes by site-specific recombination cleavage reactions should also be considered as characteristic features in the definition of herpesviruses. The sizes of the herpesvirus DNAs vary from Varicella zoster virus (VZV), of 125 kbp; Pseudorabies virus (PRV), of 150 kbp, Herpes simplex viruses type 1 and type 2 (HSV-1/2), of 155-158 kbp; Epstein Barr virus (EBV), of 179 kbp, to Cytomegalovirus (CMV), of 240 kbp.

Despite the similarity in the morphology of the capsid and envelope of different herpesviruses, the typical presence of nuclear inclusion bodies in infected cells, and the propensity of the viruses to establish latent and recurrent infections, the genomes of these viruses have undergone sequence and structural divergence among the different mammalian subgroups. The herpesvirus family is divided into three main subgroups: α, represented by HSV, PRV and VZV; β, CMV; and γ, EBV and herpes saimiri viruses. This

classification is based primarily on the size of the genome, other structure considerations, and (more recently) DNA homology. However it is equally valid from a biological standpoint; for example these groups show differences in host range, growth rate and cytopathology of infections. The best characterised herpesviruses belong to the α subgroup.

1 α-HERPESVIRUSES

The α-herpesviruses are exemplified by the intensively studied herpes simplex viruses associated with common oral (HSV-1) and genital (HSV-2) infections of man, and by the causative agent of human chicken pox and shingles (VZV). Considerable work has also been performed on pseudorabies virus (PRV), an α-herpesvirus of swine, also known as Suid herpesvirus 1, and the causative agent of Aujeszky's disease.

(i) Genome organisation

The genomes of HSV-1 and HSV-2 consist of two components, long (L) and short (S), comprising 72% and 18% of the total genome, respectively. Both L and S can invert relative to one another such that viral DNA extracted from infected cells or virions consists of 4 isomers: an arbitrarily designated prototype (P), inversion of the S component (I_S), inversion of the L component (I_L), and inversion of both components (I_{SL}) (Hayward, 1975; Roizman, 1979). Each component consists of unique sequences (U_L or U_S) flanked by inverted repeats (Fig I3). The inverted repeats of the L component were designated as ab and b'a', whereas those of the S component were designated a'c' and ca (Wadsworth et al., 1975). In addition to the inverted repeats, HSV DNA contains a direct terminal redundant sequence of approximately 400 bp. This portion is also present within the internal inverted repeats and is referred to as the sequence 'a' (Davison and Wilkie, 1981). The 'a' sequence has been shown to contain a cis-acting site for the inversion of L and S components relative to one another (Mocarski et al., 1980; Mocarski and Roizman, 1981.

Chou and Roizman, 1985), this site being involved in the cleavage of concatemeric DNA and packaging of unit-length genomes into capsids (Vlazny et al., 1982). The 'b' sequence of the inverted repeats of the L component also contains cis-acting sites for the inversion of HSV DNA sequences (Longnecker and Roizman, 1986). The structure of the genome of PRV and VZV is very similar to that of HSV-1 and HSV-2 except that L is not bounded by inverted repeats and does not invert, consequently virion DNA populations contain only two types of molecule (Stevely, 1977; Henry et al., 1981; Straus et al., 1982).

The HSV-1 genome contains approximately 80 genes (Honess and Roizman, 1973; McGeoch et al., 1985). These genes form several groups: α (immediate-early), β_1 and β_2 (early) and γ_1 and γ_2 (late).

The expression of these is coordinately regulated and sequentially ordered in a cascade fashion (Honess and Roizman 1974; 1975). The 5 α genes (see Table I4 for nomenclature) are the first genes to be expressed after infection and are transcribed by the host RNA polymerase II in the absence of viral protein synthesis de novo. A functional α4 gene product is required for the transition from α to β gene expression (Knipe et al., 1979; Dixon and Schaffer, 1980; Preston, 1981). Genes α27 and α22 play a role in late gene expression (Sacks et al., 1985; Sears et al., 1985) but the functions of α0 and α47 are unknown. Whereas HSV-1 has five α genes (Fig I4), PRV has only one, which is in a corresponding genomic location (in the R_S region) and shares

sequence homology with α4 of HSV-1 (Ben-Porat et al., 1983; Davison and Wilkie, 1983). The PRV immediate-early protein (IE 63, Mr 180,000), like α4 of HSV-1, is known to be required to initiate the early and late phases of viral gene expression (Ihara et al., 1983), but unlike α4 it does not appear to be required for multiple reinitiation events from early genes (Ahlers and Feldman, 1987).

(ii) **Comparison of the organisation of the genes in the unique long region of α-herpesviruses**

In recent years DNA sequence analysis has allowed precise comparisons between some corresponding regions in the genes of HSV-1, HSV-2, VZV and PRV. The genome of PRV is for the most part colinear with the I_L arrangement of the genome of HSV-1; however an inversion or translocation of sequences mapping on the PRV genome between 0.07 and 0.39 map units was observed and a comparison of the map positions of 5 genes with known functions confirmed these findings. The genes coding for the major immediate-early protein, the major capsid protein and the thymidine kinase occupy similar positions on the genome of PRV and on the genome of HSV-1 in the I_L arrangement. However the genes for DNA polymerase and for the major DNA binding protein appear to be inverted relative to one another on the genomes of the two viruses (Ben-Porat et al., 1983).

The complete sequence of VZV has been published and the genes are numbered from 1-71 (left to right) starting from the extreme end of the L region (Davison and Scott, 1986). In the genome of VZV the DNA polymerase (gene 28) and the major DNA binding protein (gene 29) are in the same order as in the PRV

genome. However the major capsid protein has been displaced from the position it occupies in PRV and HSV-1 relative to these two genes, so that it is VZV gene 40. The two regions of difference between the VZV and HSV-1 genomes are present at the ends of the L-segment, which have been sequenced in HSV-1. HSV-1 appears not to contain homologues to VZV gene 1, gene 2 and gene 61. Also the inverted repeats flanking U_L are much larger in HSV-1, at approximately 9000 bp, than they are in VZV (88.5 bp), and the single gene thus far identified from the HSV-1 sequence of this region specifies a spliced immediate-early mRNA encoding α0. At the present stage of analysis no homologue for this gene has been detected in VZV. Thus the differences in gene arrangement between VZV and HSV-1 in the S segment and at the ends of the L segment result in VZV apparently lacking homologues to two of five HSV-1 immediate-early genes; those encoding α47 and α0. There is only one other region for which sufficient HSV-1 data are available to indicate a difference in gene layout between VZV and HSV-1. It is almost certain that HSV-1 lacks a homologue to VZV gene 13, which encodes a protein with thymidilate synthetase activity (Thompson et al., 1987).

(iii) **Comparison of the organisation of the genes in the unique short region of α-herpesviruses**

At present the largest published HSV-1 sequence is the short region of 26,000 bp (McGeoch et al., 1985; 1986 a), comprising the short unique sequence together with the two flanking short repeat sequences. The U_S region of HSV-1 is almost 13,000 bp and contains 12 open reading frames. The genes are transcribed

as 13 mRNAs, one gene being transcribed from two different initiation sites (McGeoch et al., 1985). None of these mRNA's are spliced, the splicing of mRNAs already mentioned for α0, being a distinct rarity in α-herpesviruses. A region of the U_S portion of HSV-2 has been partially sequenced (Rixon and McGeoch, 1985; McGeoch et al., 1987). This region contains six genes (termed US2 to US7 and a part of another, US8). The HSV-1 and HSV-2 sequences were found to be colinear and related in predicted amino acid sequence, except for the US4 genes.

In PRV a section of the U_S region has also been sequenced which contains genes from positions corresponding to US4 to US8 of HSV-1 and HSV-2, except that there is no homologue of gene US5. The gene in the position corresponding to US4 has a predicted protein product homologous to that of the corresponding gene in HSV-2 and not to that of HSV-1 (see Fig I5).

In the case of VZV there are greater differences from the S segment of HSV, which in HSV-1 contains 13 unique genes and in VZV only seven. Each VZV gene has a homologue in HSV-1 but the remaining 6 HSV-1 genes have no counterparts in VZV. The missing genes include US12 (the immediate-early gene, which encodes α47) and US6 (the gene which encodes glycoprotein D). These regions of the two genomes differ substantially in layout (c.f. Table I6), but they are clearly related.

(iv) **The open reading frame US3 of HSV-1**

Several of the open reading frames that became apparent from sequencing the U_S region of HSV-1 did not correspond to known viral proteins. Examination of the predicted amino acid

sequences allowed some general conclusions (eg membrane-association, likely glycoprotein nature), but more precise prediction of function required comparison with the sequences of proteins of known function. It was found (McGeoch and Davison, 1986) that the amino acid sequence of the 53 kDa predicted product of the US3 gene of HSV-1 contained most of the sequence motifs characteristic of protein kinases already mentioned in section 1.1 (Fig I1.A). Subsequently the corresponding region in the genome of HSV-2 was analysed and it was predicted to encode a protein of identical size, exhibiting 75% amino acid sequence identity to the predicted HSV-1 US3 gene product. The predicted product of the US3 gene of HSV-1 was also homologous with the 44 kDa predicted product of gene US2 (gene 66) of VZV (Davison, 1983; Davison and McGeoch, 1986). The predicted VZV protein kinase differs completely from that of HSV-1 in the N-terminal region, but in the C-terminal catalytic domain the two predicted protein kinases show 45% identity (Fig I6). The relationship of these predicted proteins to the conserved motifs of the known protein kinases is shown in Fig I7. Because the sequence of the entire genome of VZV is known it is possible to state with certainty that US2 is the only VZV gene predicted to encode a protein kinase. As regards HSV-1 it can only be said that no open reading frame yet sequenced, other than US3, has been predicted to encode a protein with homology to protein kinases. It should be stressed that at the time of their proposal as protein kinases, none of these predicted gene products has been recognised as a protein species in infected cells.

2 COMPARISON OF GENE ORGANISATION OF α-HERPESVIRUSES WITH β-AND γ-HERPESVIRUSES

The extensive accumulation of nucleotide sequences has allowed direct genetic comparisons, not only within the herpesvirus subfamilies, but also between the different subfamilies. Baer et al. (1984) reported the first complete sequence of a herpesvirus genome, that of Epstein-Barr virus (EBV), a human pathogen belonging to the γ-herpesvirinae. Comparison with the sequences which became available for genes from HSV-1 and HSV-2 gave the first clear evidence of genetic similarity between members of different subfamilies. Of the HSV genes which have been sequenced to date many have identifiable counterparts in EBV, and these are illustrated in Table I5 and I6. Thus there is compelling evidence that HSV and EBV arose from a common progenitor.

Computer comparisons of the amino acid sequences of proteins predicted from the published complete VZV and EBV DNA sequences allowed the identification of EBV counterparts to 29 of 67 unique VZV genes. Conserved genes were detected only in the U_L component of each genome, and were located in three major regions. Within each of these regions the conserved genes are generally colinear (Davison and Taylor, 1987), but the three regions are arranged differently with respect to one another in the two genomes. No conserved genes were detected in the U_S region, in the repeated regions or in sequences near the ends of the U_L. This is not particularly surprising as these regions are also among the most distantly related in the two α-herpesviruses, VZV and HSV-1.

The 20,349 bp sequence of the human cytomegalovirus (HCMV) Hind III F fragment of the U_L segment (approximately 60-80 kb of 235 kb total genome size) has revealed 8 open reading frames with homology to HSV and/or EBV. With respect to EBV, these homologous genes can be divided into two blocks: one block contains three genes, including the DNA polymerase and glycoprotein B, and the other block contains five genes of unknown function. Although the relative organisation of genes within each block is identical in HCMV and EBV, the relative position of each block within the two genomes differs. In HCMV the two blocks are present directly adjacent to each other, whereas in EBV they are found 92 kb apart (Kouzarides et al., 1987). The U_S region of HCMV has also been determined (Weston and Barrell, 1986), but it is completely unrelated to that of EBV, consistent with the pattern already seen in other comparisons between α-herpesviruses from different subgroups.

The difference between the S regions of α-, β- and γ-herpesviruses could mean that genes from this region in α-herpesviruses have counterparts in the L region of the β- and γ-herpesviruses. However, this is not the case with HSV-1 and EBV, and in particular it should be stressed that there is no predicted protein kinase in EBV. One cannot yet be sure of the situation in β-herpesviruses, but it may be that a protein kinase gene is a feature unique to α-herpesviruses.

TABLE I.4 THE NOMENCLATURE OF HSV-1 IMMEDIATE-EARLY PROTEINS

IE number	α number	Vmw number
IE1	α0	Vmw 110
IE2	α27	Vmw 63
IE3	α4	Vmw 175
IE4	α22	Vmw 68
IE5	α47	Vmw 12

The table correlates the different nomenclatures applied to the five immediate-early proteins (IE 1-5) of HSV-1. The α-numbering system relates to the new proteins of infected cells (ICP) observed on SDS gel electrophoresis and numbered in order of decreasing size (Wilcox et al, 1980). The Vmw system numbers the proteins by their apparent Mr ($x10^{-3}$) as deduced from similar SDS gel electrophoresis. (Haar and Marsden, 1981).

TABLE I.5 COMPARISON OF THE ORGANISATION OF THE GENES IN THE UNIQUE LONG (U_L) REGION OF HERPESVIRUSES

Position in in HSV-1 genome (m.u.)	HSV-1 sequence identification of protein	VZV homologue identified (Davison and Scott, 1986)	PRV homologue identified	Possible EBV homologue (Davison and Taylor, 1987)	Refs
0.08 - 0.185	IE-1 (ICP0) α0, Vm110 Immediate-early protein				1, 32, 37, 52
0.155 - 0.185	Alkaline Exonuclease ICP19	Gene 48		Moderate BGLF 5	12, 26
0.2 - 0.3	Major capsid protein	Gene 40		Moderate BCLF 1	12, 34
0.282 - 0.308	Glycoprotein H 90 kDa	Gene 37 Glycoprotein III		Weak BXLF 2	19, 27
0.305 - 0.315	Thymidine kinase	Gene 36 (Davison and Taylor, 1987)	(0.43 – 0.45 m.u.)	Weak BXLF 1	28, 42
0.348 - 0.368	Glycoprotein B 83,845 Mr	Gene 31	Glycoprotein II (0.1 - 0.130 m.u.)	Moderate BALF 4	5, 14, 21, 24
0.36 - 0.38	ICP 18.5 Affects transport of virion glycoproteins				30
0.411 - 0.386	Major DNA Binding protein ICP 8 (130 kDa)	Gene 29		Weak BALF 2	11, 22, 23 36, 43

TABLE I.5 (cont.)

Position in in HSV-1 genome (m.u.)	HSV-1 sequence identification of protein	VZV homologue identified (Davison and Scott, 1986)	PRV homologue identified	Possible EBV homologue (Davison and Taylor, 1987)	Refs
0.411 - 0.434	DNA Polymerase	Gene 28		Strong BALF 5	7, 8, 10, 36
0.525 - 0.647	DNA Binding protein ICP 34/35				41
0.54 - 0.63	Glycoprotein C	Gene 14 Glycoprotein V	Glycoprotein III 51kDa		14, 51
0.566 - 0.602	Ribonucleotide reductase ICP 6 38 kDa 144 kDa	Small subunit = Gene 18 Large subunit = Gene 19		Strong small subunit = BARF 1 Large subunit = BORF 2	32
0.64 - 0.69	Major Tegument protein (65K)				6, 13
0.701 - 0.711	dUTPase (39K)	Gene 8		Weak BLLF2	16, 20
0.74 - 0.75	IE-2 (ICP27) α27 Vmw 63	Gene 4		weak BMLF-1	1,32,37,52
TRs	DNA binding proteins and ICP 4 (IE3)	Gene 62 Gene 71	Immediate-early (180 kDa)		38, 39

TABLE I.6 COMPARISON OF THE ORGANISATION OF THE GENES IN THE UNIQUE SHORT (U_S) REGION OF α-HERPESVIRUSES

gene (Us)	protein	No of amino acids	unprocessed Mr	identity	VZV homologue (Davison & Scott, (1986)	PRV homologue	Refs
1	IE 68 (α22)	420	46,521	Immediate-early protein α22	Gene 63 Gene 70		25,38
2	32K	291	32,468	membrane protein			25
3	53K	481	52,831	Protein kinase	Gene 66 US2, 44K		25,46
4	25K	238	25,236	Glycoprotein G			17,25 45,50
5	9K	92	9,555	possible membrane protein			25
6	gD	394	43,344	Glycoprotein D (virion surface)		Glycoprotein gp50 (50K)	15,25 44,47
7	41K	390	41,366	Glycoprotein I	Gene 67 glycoprotein IV	Glycoprotein gp63	25,47 53
8	gE	550	59,090	Glycoprotein E (virion surface)	Gene 68 glycoprotein gI	Glycoprotein (122K) gP I (gpA)	25,47 48
9	10K	90	10,026	Tegument protein	Gene 65 11K (US1)	11K Protein	25,18, 54
10	34K	312	34,053	structural protein			25
11	18K	161	17,756	DNA binding protein			25
12	IE 12 (α47)	88	9,792	Immediate-early protein α47			25
(Additional) no homologues						glycoprotein X gX	49

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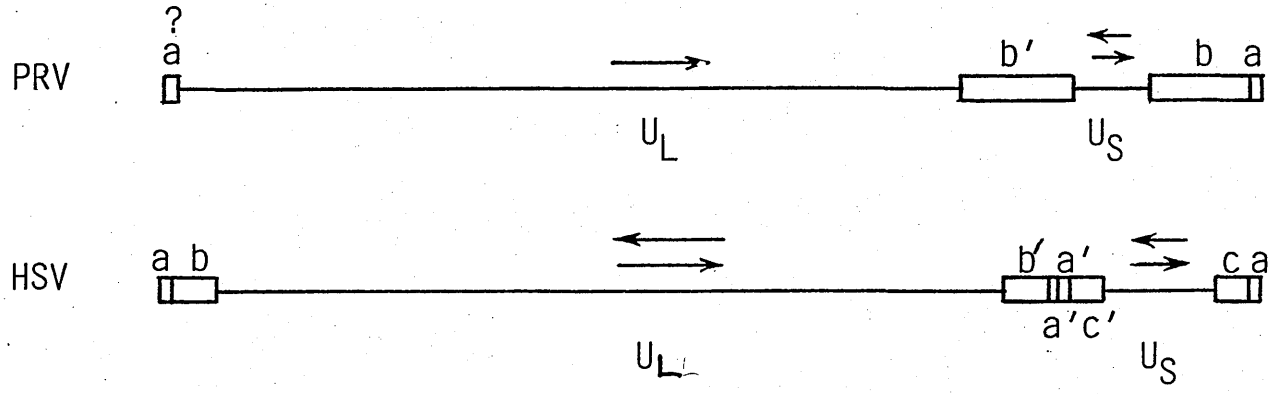


Fig. I3. Diagrammatic illustration of the genomic arrangement of α-herpes viruses. The horizontal arrows above the linear forms indicate the possible orientations of the U_S and the U_L regions.

Fig. I4. Location of immediate early genes in the HSV-1 genome.

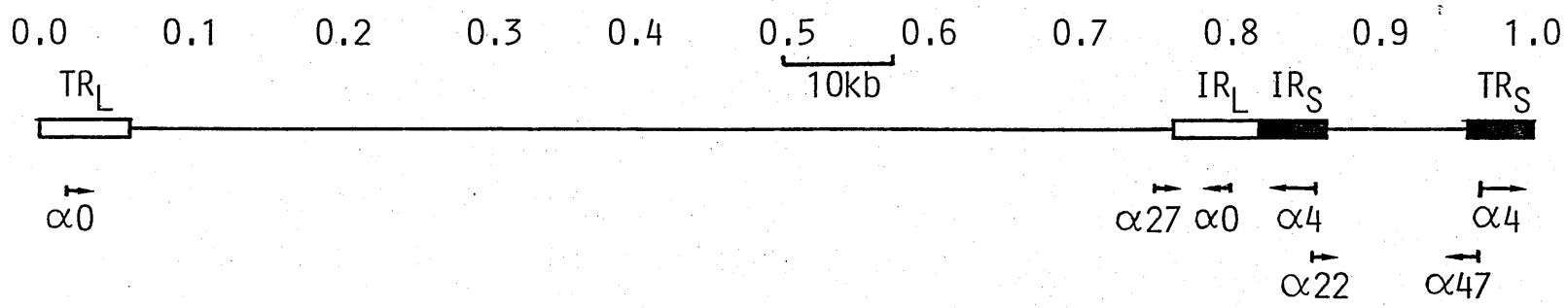
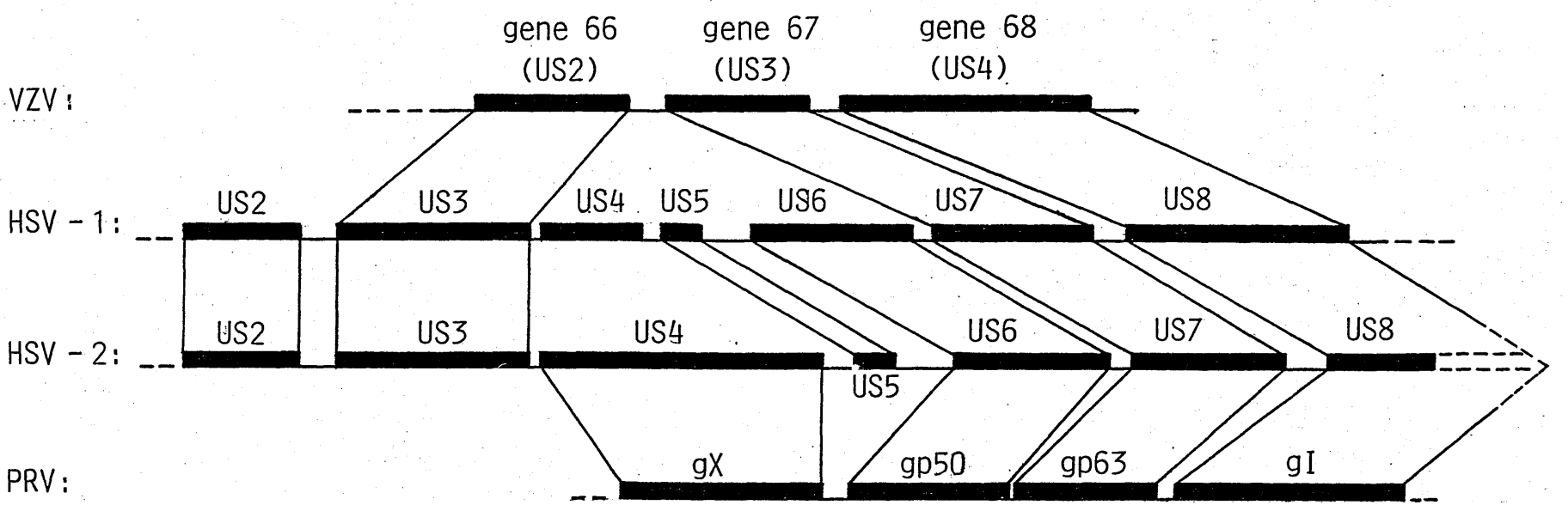


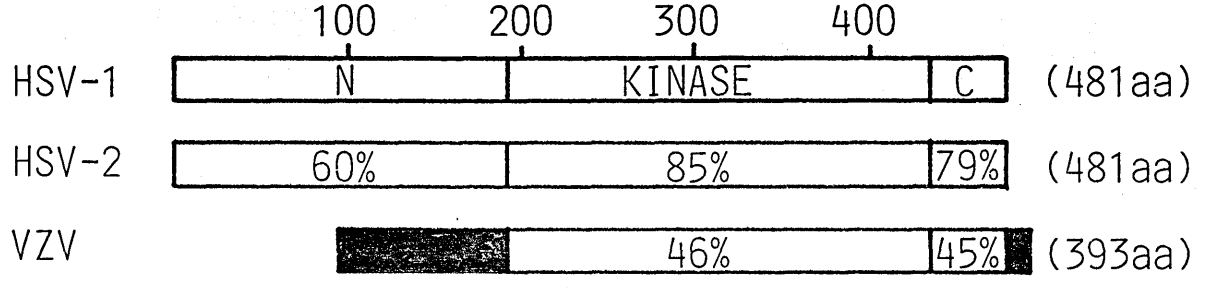
Fig. I5. Comparison of genes in the U_S region of different α-herpesviruses.



These genes the conceptual translation products of which are homologous have been joined together by vertical lines.

Fig. I6.

Comparison of protein kinases from three α-herpesviruses



The sizes (the scale is of numbers of amino acids) are those deduced from the nucleotide sequences, and the proteins have been divided into N-terminal, kinase and C-terminal domains. The percentage identity of the HSV-2 and VZV sequences to that of HSV-1 are indicated numerically, completely different regions being represented by solid shading.

Fig. I.7. Status of conserved amino acid motifs of protein kinases in the conceptual translation products of the US3 genes of HSV-1 and HSV-2, and gene 66 of VZV.

ATP binding domain

Consensus:	....G-G--G-V.......A-K.......
HSV-1 US3:	....TPGSEGCV.......IVK.......
HSV-2 US3:	....IPGSEGCV.......IVK.......
VZV Gene 66:	....TPGAEGVA.......VIK.......

Other conserved motifs

Consensus:	..H------RDL---N......APE.......D-WS-G..
HSV-1 US3:	..H------RDIKTEN......APE.......DIWSAG..
HSV-2 US3:	..H------RDIKTEN......APE.......DIWSAG..
VZV Gene 66:	..H------RDIKSEN......SPE.......DIWSAG..

3 PHOSPHORYLATION IN CELLS INFECTED WITH HERPESVIRUSES

The fact that the US3 gene of HSV-1 is homologous to known protein kinases makes it necessary to consider what is known regarding phosphorylation in cells infected with herpesviruses. In other viruses phosphorylation of proteins has been implicated in their replicative cycles: for example, in the interaction of structural virion proteins with the RNA of murine retroviruses (Sen, 1977), and in the control of the activity of the RNA polymerases of vesicular stomatitis virus (Kingsford and Emerson, 1980), and influenza virus (Kamata and Watanabe, 1977). Although there are only a small number of examples of viral protein phosphorylations for which specific functions have been assigned, there are very many phosphorylated regulatory and structural proteins in animal viruses for which the phosphorylation may turn out to be functionally important (Jeng et al., 1977; Russell and Blair, 1977; Clinton et al., 1979; Wilcox et al., 1980).

During the lytic infection of cells by herpesviruses the phosphorylation of many viral proteins occurs (Pereira et al., 1977; Marsden et al., 1978; Wilcox et al., 1980; Faber and Wilcox, 1986; Perry et al., 1986). Examples of the known HSV-1 phosphoproteins are shown in Table I7. Some cellular proteins are also phosphorylated (Fenwick and Walker 1979), one of which has been identified as being the ribosomal protein S6 (Kennedy et al., 1981).

Important to the understanding of the significance of these different phosphorylations is the identification of the protein kinases responsible for catalysing them. Such protein kinases might be of cellular origin or be specified by the particular virus itself. As

already mentioned in the Introduction (Section I), the cellular protein kinases constitute a large and diverse family of enzymes, differing in their substrate specificities, regulatory control and the amino acids that they phosphorylate. Cellular enzymes are thought to be responsible for several of the protein phosphorylations that occur in infected cells. The protein kinase activity responsible for the phosphorylation of the major phosphorylated species of the virions of PRV and HSV-1 is indistinguishable from host casein kinase II (Stevely et al., 1985), and the kinase responsible for the increased phosphorylation of ribosomal protein S6 during infection of BHK cells with PRV is almost certainly the specific cellular S6 kinase, which is also activated by growth stimuli (Jakubowicz and Leader, 1987 (a) and (c)).

As regards virally-coded protein kinases in general, it is well-established that the tyrosine protein kinase, pp60 $^{v\text{-}src}$, is the product of the src gene of Rous Sarcoma Virus (Collett and Erikson, 1978; Levinson et al., 1978). Such highly oncogenic retroviruses as Rous sarcoma virus represent a distinct group in the retrovirus family with respect to the organisation of their genomes, and their ability to cause acute diseases with a short latent period (Cooper and Hunter, 1983). These genomes are chimeric, with both termini being derived from a non-defective weakly-oncogenic retrovirus but the central part of the genome containing sequences originating from a transduced cellular gene or genes. This part of the genome, which is responsible for transformation, is termed the viral oncogene. Many of the viral oncogenes encode tyrosine-specific protein kinases (Sefton, 1985), although other viral oncogenes such as v-mos and

v-raf encode proteins that are serine/threonine protein kinases. However these retroviral protein kinases cannot be regarded as normal viral proteins. The genes which code for them originate from host cells, and in most cases have actually inactivated or replaced a retroviral gene, so that a "helper" virus is required for infection. Although it can be argued that viral enzymes such as thymidine kinase were probably also originally aquired from the host genome, the situation with the oncogenic retroviral protein kinases is clearly different. What is important here is not a single enzymic function but an ability to induce cellular transformation, as exemplified by the fact that other viral oncogene products such as those of ras and fos are not protein kinases at all.

Until the identification of the open reading frame US3 in the genome of herpesviruses there was no convincing evidence for a protein kinase encoded by a eukaryotic virus as an inherently viral protein. Attempts had previously however been made to identify protein kinases that might be encoded by herpesviruses, and these employed three main approaches, which will be considered in turn in relation to possible candidates for the product of the US3 gene.

One approach has been to examine the phosphorylating ability of immune-precipitated viral antigens, the substrate being either the putative kinase itself ("autophosphorylation reaction"), the immunoglobulin, or added exogenous protein. Although this approach was originally applied successfully to the v-src kinase (Collett and Erikson, 1978), its main drawback has been the possible contamination of the antigen with minute amounts of catalytically active cellular protein kinase(s). This is illustrated with human cytomegalovirus,

where the 68 kDa viral tegument protein of the virus was identified by immunoprecipitation of extracts of infected cells with a monoclonal antibody (Michelson et al., 1984). The immunoprecipitates of this protein had an associated protein kinase activity which was found almost exclusively in the nuclear fraction of the infected cells. This protein kinase phosphorylated casein, used GTP as well as ATP and was inhibited by heparin, although at ten times higher concentrations than cytoplasmic casein kinase II for which these properties are otherwise diagnostic (Michelson et al., 1985). This requirement for a higher concentration of heparin to inhibit protein kinase activity is also a property of a cellularly-specified nuclear casein kinase II (Baydoun et al., 1986). Thus there are clear grounds for suspecting that the immunopreciptate is contaminated with nuclear casein kinase II. Further evidence supporting the suspicion that this enzyme activity is not intrinsic to the virally-coded tegument protein came from analysis of the gene specifying it. This gene was predicted to encode a protein with an amino acid sequence bearing no relationship to the protein kinase family (Davis and Huang, 1985).

It is worth mentioning that with other viruses there have been similar experiences. The large transforming (T) antigen of Simian Virus 40 which was phosphorylated in vivo, was originally thought to have intrinsic seryl protein kinase activity. However the activity has now been separated from the antigen, indicating that the antigen is not itself a protein kinase (Walser and Deppert, 1986). Similarly immunoprecipitates of the transforming protein (middle T antigen) of Polyoma Virus contain a tyrosine protein kinase activity. However

this activity can be attributed to $pp60^{c\text{-}src}$ (Courtneidge and Smith, 1983) which exists in a stable complex with the middle T antigen.

As regards α-herpesviruses it has been reported that an immunoprecipitated antigen of VZV has protein kinase activity. This was a protein of Mr 50,000 designated p50 which could phosphorylate glycoprotein I (gI) (which is also phosphorylated in vivo) in vitro, but not other VZV glycoproteins, for example gII and gIII (Montalvo and Grose, 1986). Protein p50 could not be detected in purified virion preparations or in uninfected cells. Using the monoclonal antibody, indirect immunofluorescence microscopy revealed that the p50 protein was located on the outer-membrane surface of infected cells. It was postulated that the p50 with kinase activity was either a virally coded non-structural protein, or a cellular product the synthesis of which was activated by viral infection. However the positioning of the protein on the outer-membrane surface would be most unusual for a seryl/threonyl protein kinase, all previous examples of which are intracellular. Thus p50 may not be a protein kinase but the immunoprecipitates of this protein may be contaminated with a small amount of a cellular protein kinase.

Another approach to identifying possible protein kinases encoded by herpesviruses (and other viruses) has been to examine purified virions. The rationale here was that certain virion proteins were found to be phosphorylated in vivo, and it was thought that the enzymes responsible might be viral and specifically localised in the virion. Such protein kinases were envisaged as being important for the phosphorylation and regulation of the functions of virion

proteins. Indeed in HSV-1 (Lemaster and Roizman, 1980) and later in PRV (Stevely et al., 1985) protein kinase activity was found in association with the virion and could catalyse the phosphorylation of virus proteins in vitro. However this, in itself, did not prove the protein kinase activites in question to be virally coded. The chance incorporation of cellular protein kinases into virions is also a possibility. Indeed fractionation of soluble extracts of purified virions showed that they contained protein kinase activities with properties similar to cellular casein kinase II (Stevely et al., 1985). This phenomenon is not unique to herpesviruses, for cellular protein kinases have been found in the virions of vesicular stomatitis virus (Harmon et al., 1983) and Rous sarcoma virus (Weis and Faras, 1983).

A third approach has been to screen extracts of infected cells for new protein kinase activity, using artificial substrates such as histones, protamine, casein and phosvitin. Adoption of this technique lead to the identification of two possible candidates for an α-herpesvirus protein kinase in addition to the previously mentioned p50 of VZV. A new phosvitin/casein kinase activity was reported to be present in HeLa cells infected with HSV-1 (Blue and Stobbs, 1981), and a new protamine kinase activity was detected in BHK cells infected by PRV (Katan et al., 1985).

The kinase activity reported by Blue and Stobbs was specific for acidic rather than basic substrates. This activity was very different from the protein kinase reported by Katan et al. (1985), which could phosphorylate basic proteins but was incapable of using acidic proteins such as casein and phosvitin as artificial

substrates. The two enzymes also differed in size and time of appearance. The appearance of the activity described by Blue and Stobbs (1981) appeared to be balanced by the disappearance of a pre-existing cellular kinase activity, suggesting that it may be a derivative of a pre-existing cellular protein kinase such as a casein kinase.

As previously mentioned, the location of the VZV p50 protein on the surface of the cell membrane would seem to make this an unlikely candidate for the viral protein kinase.

The most likely candidate for the protein kinase encoded by α-herpesviruses seemed to be that designated "virus-induced protein kinase" (ViPK) by Katan et al. (1985), as this was shown to be unrelated to a range of known cellular protein kinases.

The objective of this work was to purify, characterise, determine the substrate-specificity and genomic origin for this protein kinase.

TABLE I.7. HSV-1 PHOSPHOPROTEINS

Name/Description	Class	Genomic Location
ICP 0 111K	α IE1	0.02-0.04 (TL_L) 0.95-1
ICP 27 63K	α IE2	0.74-0.75
ICP 4 175K (transcriptional activator)	α IE3	0.82-0.86 (IRs) 0.96-1 (TRs)
ICP 22 68K	α IE4	0.86-0.87 (US1)
ICP 6 148K (Ribonucleotide reductase)	β	0.57-0.6
ICP 8 127 (major DNA binding protein)	β	0.386-0.411
ICP 19 (18?) 82K (Alkaline Exonuclease)	β	0.16-0.175
ICP 150K (DNA polymerase)	β	0.41-0.43
ICP 31/VP16 65K	γ	0.669-0.685
ICP 39.3 38K (VP23)		0.66-0.76
US9 10K (Virion tegument protein)		(US9)

references: Frame et al. (1986)
Lemaster and Roizman (1980)
Marsden et al. (1978)
McGeoch et al. (1985; 1986a; 1986b)
Pereira et al. (1977)
Preston (1979a; 1979b)
Quinn and McGeoch (1986)
Stevely et al. (1985)
Swain and Galloway (1985)
Watson et al. (1979)

1 BIOLOGICAL

1.1 Cells

BHK 21/C13 cells are an established line of Hamster Kidney fibroblasts (MacPherson and Stoker, 1962). These cells contained an intact thymidine kinase gene. Cell stocks were obtained from the MRC Virology Unit, Institute of Virology, University of Glasgow. They were thus termed BHK (TK^+) (Glasgow cells).

The following cell lines were gifts from Prof. B. Roizman, Marjorie B. Kovler Viral Oncology Laboratories, University of Chicago, Chicago, Illinois, U.S.A.

BHK (TK^-) cells

Vero (TK^-) cells: African green monkey kidney cells

Rabbit Skin cells: Originally obtained from J. McLaren, were used for transfection of cells with viral DNA (Longnecker and Roizman, 1986).

Human 143 (TK^-) cells were used for selection of TK^+ and TK^- recombinant viruses. (Longnecker and Roizman, 1986).

1.2 Viruses

Pseudorabies virus (PRV), a member of the Alpha-Herpesvirus group, was originally obtained from Kaplan and Vatter (1959), via Dr. W.S. Stevely.

Herpes simplex virus type 1 (HSV-1) Glasgow Strain 17 was provided by Dr. J. MacNab, MRC Virology Unit, Institute of Virology, University of Glasgow, Glasgow. It was termed HSV-1(G).

Herpes simplex virus type 1 (HSV-1)F strain (Ejercito and Kieff, 1968), was provided by Prof. B. Roizman (University of Chicago). It was termed HSV-1(F).

1.3 Viral Mutants

tsK mutant of HSV-1(G) (Marsden et al., 1976) was a gift from Dr. J. MacNab.

Recombinant viruses of HSV-1(F), R7035, R7041, R7050, R7051 were gifts from Dr. R. Longnecker and Prof. B. Roizman, University of Chicago.

HSV-1(F) Δ305(TK^-) (Post et al., 1981, Post and Roizman., 1981) was provided by Prof. B. Roizman.

1.4 Plasmids

These were used during the construction of HSV-1(F) recombinant viruses.

pRB103 - Plasmid containing the Bam HI Q fragment of HSV-1(F).

pRB3446 - 4.89 kp fragment of HSV-1(F) Sac I fragment cloned in vector pUC19.

pRB425 - 4.47 kbp fragment of HSV-1(F) Hind III - Sac I fragment cloned in vector pUC19.

pRB3635 - 4.89 kbp Sac I fragment from plasmid pRB3446 with α27-TK chimeric gene inserted in place of a small Nco I fragment within the US3 gene.

pRB3696 - The US3 gene sequence of HSV-1(F) is deleted in this plasmid. The deletion is in the 4.47 kbp Hind III - Sac I fragment (cloned as pRB425). The deletion extends from a Pst I site located 69 amino acids from the 5' end of US3 to a Bam HI site located at amino acid 357 or US3.

The plasmids described above were constructed by Dr. R. Longnecker and were gifts from him and Prof. B. Roizman.

2 CHEMICAL

2.1 General

General laboratory chemicals were of analytical grade, where appropriate and obtained from standard commercial suppliers unless a particular source is specified.

2.2 Radiochemicals

[^{35}S]methionine, [^{125}I]NaI and ^{32}[P]orthophosphate were purchased from Amersham International Ltd., England.

[γ^{32}P]ATP was either from Amersham International or synthesised according to Maxam and Gilbert (1980).

[^{125}I]NaI was used to prepare radiolabelled protein-A.

2.3 Enzymes, Other Proteins, Amino Acids and Additional Compounds

Protein kinase C, homogeneous preparation isolated from bovine brain according to Parker et al. (1984) was a gift from Dr. P.J. Parker, ICRF, London.

Protein kinase C, partially pure preparation, was purified from BHK cells according to the method of Kikkawa et al. (1986). Cyclic AMP-dependent protein kinase was from rabbit muscle and was purchased from Sigma Chemical Co., U.S.A.

Casein Kinase II was a gift from Dr. O-G. Issinger Department of Human Genetics, University of the Saarland.

DNase (prepared according to Banks et al., 1985) was a gift from Dr. K.L. Powell, University of Leeds.

Protamine sulphate, casein and mixed histones type II-A, lysine rich histones III-S, histone type V-S, VI-S and VII-S were from Sigma Chemical Co., U.S.A.

Low molecular weight protein standards were from Pharmacia, Sweden. High molecular weight protein calibration kit was from Boehringer Mannheim GMBH, West Germany. L-threonine, O-phosphoserine, O-phosphothreonine and O-phosphotyrosine were from Sigma Chemical Co., U.S.A.

Cycloheximide, 1, ethyl-3-(3-dimethylaminopiopyl)-carbodiimide hydrochloride, phenyl methyl sulphonyl fluoride, Lubrol-PX, β-glycerophosphate and EGTA were obtained from Sigma Chemical Co., U.S.A. Aquacide (Type II) was from Calbiochem Behring Corporation, U.S.A. Puromycin was obtained from Boehringer Mannheim, West Germany.

2.4 Nucleic Acids and Nucleotides

ATP was from P-L Biochemicals Inc., U.S.A. Cyclic AMP was from Boehringer Mannheim, West Germany.

2.5 Peptides

Synthetic peptides for determination of substrate specificity were synthesised according to Marchiori et al. (1979; 1982), Meggio et al, (1981) and Chessa et al. (1983).

All were gifts from Prof. F. Marchiori and Prof. L.A. Pinna of the Organic Chemistry and Biochemistry Departments, respectively, of Padua University, Padua, Italy.

A synthetic peptide of the eight C-terminal amino acids of the predicted product of the US3 gene of HSV-1 (with an additional tyrosine amino acid at the N-terminal side) was synthesised by Cambridge Research Biochemicals, Cambridge, England.

2.6 Antibodies

Monoclonal, polyclonal and peptide antibodies to protein kinase C were gifts from Dr. P.J. Parker, ICRF, London.

A Synthetic oligo-peptide antiserum to the predicted product of the US3 gene (anti-US3 serum) was provided by Dr. M. Frame and Prof. J. Subak-Sharpe of the MRC Virology Unit, Institute of Virology, University of Glasgow, Glasgow.

Monoclonal antibody H1163 to HSV-1(F) glycoprotein B was a gift from Dr. L. Pereira, Rickettsial Disease Laboratory, California, Department of Public Health, Berkeley, California, 94704, U.S.A.

2.7 Chromatographic Material

DEAE-cellulose (DE-52) was from Whatman Biochemicals Ltd., England. DEAE-Sephacel, Phenyl-Sepharose, Sephacryl S-200, AH-Sepharose 4B, Mono Q HR 5/5 column (5mm x 50mm), Superose 6 (10 x 300mm), and Superose 12 (10 x 300mm), were all from Pharmacia, Sweden.

Protamine-agarose was from Sigma Chemical Co., U.S.A.

Threonine-sepharose was synthesised according to Kikkawa et al. (1986). **Sepharose is a registered trademark of Pharmacia AB.**

The TSK phenyl-5PW column was a product of Toyo Soda Manufacturing Co., (Japan) and obtained through Anachem, Luton, England.

2.8 Reagents for electrophoresis

Acrylamide, N-methylene-bis-acrylamide, diallytartardiamide and ammonium persulphate were from Biorad Laboratories, U.S.A.

Urea was of Aristar grade, from British Drug House Ltd., England.

Ampholines pH 4-6 were from LKB, Sweden.

Agarose (type VI) and, Coomassie Brilliant Blue R250 and G250 were from Sigma Chemical Co., U.S.A.

NNN'N'tetraethylmethylethylenediamine (TEMED) and Bromophenol Blue were from Eastman Kodak Co., U.S.A.

Pyronine Y was from G.T. Gurr Ltd., England.

2.9 Reagents for Immunology

Nitrocellulose (BA or BA85) was from Schleicher and Shuell; Anderman and Co., Ltd., Surrey, England. Tween 20 was from Sigma Chemical Co., U.S.A.

The electrotrans-blot apparatus was from Biorad Laboratories, U.S.A.

Horseradish peroxidase-coupled rabbit anti-mouse immunoglobulin G was from Miles Laboratories Inc., Elkhart, Ind., U.S.A.

Ortho-dianisidine dihydrochloride and hydrogen peroxide were from Sigma Chemical Co., U.S.A.

2.10 Reagents and Tissue Culture

Eagle's minimum essential medium (MEM) Glasgow Modification (G-MEM) (Eagle, 1959, as modified by MacPherson and Stocker, 1962), MEM vitamins, MEM amino acids, MEM amino acids minus methionine, newborn calf serum, sodium bicarbonate, L-glutamine and trypsin were from Gibco Bio-Cult, Scotland. Dulbecco modified Eagle minimum essential medium, maintenance medium 199, foetal calf serum were from Gibco Bio-Cult, U.S.A.

Trypsin and PPLO agar were from Difco Laboratories, U.S.A. Blood agar, brain and heart infusion broth, tryptose phosphate broth were from Oxiod Ltd., England. Penicillin and streptomycin were from Glaxo Pharmaceuticals Ltd., England.

Bromouracil deoxyribose and methotrexate were from Gibco, U.S.A.

Phenol red and Giemsa stain were from BDH Chemicals, England.

Dimethylsulphoxide (DMSO) and L-glutamine were from Sigma Chemical Co., U.S.A.

Plastic ware was from Nunc, England and U.S.A.

2.11 Photographic Materials and Reagents

DX10 photographic developer, FX40 photographic fixer, X-ray (screen) film (X-Omat XAR5), X-ray (screen) film (X-Omat S) were from Eastman Kodak Co., U.S.A.

Cronex intensifying screens and cassettes were from du Pont de Nemours and Co. Inc., U.S.A.

2.12 Scintillation Reagents

2,5-diphenyloxazole (PPO), p-bis-[2-5 phenyloxazole] - benzene (POPOP) were from New England Nuclear, U.S.A.

Ecoscint was from National Diagnostics, New Jersey, U.S.A.

2.13 DNA Cloning Reagents

Restriction endonucleases were purchased from New England Biolabs, Beverly, Mass., U.S.A. T4 DNA ligase was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind., U.S.A.

Agarose, low and high melting point, was from Sea Kerm Me agarose (FMC Corp., Marine Colloids Div., Rockland, Maine, U.S.A.).

3 COMPOSITION OF STANDARD SOLUTIONS

3.1 Phosphate-Buffered Saline (PBS)

This buffer was prepared essentially according to Dulbecco and Vogt (1954) and contained 0.17M NaCl, 3.4mM KCl, 10mM $NaHPO_4$, 2.4mM KH_2PO_4, 0.49mM $MgCl_2$ and 0.68mM $CaCl_2$ at a pH of 7.5.

3.2 Scintillation Fluid

A. 66% (v/v) toluene, 33% (v/v)2-methoxyethanol,
0.4% (w/v) 2,5 - diphenyloxazole,
0.01% (w/v) p-bis-[2-(5-phenyloxazoyl)]-benzene.

B. ECOSCINT

3.3 Cell Culture Media

A. ETC - Glasgow modified Eagles medium containing tryptose phosphate and bovine calf serum.

300ml	sterile distilled water
40ml	Eagles minimum essential medium, Glasgow modification (G.MEM)(X10)
40ml	tryptose phosphate
14ml	sodium bicarbonate (7.5% ; 200mM)
4ml	penicillin/streptomycin (10,000 units)
4ml	L-glutamine (200mM)

This medium was supplemented with 1%(v/v), 5%(v/v) or 10%(v/v) newborn calf serum as indicated.

B. DME

Dulbecco modified minimum essential medium was supplemented with 5%(v/v) or 10%(v/v) foetal calf serum and antibiotics. To make 5 litres of this growth media, one 5 litre packet of Hazleton DME was added to 4 litres of sterile distilled water and mixed for

20 min. 7.5g of $NaHCO_3$ was added till the colour changed from orange to red. A further litre of distilled water was added and the media was mixed for 1 to 2h.

For transportation of cells, flasks of confluent Vero cells were filled to capacity with DME supplemented with 10% (v/v) foetal calf serum and 4.5g of glucose. For BHK cells the flasks were filled with ETC containing 10% (v/v) newborn calf serum.

C. **199 Media for Propagation and titration of Viruses**

199V Medium

400ml	sterile distilled water
50ml	concentrated 199 medium (Hank's BSS 10X)
0.5ml	penicillin/streptomycin (10,000 units)
5ml	inactivated foetal calf serum*
5-6ml	$NaHCO_3$ (7.5% ; 200mM)**
1-5ml	glutamine (200mM)***

* Inactivated by heating the serum for 30 min. at 56°C.

** $NaHCO_3$ was added till the colour of the medium turned cherry red.

*** The 199 (10X concentrate) obtained from commercial sources, contained glutamine. However the glutamine only remains stable for 8-12 weeks. During this time, and after, it was necessary to supplement the medium with the amino acid. For each month after the arrival of 199 from the supplier 1ml of 1% (w/v) glutamine was added to the medium.

199V medium minus specific amino acids was prepared as described above, except that, dialysed, inactivated foetal calf serum was used.

1990 Medium

This medium was used for the titration of HSV-1(F) and R7035, R7041, R7050 and R7051 recombinant viruses on Vero cells. It consisted of 500ml of 199V medium with an additional 0.5ml of pooled human γ-globulin (0.5-1mg).

D. **Pre-Freeze Medium (PFM)**

The formulation used depended on the cell type.

Vero cells BHK TK⁻ cells (USA)	BHK TK⁻ cells (Glasgow)
360ml sterile distilled water	360ml sterile distilled water
40ml 199 medium ×10	40ml G-MEM ×10
100ml inactivated foetal calf serum	100ml newborn calf serum

The pH of each solution was adjusted till the medium became a bright tomato red colour by the addition of sterile 6M NaOH dropwise.

E. **Freeze Medium**

The appropriate pre-freeze medium (PFM) was used with Dimethylsulphoxide (DMSO) in a ratio of 4 parts PFM : 1 part DMSO.

F. **Tryptose Phosphate Broth**

For 5 litres of broth, 147.5g of Tryptose phosphate broth (Difco bacto) was added to 5 litres of distilled water. The mixture was divided into 40ml aliquots and sterilized by autoclaving at 15 lbs/sq in pressure for 15 min.

G. **Versene**

Versene (EDTA, disodium salt) was prepared in PBS. The total solution contained, in 1 litre : 0.14M NaCl, 2.7mM KCl, 10mM $NaHPO_4$, 2.4mM KH_2PO_4, 2mM EDTA, 0.015% phenol red. The versene mixture was aliquoted and autoclaved and stored at room temperature.

Trypsin/Versene

Just before use a trypsin/versene mixture was prepared containing 1 volume of trypsin (0.25% (w/v)) and 4 volumes of versene.

H. **Giemsa Stain**

To stain infected monolayers Giemsa stain was prepared as follows:

30g of Giemsa was added to 1,980ml of glycerol and heated at 56^{o} for 90 to 120 min, after which 1,980ml of methanol was added and the solution was mixed well. The stain was allowed to stand at room temperature for 7 days and then filtered through Green's 904.5 filter paper. The stain was bottled and diluted 1 in 10 with distilled water before use.

1 TISSUE CULTURE METHODS

1.1 Growth of Cells

A. BHK 21/C13 TK^+ (Glasgow) or TK^- (USA) Cells.

These were grown as monolayers in rotating 2.5 litre roller bottles (glass or plastic) in 150ml of pre-warmed ETC supplemented with 10% newborn calf serum or, in the case of the BHK TK^- (USA) cells, DME supplemented with 10% foetal calf serum. Cultures were seeded at 2-3 x 10^7 cells per roller bottle and "gassed" with 100ml of 5% (v/v) CO_2 in air to maintain buffering capacity. The bottles were rotated at 37°C for three days, by which time the cells had generally just reached confluence.

B. Vero Cells, TK^- (African green monkey kidney cells).

These were transferred from 500ml (150cm^2) plastic flasks to 2.5 litre roller bottles in 150ml of pre-warmed DME supplemented with 10% foetal calf serum. The cultures were seeded at 5-6 x 10^7 cells per roller bottle and "gassed" as described in 1.1A. The cells were confluent within two to three days at 37°C. Vero cells were also grown in small screw-topped plaque dishes. The cultures were seeded at 2-3 x 10^6 cells per dish and the cells were grown in 10ml of DME supplemented with 10% foetal calf serum and gassed with 10mls of 5% CO_2 in air. The cells reached confluence within 12-24h at 37°C.

C. Rabbit Skin Cells

These were grown under similar conditions to the Vero cells in plaque dishes or 150cm^2 plastic flasks.

D. **Cell Density**

The number of cells obtained from confluent monolayers from the various flasks were:

plastic screw-topped plaque dish; $4\text{-}5 \times 10^6$

plastic 500ml flask ($150cm^2$); 5×10^7

roller bottle 2.5l; 2.5×10^8

E. **Passaging Cells**

When cells had become confluent they were removed from the plastic or glass vessel by trypsinization. Firstly the medium was discarded and the cells were rinsed with approximately 10-20ml of a trypsin/versene mixture (1 part trypsin (0.05%), 4 parts versene), prepared as described in Materials, Section 3.3G, and pre-warmed to 37°C. After 2 min this mixture was discarded and another 10-20ml of the trypsin/versene mixture was added. The cells were rinsed again for 2 min and this mixture was then poured off, leaving 2-5ml of the trypsin/versene mixture in the flask. The flask was then rotated at 37°C until the cells were clearly seen detaching from the walls. This usually took 5-10 min. Approximately 20ml of the appropriate pre-heated medium was then added to the flask of cells and the cells were suspended in this medium using an automatic pipette aid and a 10ml sterile glass pipette. The cells were transferred to a sterile vessel and either seeded immediately, or kept at 4°C for up to 24h before seeding.

F. **Freezing and Storage of Cells**

a. <u>Small Scale</u>

Cell cultures were grown and harvested by trypsinization when they were between 80-90% confluent. They were resuspended in

the appropriate grown medium, (this was determined by the particular cell type) and the suspension was centrifuged at 2000 rpm (500g) for 5 min. The cells were resuspended at 4-12 x 10^6 per 1ml of the appropriate pre-freeze medium (PFM) described in Methods (Section 3.3D). This suspension was left on ice for 30 min and was mixed at 10 min intervals. Then an equal volume of cold PFM-DMSO mixture (Methods, Section 3.3E) was added slowly. The cell suspension was again left on ice for 30 min and mixed at 10 min intervals. Aliquots of approximately 1ml containing 2 x 10^5 - 5 x 10^6 cells were placed in sterile screw-topped vials and frozen at -80°C or in liquid nitrogen.

b. Large Scale

BHK TK^+ (Glasgow) cells from one roller bottle were harvested by trypsinization when 90% confluent. They were resuspended in ETC supplemented with 10% newborn calf serum. The suspension (approximately 5 x 10^6 - 1 x 10^7 cells/ml) was centrifuged at 2000 rpm (500g) in a Beckman bench centrifuge, to sediment the cells. The medium was removed and 20ml of freeze medium were added to the cells. The cells were then cooled to 4°C and 5 x 10^6 - 1 x 10^7 cells in 10-20ml of cold ETC freeze medium were dispensed in 20ml screw-topped sterile Universal Bottles. These were then frozen directly at -80°C. Cells stored in this way remained viable for 6 months to 1 year.

G. **Recovery of Cells After Freezing**

a. Small Scale:

Each vial of cells was quickly thawed to 37°C in a water bath and diluted by adding 10ml of pre-warmed growth medium. The cells were then placed in a small screw-topped plaque dish. Cells from 1 vial became confluent within 1h. Alternatively, cells from 1 vial were sometimes diluted with 50ml of pre-warmed growth medium and transferred to a single 150cm^2 (500ml) plastic flask, generally reaching confluence within 24h.

b. Large Scale:

Each Universal Bottle of cells was quickly thawed to 37°C in a water bath; the cells were transferred to a 150cm^2 flask and, when they were confluent, trypsinized and placed in a 2.5l roller bottle containing 150ml of the appropriate growth medium (as described in Methods, Section 1.1A).

1.2 **Infection of BHK Cells with Pseudorabies Virus**

BHK TK$^+$ (Glasgow) cells were grown to confluence in roller bottles for 3 days at 37°C. Each bottle was infected with PRV at a multiplicity of approximately 20 p.f.u./cell in 20ml of the original medium or fresh medium with the calf serum reduced to 1%. The virus was allowed to adsorb onto the cells for 1h at 37°C. After this adsorption period the medium was removed and replaced with 50ml of the original medium or fresh ETC medium containing 1% calf serum, and the infection allowed to continue at 37°C until harvesting. This was usually 8h after infection, when 95% - 100% of the cells were rounded up but still remained on the surface of the roller bottle or flask.

1.3 Infection of BHK Cells and Vero Cells with Herpes Simplex Virus Type 1

BHK cells were infected when confluent with 10 p.f.u./cell of HSV-1(G) in 20ml of ETC/roller bottle, with the calf serum reduced to 1%. The virus was allowed to adsorb onto the cells for 1h at 37°C. After this time the medium was discarded and 50ml of ETC containing 1% calf serum was added. The infection was allowed to continue at 37°C until harvesting 17-18h after infection. BHK TK^- (USA) and Vero cells were grown to confluence at 37°C. They were infected at 10 p.f.u./cell with HSV-1(F), R7035, R7041 or R7050 in 25ml per roller bottle of 199V growth medium. The particular virus was discarded and 50ml of 199V was added, and infection continued for 17-18h, when the cells were harvested.

1.4 Growth of Pseudorabies Virus

PRV for subsequent bulk preparation of protein kinase was prepared as follows: BHK cells were grown to confluence in 2.5 litre roller bottles. Then they were infected at 1 p.f.u./200 cell with PRV in 20ml of ETC medium containing 1% calf serum per roller bottle. After an adsorption period of 1h at 37°C the cells were overlayed with 100ml of ETC containing 1% calf serum. The infection was allowed to continue at 37°C until the cytopathic effect was complete (36-48h). The roller bottles were shaken gently and the virus and cell debris were aliquoted in 250-300ml quantities and stored frozen at -80°C. This virus was used to infect cells for the preparation of the protein kinase. PRV stocks were produced as described above but once the cytopathic effect was complete the bottles were shaken gently to detach the remaining cells from the glass surface, and the cell debris removed by centrifugation at 2000 rpm (500g) for 10 min. The

virus was sedimented by centrifugation at 10,000 rpm (20,000g) for 90 min in an MSE-18 centrifuge. The sedimented virus was resuspended in growth medium containing 1% calf serum, titred and stored in aliquots over liquid nitrogen.

1.5 **Growth of Herpes Simplex-1(G)**

Virus for bulk preparation of protein kinase was prepared essentially according to the method described above for PRV (Methods, Section 1.4) but with the following amendments. Infection was at 1 p.f.u. per 100 cells with an original stock of HSV-1 of titre 5×10^7 p.f.u./ml. A complete cytopathic effect was achieved in 48-72h under these conditions. Supernatant viral stocks were also prepared as for PRV, however the majority of HSV-1 virus remains associated with the cell pellet after the first centrifugation. This pellet was therefore resuspended in approximately 6ml of ETC (1% calf serum) and sonicated. The virus was titred and shock-frozen in 2ml aliquots at $-80^{\circ}C$.

1.6 **Growth of the Temperature Sensitive Mutant of HSV-1(G): tsK**

Approximately 7 roller bottles of confluent BHK cells were infected at a multiplicity of 1 p.f.u./300 cells. The titre of the stock of tsK was 1.3×10^7 p.f.u./ml and 0.4ml was added to 350ml of ETC containing 5% calf serum which had been prewarmed to $31^{\circ}C$. 50ml of this was added to each roller bottle of cells. The cells were transferred to $31^{\circ}C$ and after 3 days a complete cytopathic effect was observed. The bottles were shaken gently and the cells were sedimented by centrifugation for 5 min at 2000 rpm (500g). The cell pellet was resuspended in 6ml of ETC and sonicated to release the virus still associated with the cells. The supernatant was centrifugated at 10,000 rpm (20,000g) for 90 min in a MSE-18

centrifuge. The pellet of virus was resuspended in approximately 10ml of ETC and titred before use.

1.7 **Infection of Cells with tsK**

Plastic flasks ($150cm^2$) of confluent BHK cells were infected at a multiplicity of 10 p.f.u./cell with tsK in ETC medium containing 1% calf serum. The flasks of cells, medium, pipettes and virus were all pre-warmed to the appropriate temperatures: 31^oC, the permissive temperature, or 38.5^oC, the restrictive temperature. The virus was allowed to adsorb onto the cells in 5ml of ETC (1% calf serum) for 1h. The medium was then removed and 20ml of ETC (1% calf serum) was added. The infections were then allowed to continue for 18h at the appropriate temperatures in carefully monitored incubators to prevent any escape of the mutant into β protein synthesis, which could occur if the temperature fell below 38.5^oC. The cells were rounded up by 18h after infection and were harvested at this time.

1.8 **Growth of HSV-1(F) and Recombinant Viruses (R7035, R7041, R7050 and R7051)**

Ten plastic roller bottles of confluent Vero cells were used routinely to make stocks of each virus. Each roller bottle of cells was infected at a multiplicity of 1 p.f.u./100 cells with the appropriate virus in 25ml of pre-warmed 199V. The virus was allowed to adsorb for 1h at 37^oC. The cells were then overlayed with a further 50ml of pre-warmed 199V. The virus was allowed to grow for approximately 48-72h at 34^oC, by which time a good cytopathic effect was observed. The bottles were shaken gently to dislodge any cells and virus from the plastic surface. The virus and cells were then poured into sterile screw-capped bottles and sedimented at 5000 rpm (10,000g) for 10 min. The supernatants were discarded and the

pellets were resuspended in a final volume of approximately 10ml of 199V. The suspension was then sonicated to release the majority of the virus associated with the cell. Each virus preparation was aliquoted into 2ml fractions and stored at -80°C. A portion of each was used for immediate titration. The stocks of virus used for the original innoculation were prepared from a mother stock as follows:

Screw-topped plaque dishes each containing 4 x 10^6 Vero cells were infected at 1 p.f.u./100 cells with an aliquot of the mother seed stock in 1ml of 199V medium. After 1h the cells were overlayed with 5ml of 199V and infection was allowed to continue for 36-48h, when a complete cytopathic effect was observed. The dishes were shaken gently and frozen at -80°C. An aliquot of these seed stocks was titred.

1.9 Titration of Viruses

A. PRV

Serial logarithmic dilutions of PRV were prepared in ETC (1% calf serum). Screw-topped plaque dishes were seeded with 2-3 x 10^6 BHK cells in 5ml of ETC (10% calf serum) and the cells were allowed to grow to confluence in a CO_2 incubator at 37°C. Three to four dishes were used per dilution. After the cells had grown for 12-24h the medium was aspirated off the cells. The cell monolayers were infected with 0.1ml of the various virus dilutions: routinely 10^{-4} -10^{-10} were used. After adsorption of the virus onto the cells for 1h, 5ml of ETC (1% calf serum) was added and the incubation continued for a further 2h. Then approximately 250µg of heparin was added to each dish to prevent the virus spreading and the cultures returned to the 37°C incubator for a further 24-30h. After this time the medium was

removed and the infected monolayers fixed with 4% formal saline for 10-20 min. After fixing, the monolayers were stained with Giemsa stain for 20 min and then washed with distilled water. The plaques were clearly visible by eye but were viewed also with a plate microscope and counted. Plates containing 10-50 and 100-200 plaques were chosen and the average number taken over the 4 dishes was used to calculate the titre of the virus.

B. **HSV-1(G) and tsK**

HSV-1(G) was titred essentially as already described for PRV. Serial dilutions of HSV-1(G) were prepared in ETC (1% calf serum). Plaque dishes with approximately 3 x 10^6 cells were set up and grown for 12h at 37°C. The monolayers were then examined to ensure an even cell layer was achieved. HSV-1(G) was titred at 37°C and 31°C, the titres obtained at 31°C being routinely a factor of 10 lower than that obtained at 37°C. tsK was titred at 31°C and 37°C also. To each plaque dish 0.1ml of the appropriately diluted virus (10^{-4} -10^{-10} range) was added. The virus was allowed to adsorb onto the cells for 1h, and then overlayed with 5ml of ETC (1% calf serum) and left for 48-72h in an incubator maintained at the appropriate temperature. The medium was then removed and the infected monolayers were fixed and stained as before.

C. **HSV-1(F), R7035, R7041, R7050 and R7051**

Vero cells were used for the preparation of these viral stocks and also for the titration of these viruses. Screw-topped plaque dishes with confluent Vero cells (approximately 4 x 10^6) were used. Serial logarithmic dilutions of each virus were prepared in 199V. (Care was taken to mix each dilution thoroughly and new

pipettes were used throughout.) The medium was aspirated off the cells and the cell monolayers were infected with 0.1ml of the various dilutions of virus (routinely 10^{-5} -10 $^{-10}$), each condition being performed in triplicate. The virus was allowed to adsorb onto the cells for 1h at 37°C. The cells were then overlayed with 5ml of 199V medium and the cells returned to 37°C for 36-48h. When the monolayers were sufficiently infected, the plaques were clearly visible even before staining. The medium was removed from each dish and 2-5ml of methanol was added. After 10 min, this was removed and the infected monolayers were stained with Giemsa stain for 30 min.

The monolayers were then washed with distilled water. The plaques were clearly visible as clear holes in the cell sheet. They were counted and the average values used to calculate the concentrations of HSV-1(F), R7035, R7041 and R7050 viral stocks. As a control, viruses of known titre were re-titred in every viral titration.

D. **Titres**

The typical titres obtained routinely for the various viral stocks were:

PRV : $1 \times 10^9 - 5 \times 10^9$ p.f.u./ml

HSV-1(G) : $1 \times 10^8 - 5 \times 10^8$ p.f.u./ml

tsK : 1×10^8 p.f.u./ml

HSV-1(F) : $2 \times 10^9 - 5 \times 10^9$ p.f.u./ml

R7035 : $1 \times 10^8 - 1 \times 10^9$ p.f.u./ml

R7041 : $1 \times 10^8 - 1 \times 10^9$ p.f.u./ml

R7050 : $1 \times 10^8 - 1 \times 10^9$ p.f.u./ml

R7051 : 1×10^9 p.f.u./ml

1.10 **Labelling of BHK and Vero Cells with [^{32}P]Orthophosphate**

Screw-topped plaque dishes containing 4 x 10^6 Vero or BHK cells were labelled with ^{32}P during infection with HSV-1(F), R7041 or during mock infection. The cells were infected as described in Methods, Sections 1.3 and 1.14. One hour before the chosen time for labelling, the medium was removed from the cells and 1ml of growth medium in which the phosphate had been decreased to one tenth (0.09mM) of its normal concentration and from which tryptose phosphate broth had been excluded was added. After the 1h incubation period, 200-500µCi of [^{32}P]orthophosphate was added to the cells. The cells were either harvested after short intervals or they were rinsed with 199V medium (with the phosphate concentration decreased to 10% of its normal concentration) and then overlayed with 2ml of this medium. The labelled cultures were then further incubated for 2-4h and then harvested.

1.11 **Labelling of BHK and Vero Cells with [^{35}S]Methionine**

4 x 10^6 cells in screw-topped plaque dishes were labelled with [^{35}S]methionine during viral infection. After infection for 3h the cells were washed with medium lacking methionine, and 30 min later 200-500µCi of [^{35}S]methionine was added to the cells in 2ml of methionine-free medium diluted with 199V in a ratio of 9:1. After 1h a further 200-500µCi of [^{35}S]methionine was added, and after 1h the cells were harvested.

1.12 **Inactivation of PRV and HSV-1(G) by Ultra-Violet Radiation**

Irradiation of each virus by ultra-violet light was as follows:

Stock virus was diluted tenfold with phosphate-buffered saline containing 1% (w/v) glucose. Aliquots (2.5ml) received 5kJ/m^2 of radiation in 50mM petri dishes under a UVSL-58 Mineralight

(Ultra-Violet Products, San Gabriel, CA., U.S.A.). Dosage was calculated from the distance between source and sample and the time of irradiation.

1.13 **Assay of DNA Polymerase**

This was performed according to Weissbach et al. (1973).

1.14 **Mock Infection**

This was performed under the same conditions as the particular viral infection, except that no virus was added. The appropriate reduced volume of medium was added to the cells and 1h later this was removed and new medium added. The volume of this medium was again the same volume used in viral infection. The cells were harvested at the same time as the particular virally infected cells.

1.15 **Preparation of Plasmids**

Plasmids pRB3446, pRB3447, pRB425, pRB3696 were prepared according to a modification of the methods of Birnboim and Doly (1979) and Maniatis et al. (1982). A single bacterial colony was inoculated into 1 litre of sterile L-broth (20g/litre) containing glucose (1g/litre) and ampicillin in a 2.5 litre flask, and shaken overnight at 37°C. The bacteria were centrifuged in a Sorval centrifuge at 8000 rpm (7000g) for 30 min at room temperature. Material from 1 litre of original culture was resuspended in 20ml of a solution containing 25mM Tris-HCl (pH8), 50mM EDTA (di-sodium salt), 1% (w/v) glucose and 5mg/ml lysozyme, and then left at room temperature for 5 min. A wide-bore pipette was used to add 40ml of a solution containing 0.2M NaOH and 1% SDS, and the mixture was mixed and left on ice for 10 min. Again using a wide-bore pipette, 45ml of a cold solution of 3M potassium acetate (pH4.8) was added. The mixture was left on ice for 5 min and then centrifuged at 10,000 rpm (20,000g) for 30 min.

The supernatant was transferred to a new tube by straining it through Kleenex tissues. Then 0.6 vol of isopropanol was added and the solution mixed and left at room temperature for 15 min. The mixture was then centrifuged at 8000 rpm (7000g) in a Sorval centrifuge for 10 min at room temperature. The precipitated DNA was resuspended in a solution of 10mM Tris-HCl (pH7.6) and 1mM EDTA or glass-distilled water. Plasmid DNA was separated from chromosomal DNA by caesium chloride density gradient centrifugation. Each gradient contained 24ml CsCl (175% w/v) 12ml of DNA solution and 1ml of ethidium bromide (5mg/ml). The tubes were topped up with extra CsCl and water in the same ratio as CsCl to DNA solution. The tubes were centrifuged at 45,000 rpm for 15-18h at 23°C in the VTi75 rotor of a Beckman ultracentrifuge. The centrifuge was allowed to stop without use of the break and the DNA visualised using ultra-violet light. The lower of the two bands observed was carefully removed using a 10ml syringe and needle.

The ethidium bromide was then removed from the DNA by three to four successive washings with isopropanol. The top phase was aspirated off after each wash. The DNA was then dialysed against a solution containing 10mM Tris-HCl (pH7.6-8) and 1mM EDTA for 12h with several changes of buffer. The concentration of the DNA was then determined using the relationship that 50µg/ml DNA has an A_{260} value of 1 when measured in a cell with a 1 cm light path. Yields of 1.8-2mg of DNA were obtained. The DNA was divided into aliquots and stored at -20°C.

1.16 Transfection of BHK TK$^-$ Cells with Plasmid DNA (for Transient Expression)

Plasmid DNA (40-80µg) was transfected into cells which had been grown until they were 90% confluent in (150cm^2) flasks. The DNA was diluted to 2.25ml in Tris-HCl (pH8) and 1mM EDTA. 2.25ml of a 2 X concentrated solution of saline buffered with Hepes was then added. This contained 8g NaCl, 0.37g KCl, 1g glucose, 0.125g $Na_2HPO_4.2H_2O$, 5g HEPES (free-acid) dissolved in 250ml of distilled water and the pH adjusted to 7.05 with NaOH. The final volume was adjusted to 500ml and the solution was autoclaved or filtered. The DNA mixture was then thoroughly mixed and approximately 0.25ml of 2.2M $CaCl_2$ was added causing the DNA to precipitate. This mixture was allowed to remain at room temperature for no longer than 10 min. The medium was aspirated off the cells and the cells were washed once with fresh DME containing 10% foetal calf serum (Materials, Section 3.3B). Approximately 4.5ml of the $CaCl_2$/DNA suspension was added to each flask of cells, and the cells were incubated at room temperature for 10-15 min, 45ml of DME (10% foetal calf serum) was added to the cells and they were incubated at 37^{o}C for 4h. After this time the DNA and medium was aspirated off the cells and they were washed with DME containing no serum, care being taken that the cells were not lost at this time.

Approximately 10ml of DME with no serum but containing 15-20% (v/v) glycerol was next added to the cells and they were incubated for 1-1.5 min. All the glycerol medium was then removed and the cells were washed once with DME containing no serum. Again care was taken not to lose the cells at this stage. 65ml of DME supplemented with

10% foetal calf serum was added to each flask of cells and they were incubated at 37°C for 40-48h. To aid recovery of the cells after the shock, the medium was changed about every 4h.

1.17 **Preparation of Viral DNA**

(1) Small Scale : For the preparation of viral DNA from a 150cm^2 flask of infected cells

Cells were harvested by scraping into PBS when the cytopathic effect was 100%. The cells were centrifuged at 2000 rpm (500g) for 5 min and the supernatant was then removed. Approximately 2ml of 0.5% (v/v) NP40 in 10mM Tris-HCl (pH8) and 1mM EDTA was added to the sedimented cells. This mixture was vortexed and kept on ice for 5-10 min. 10µl of RNase (10mg/ml) was added. The mixture was centrifuged at 2000 rpm for 5 min in an Eppendorf centrifuge to sediment the nuclei. The supernatant was removed and 50µl of 20% (w/v) SDS and 50µl of proteinase K (10mg/ml) was added. This mixture was then incubated for 30 min to 2h, extracted with phenol and then ether, and the DNA precipitated by addition of 1/20 vol of 4.5M sodium acetate and 5 vols of ethanol. The yield of DNA was usually sufficient for approximately 10 restriction endonuclease digestions.

(2) Large Scale : For the preparation of viral DNA from 1-5 roller bottles of infected cells

The cells were harvested when the cytopathic effect was complete, and centrifuged for 10 mins at 2000 rpm (500g).

They were resuspended in 12ml of a solution containing 10mM Tris-HCl (pH8) and 1mM EDTA. 0.3ml of 20% (v/v) NP40 was then

added and the mixture was vortexed and kept on ice for 10 min. The nuclei were removed by centrifugation of this mixture for 10 min at 2000 rpm. 0.5ml of 5M EDTA, 0.5ml of 20% (w/v) SDS and 0.1ml of Proteinase K (10mg/ml) were added to the supernatant. This mixture was incubated at 37°C for 2-12h, before being subjected to NaI density gradient centrifugation essentially according to Walboomers and Ter Schegget (1976). Approximately 1.5ml of NaI which had been saturated at room temperature was added for each ml of supernatant, and this should give a solution with a refractive index of 1.434-1.435. The centrifuge tube was then filled to capacity with extra NaI at the correct refractive index after 0.5ml of EtBr (5mg/ml) had been added to the mixture. The tubes were centrifuged at 45,000 rpm for 18-36h in the VTi50 rotor of a Beckman ultracentrifuge. After centrifugation the lower band in the gradient which was visualised by ultra-violet light was collected in a syringe. This was subjected to extraction with phenol and ether before being dialysed against 10mM Tris-HCl (pH8) and 1mM EDTA.

1.18 **DNA Co-transfections**

These were performed as described by Graham and Vander Eb (1973) as modified by Kousoulas et al. (1984). Essentially rabbit skin cells were treated with 0.2mg of DEAE-dextran per ml in PBS for 5 min and washed with 10mM Tris HCl (pH7.5) containing 1mM EDTA and 150mM NaCl immediately before adding the precipitated DNA. DNA in 0.5ml of N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid buffer was precipitated onto rabbit skin cells by the addition of 30µl of 2.2M $CaCl_2$ and incubation at room temperature for 30 min. Growth medium (DME containing 10% foetal calf serum: Materials,

Section 3.3B) was added 10 min after the addition of the $CaCl_2$-precipitated DNA to the rabbit skin cells. After incubation for 6-10h the medium was replaced and the cells were further incubated.

1.19 **Selection of Recombinant Viruses**

TK^- progeny was selected on 143 TK^- cells maintained in medium containing a mixture of 199V supplemented with 3% foetal calf serum and bromouracil deoxyribose (40µg/ml). TK^+ progeny was selected on 143 TK^- cells maintained in medium consisting of a mixture of DME supplemented with 10% foetal calf serum, hypoxanthine aminopterin and thymidine (HAT).

2 PREPARATION OF CELLULAR FRACTIONS

All the following manipulations were conducted at 0-4°C.

2.1 Total Extracts from Infected BHK and Vero Cells

Infected monolayers were washed once with PBS, harvested and sedimented by centrifugation at 2000 rpm (500g) for 5 min. The cells were solubilised in a disruption buffer containing: 7.5mM $MgCl_2$, 10mM 2-mercaptoethanol, 10mM EGTA, 40µg/ml PMSF, 40mM β-glycerophosphate (pH7.4), 8.5% (w/v) sucrose, 2% (w/v) SDS and then sonicated.

2.2 Nuclear Extracts from Infected BHK and Vero Cells

Infected cells were washed twice with PBS, scraped off and sedimented by centrifugation at 2000 rpm (500g) for 5 min. The cells were disrupted by addition of the following buffer: 40mM β-glycerophosphate (pH7.4), 7.5mM $MgCl_2$, 10mM 2-mercaptoethanol, 10mM EGTA and 40µg/ml PMSF. The nuclear and membrane fractions were separated from the cytoplasmic extract by subsequent centrifugation in a micro-centrifuge for 10 min. The sediment containing the nuclei was then solubilised in the disruption buffer described above.

2.3 Cellular Cytoplasmic Extracts (Post-Ribosomal Supernatant)

BHK or Vero cells (either infected or mock-infected) were scraped into PBS, washed twice with PBS, and then resuspended in an equal volume of a solution containing 10mM KCl, 1.5mM magnesium acetate, 10mM Tris-HCl (pH7.6), 1mM EGTA and 40µg/ml PMSF, and then allowed to stand on ice for 10 min. The cells were then broken open

by 20 strokes of a Teflon-glass homogenizer and the ionic composition adjusted to 125mM KCl, 5mM magnesium acetate, 5mM 2-mercaptoethanol and 25mM Tris-HCl (pH7.5). The homogenate was sedimented by centrifugation at 10,000 rpm (30,000g) for 30 min, and the supernatant subjected to ultracentrifugation at 50,000 rpm (165,000g) for 2.5h in the Ti50 or Ti75 rotor of a Beckman ultracentrifuge. This removes mitochondria and ribosomes. The supernatant (approximately 4-5mg protein per ml) was dialysed overnight against Buffer A (20mM Tris-HCl pH7.6, 1mM EDTA, 1mM EGTA, 10mM 2-mercaptoethanol, 40µg/ml PMSF, 10% glycerol) and either subjected to immediate anion-exchange chromatography or shock-frozen and stored at -80°C.

2.4 Ribosomes from BHK Cells

A modified version of the methods described by Ascione and Arlinghaus (1970) and Thomas et al. (1977) was used. Sedimented cells were resuspended in an ice-cold buffer containing 20mM Tris-HCl (pH7.5), 50mM KCl, 5mM $MgCl_2$ and 5mM $CaCl_2$, and kept on ice for 10 min. An equal volume of a buffer containing 0.5M Sucrose, 0.55M KCl, 10mM $MgCl_2$, 5mM $CaCl_2$, 1mM EDTA, 40mM Tris-HCl (pH7.5), 1% Triton X-100 was then added and cells left on ice for another 5 min. The cells were homogenized with 6 strokes of a Teflon-glass homogenizer. The homogenate was sedimented by centrifugation at 2000 rpm (500g) for 10 min. Sodium deoxycholate was then added to the supernatant to give a final concentration of 0.5% (w/v). The supernatant was layered over a step gradient of 8ml of a buffer containing 0.7M sucrose in 20mM Tris-HCl (pH7.6), 100mM KCl, 5mM $MgCl_2$, 1mM 2-mercaptoethanol. The gradients were centrifuged at 27,000 rpm (96,000g) for 16h at 4°C in the SW27 rotor. Any light material was

washed off the pellet and, if not used immediately, ribosomes were stored at -80°C.

2.5 Ribosomal Subunits

Ribosomal subunits were prepared by dissociation at high ionic strength using a modification of the method of Leader and Wool (1972). Ribosomes were suspended at 4°C in 10mM Tris-HCl (pH7.5), 80mM KCl and 5mM $MgCl_2$. The concentration of KCl was adjusted to 500mM and the supernatant clarified by centrifugation at 2000 rpm (500g) for 5 min. To promote separation of the subunits the clarified suspension was incubated at 37°C for 15 min with 0.1mM puromycin and 20mM 2-mercaptoethanol. Aliquots containing approx $80A_{260}$ units of ribosomes were then layered directly onto 37ml of linear 10-30% (w/v) sucrose gradient containing 10mM Tris-HCl (pH7.6), 300mM KCl, 5mM $MgCl_2$ and 20mM 2-mercaptoethanol. After centrifugation at 27,000 rpm (96,000g) for 4h at 28°C in a Beckman SW27 (or SW28) rotor, the gradients were pumped through a Gilford flow-cell (Gilford model 240) and the absorbance at 260nm was monitored. The subunit fractions were collected and the total 40S and 60S subunits obtained from 6-12 were separately pooled and sedimented at 177,000g for 16h at 4°C in a Beckman Ti60 rotor. The sedimented ribosomes were resuspended in a buffer containing 20mM Tris-HCl (pH7.6), 100mM KCl, 4mM magnesium acetate, 7mM 2-mercaptoethanol and 250mM sucrose, so that the final concentration of 40S or 60S subunits was $100A_{260}$ units per ml. Aliquots (0.1ml) were stored at -80°C.

3 SEPARATION OF PROTEINS BY CHROMATOGRAPHY

3.1 DEAE-Cellulose (DE-52) and DEAE-Sephacel Anion-Exchange Column Chromatography.

A. Analytical I

This was used for analytical studies of protein kinases. Chromatography was on DEAE-Sephacel packed in a column of 2.5 x 1cm (height x diameter) and equilibrated with Buffer A (20mM Tris-HCl pH7.6, 1mM EDTA, 1mM EGTA, 40µg/ml PMSF, 10mM 2-mercaptoethanol, 10% glycerol). Cellular cytoplasmic extract (prepared as described in Methods, Section 2.3) containing 4-15mg protein was applied, the column washed with 25ml of Buffer A, and then eluted with 84ml of a linear gradient of 0-0.4M KCl in the same buffer at a flow rate of 12ml/h, 1ml fractions being collected and assayed for protein kinase activity.

B. Analytical II

This was used in studies with mutant viruses derived from HSV-1(F). The cellular cytoplasmic extract (prepared according to Methods, Section 2.3) from each condition (30mg protein) was applied to pre-equilibrated DEAE-cellulose (DE-52) packed in a 5 x 1.6cm column. The column was washed with 165ml of Buffer A and eluted with 500ml of a linear gradient of 0-0.4M KCl in the same buffer at a flow rate of 66ml/h, 5.5ml fractions being collected and assayed for protein kinase activity immediately.

C. Preparative

The cellular cytoplasmic extract (prepared according to Methods, Section 2.3) from a single preparation (generally 75-100mg protein) was applied to pre-equilibrated DEAE-cellulose (DE-52) packed in a 6 x 1.6cm column. The column was washed with 150ml

Buffer A and eluted with 500ml of a linear gradient of 0-0.4M KCl in the same buffer at a flow rate of 72ml/h, 6ml fractions being collected. Fractions containing the viral protein kinase activity, eluting at approximately 220mM KCl were combined, shock-frozen, and stored at -80°C until the next stage of purification.

3.2 High-Performance Hydrophobic Interaction Chromatography: TSK Phenyl-5PW

Four or five DEAE-cellulose preparations (derived from 4-5 x 10^{10} infected cells) were allowed to thaw at 4°C and concentrated 10-15 times in aquacide II for 36h. The concentrated material was dialysed for 12h against several changes of Buffer A lacking glycerol and containing 1M ammonium sulphate (Buffer B). The dialysed material (35-40ml containing 90-100mg protein) was applied by repeated injection to a 7.5 x 0.75cm TSK phenyl-5PW column, pre-equilibrated with Buffer B and linked to an HPLC system, and the column washed at 1ml/min with Buffer B until all unbound protein had eluted. The column was 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 Buffer at the final concentration of 0M ammonium sulphate. Fractions (1ml) were collected and those containing the viral protein kinase were combined and subjected immediately (without freezing) to the next stage of purification.

3.3 Affinity Chromatography : Threonine-Sepharose

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 of the gel with distilled water, a 7 x 1cm column of the chromedium was

prepared and equilibrated with Buffer A. The viral protein kinase (20-25ml, containing 5-6mg protein) from TSK phenyl-5PW was applied directly (i.e. without dialysis) to the column, which was washed with 25ml of 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 of 0.4M KCl, all in the same buffer. The viral protein kinase was then eluted from the column with 50ml of a linear gradient of 0.4M-1.0M KCl in Buffer A, 2.5ml fractions being collected. Finally the column was washed with 15ml of 1.0M KCl. The fractions containing the bulk of enzyme activity eluted at approximately 0.7M KCl. They were combined and diluted with an equal volume of Buffer A to decrease the ionic strength to a value which would allow immediate further chromatography (Methods, Section 3.4A) without prior dialysis.

3.4 **Affinity Chromatography: Protamine-Agarose**

A. The diluted viral protein kinase (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 of Buffer A the column was eluted with 12ml steps of 0.5M, 0.8M, 1.0M, 1.3M, 1.5M and 2.0M KCl at a flow rate of 12ml/h, 1.2ml fractions being collected. The enzyme induced by infection with PRV eluted at 0.8M KCl, in contrast to the enzyme induced by infection with HSV-1, which eluted at the 0.5M KCl step. Peak fractions of enzyme activity were combined and adjusted to 0.05% Lubrol-PX. The purified enzyme was divided into aliquots, shock-frozen and stored at -80°C, where it was stable for several weeks at least.

B. In some instances after hydrophobic interaction chromatography the viral protein kinase was applied directly to the protamine-agarose column. The conditions of the chromatography were the same as those already described above (Methods, Section 3.4A) for the enzyme induced by infection with PRV, however several modifications were made for chromatography of the enzyme induced by infection with HSV-1. The HSV-1 enzyme was applied directly (after the hydrophobic stage) to a 3 x 1cm column of protamine-agarose pre-equilibrated with Buffer A. The column was washed with 30ml of Buffer A at a flow rate of 12ml/h and 1.2ml fractions were collected. It was then eluted with a linear gradient of 0.3-0.8M KCl in Buffer A and then with 10ml steps of 1.0M, 1.5M and 2.0M KCl. The HSV-1 enzyme eluted at approximately 0.5M KCl. The peak fractions of enzyme activity were combined and adjusted to 0.05% Lubrol-PX. The partially purified enzyme was then aliquoted, shock-frozen and stored at -80°C.

3.5 High-Performance Gel-Exclusion Chromatography

A. Superose 12

The protein kinase induced by infection with PRV was purified (as described in Methods, Sections 3.1 - 3.4), concentrated 5-fold, and then dialysed for 4h against Buffer A supplemented with 500mM KCl and 0.05% Lubrol-PX, but with glycerol omitted (Buffer C). The concentrated enzyme preparation (15-20µg protein in 0.2ml) was applied to a Superose 12 column equilibrated with Buffer C and eluted at a flow rate of 0.5ml/min, 0.5ml fractions being collected.

B. Superose 6

A sample of the protein kinase induced by infection with HSV-1 from the TSK phenyl-5PW column was concentrated 10-fold by aquacide II. The concentrated enzyme preparation (0.5mg in 0.2ml) was applied to a Superose 6 column equilibrated with Buffer C and eluted at a flow rate of 0.5ml/min, 0.5ml fractions were collected.

3.6 **High-Performance Anion-Exchange Chromatography**

The viral protein kinases partially purified from BHK cells infected with PRV or HSV-1(G) by chromatography of the post-ribosomal supernatants on DEAE-Sephacel were concentrated and dialysed against a buffer containing 20mM Tris-HCl (pH 7.6), 10mM 2-mercaptoethanol and 1mM EDTA. Equal activities of the enzymes were either loaded together or separately onto a Mono Q column, pre-equilibrated in the above buffer. The column was washed with 10ml of 0.25M NaCl in the same buffer and eluted with 30ml of a linear gradient of 0.25-0.8M NaCl at a flow rate of 1ml/min, 0.5ml fractions being collected.

3.7 **Hydrophobic-Interaction Chromatography : Phenyl-Sepharose CL-4B**

This was used initially for the preparation of the viral protein kinases and was also employed for the purification of protein kinase C from BHK cells. Chromatography was performed on pre-equilibrated phenyl-Sepharose CL-4B packed in a 6 x 1.6cm column. Fractions containing the appropriate enzyme activity from the DEAE-cellulose stage were concentrated approximately 10-fold in aquacide II and dialysed against Buffer A containing 1M ammonium sulphate (Buffer D). The sample (containing 50-100mg protein) was applied to the column, which was then washed with 150ml of Buffer D. Next the column was eluted with 500ml of a linear gradient of 1.0M-0M ammonium sulphate in Buffer A, at a flow rate of 70ml/h, 6ml fractions being collected.

Finally the column was washed with a further 60ml of Buffer A. The column fractions were assayed for protein kinase activity and the peak fractions of activity were pooled, concentrated and either subjected to further chromatography or shock-frozen and, in the case of the viral enzymes, stored at -80°C. Protein kinase C was concentrated and either subjected to immediate chromatography on threonine-sepharose (as described in Methods, Section 3.3) or dialysed against Buffer A containing 50% glycerol and 0.01% triton -X100, and stored in aliquots at -20°C.

3.8 Gel-Permeation Chromatography : Sephacryl S-200

Samples of the viral protein kinases obtained after chromatography on DEAE-cellulose were concentrated and dialysed for 12h against Buffer A containing 0.5M KCl. The same buffer was used for equilibration and elution of the column. A sample containing approximately 1mg of protein in 0.5ml was loaded on to a pre-equilibrated column (40 x 1cm) of Sephacryl S-200. The elution was performed at a flow rate of 0.5ml/min, and 0.5ml fractions were collected. Blue Dextran (1mg) was used to determine the void volume of the column. The positions of elution of proteins of known relative molecular mass were determined. Aldolase (2mg), bovine serum albumin (2mg), ovalbumin (2mg) and cytochrome c (2mg) were used as protein standards.

4 SEPARATION OF PROTEINS BY POLYACRYLAMIDE GEL ELECTROPHORESIS

4.1 Separation Methods

A. One-dimensional gel-electrophoresis in the presence of sodium dodecyl sulphate

This was performed by the method of Laemmli (1970) on SDS slab gels. The dimension of the gel plates was 19 x 16cm to fit a BRL-V-16-2 slab apparatus, and the thickness of gels was 1.5mm or 0.5mm. The separation gels contained 10% (w/v) acrylamide and 0.27% (w/v) bisacrylamide. When optimal separation of proteins of approximately 30kDa was required gels containing 15% (w/v) acrylamide, 0.09% (w/v) bisacrylamide were prepared. The separation gel also contained 0.375M Tris-HCl (pH8.8), 0.1% (w/v) SDS, 0.03% (v/v) TEMED and 0.1% (w/v) ammonium persulphate. The upper stacking gels contained 5% (w/v) acrylamide, 0.13% (w/v) bisacrylamide, 0.12M Tris-HCl (pH6.8), 0.1% (w/v) SDS, 0.03% (v/v) TEMED and 0.2% ammonium persulphate. For better resolution of viral infected cell proteins labelled with [^{32}P]orthophosphate or [^{35}S]methionine in vivo electrophoresis conditions were slightly different. Essentially the proteins were separated on 9.25% (v/v) or 11% (v/v) polyacrylamide gels containing SDS and crosslinked with diallytartardiamide as described by Morse et al.(1978).

Samples were mixed with 0.5 volumes of sample buffer containing 0.05M Tris-HCl (pH6.8), 4.5% (w/v) SDS, 45% (w/v) glycerol, 10% (w/v) 2-mercaptoethanol or 10mM dithiothreitol and 0.002% (w/v) Bromophenol Blue or Pyronine Y. The maximum amount of protein in samples was 100µg, and the maximum volume of the sample loaded was 120µl. The protein samples were completely reduced and denatured by heating at 100°C for 2 min. The electrophoresis

buffer contained 0.05M Tris base, 0.192M glycine, and 0.1% (w/v) SDS at a pH of 8.5. Electrophoresis was towards the anode at 25mA per gel for 1h and then 40mA per gel for about 4h, until the dye front was about 0.5cm from the bottom of the gel. The gels were then processed as described in Methods section 4.2.

B. Two-Dimensional gel electrophoresis

This was performed essentially according to O'Farrell et al. (1977), with the first (iso-electric focussing) dimension containing 1% ampholines in the pH 4-6 range. Samples either contained 200µg of protein or were highly radiolabelled after reaction with [γ^{32}P]ATP. The samples were concentrated by acetone precipitation and resuspended in no more than 20µl of sample buffer containing 9M urea, 5% (v/v) 2-mercaptoethanol or 10mM dithiothreitol, 2% Nonidet P40 and 2% (v/v) ampholines (pH4-6).

The first-dimension gels were poured to a height of 11cm in glass tubes of 2mm inside diameter. The gel mixture was composed of 9.2M urea, 2% (v/v) Nonidet P40, 7.3% (w/v) acrylamide, 0.1% (w/v) bisacrylamide, 3% (v/v) ampholines and 0.06% (w/v) ammonium persulphate. The gels were overlayed with a solution containing 9.2M urea and 0.1% (v/v) ampholines. The lower reservoir of the gel tank was filled with 2% (v/v) orthophosphoric acid, the tube gels were placed in the electrophoresis chamber and the samples were loaded and then overlayed with 10µl of the overlay solution. The tubes were filled with 4% (v/v) ethanolamine and the upper reservoir was then filled with this same solution. Electrophoresis was towards the anode at 200V for the first hour, then 300V overnight for at least 12h. The voltage was adjusted to 400V for the last hour of

electrophoresis to sharpen the protein bands. At the end of electrophoresis the gels were removed from the tubes and equilibrated for 1h in a two-dimensional SDS interdimensional soaking buffer containing 10% (v/v) glycerol, 10mM dithiothreitol, 3% (w/v) SDS, 0.1% Bromophenol Blue and 0.05M Tris-HCl (pH6.8). After the equilibration the gels were subjected immediately to the second-dimension electrophoresis. The second dimension was on SDS slab gels containing 10% (w/v) acrylamide and 0.27% (w/v) bisacrylamide, with a stacker gel containing 5% (w/v) acrylamide, 0.13% (w/v) bisacrylamide, as described in Methods, Section 4.1A. The first-dimension gel was placed on the top surface of the stacking gel and overlayed with 0.1% (w/v) agarose prepared in the SDS interdimensional soaking buffer. Electrophoresis was performed according to Methods, Section 4.1A.

4.2 Processing of Gels

A. Staining of gels with Coomassie Brilliant Blue

Gels were stained for 1-2h at room temperature in 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid. They were then destained for 12-24h at 37°C with several changes of the destaining solution which contained 10% (v/v) acetic acid and 10% (v/v) methanol.

B. Silver Staining

When Coomassie Blue staining was inadequate for visualisation of the proteins, the same gels were re-stained using the Biorad silver stain derived from the method of Merril et al. (1981). This method is 10-50 times more sensitive than staining with Coomassie Brilliant Blue R-250. All solutions for silver staining were prepared in deionised water (conductivity less than

1μmho, and they were all warmed to 25°C before use. Staining was performed in a glass container placed in a water bath with a shaker platform at 25°C. Gels 0.5mm - 1mm thick were kept in 40% (v/v) methanol/10% (v/v) acetic acid for at least 30 min until all traces of the Coomassie Brilliant Blue R-250 were removed. Gels were then treated with 10% (v/v) ethanol/5% (v/v) acetic acid for 30 min and then incubated in the presence of oxidiser (containing potassium dichromate and nitric acid) for 5 min. The gels were then subjected to three 5 min washes in deionised water before addition of the silver reagent (containing silver nitrate). After 20 min the silver reagent was removed and the gels were washed quickly in deionised water. The developer (containing sodium carbonate and paraformaldehyde) was added for 3-10 min. The reaction was stopped by incubation of the gels in 5% (v/v) acetic acid for 5 min. The gels were stored in deionised water or 40% (v/v) methanol to prevent swelling.

C. Autoradiography

Destained gels containing [^{32}P]labelled proteins were incubated in destaining solution with 3% (v/v) glycerol for 1h and then dried on to Whatman 3MM filter paper under suction from an electric vacuum pump. Autoradiography was at -80°C on Kodak X-Omat XAR5 or X-Omat S film, using a Cronex intensifying screen. In some instances gels were dried immediately after electrophoresis (i.e. without staining) on the same Whatman 3MM paper and autoradiographed as above.

D. Measurement of pH gradients

Measurement of the pH gradient in the first-dimensional gel in two-dimensional gel electrophoresis was as follows. An additional

first-dimensional gel was prepared in parallel with the gel on which the sample was loaded. This gel was removed from the gel tube after electrophoresis in the first dimension and it was cut into 1cm pieces. Each piece was placed directly into a stoppered vial. To each piece of gel 1ml of boiling distilled water was added and the vials stoppered securely. After 2h the vials were vortexed and the pH of the solution was measured with a thin pH electrode.

4.3 Immunological Methods

TRANSFER OF PROTEINS TO NITROCELLULOSE MEMBRANES AND DETECTION OF ANTIGENS WITH ANTISERA AND PROTEIN A OR A SECOND ANTIBODY CONJUGATED TO HORSERADISH PEROXIDASE.

Proteins separated by one-dimensional electrophoresis were transferred to nitrocellulose paper, essentially according to Towbin el al. (1979) Bowen et al. (1980) and Renart et al. (1979). Nitrocellulose was either placed in a single large sheet over the entire gel or it was cut into strips (15cm x 0.6cm) and placed over the specific proteins to be transferred. To remove any air bubbles, the nitrocellulose filters were soaked in the Blotting Buffer of Towbin et al. (1979), which consisted of 25mM Tris-HCl (pH8.3), 192mM glycine and 20% methanol. The nitrocellulose was allowed to wet by capillary action as abrupt wetting led to entrapment of air bubbles in the nitrocellulose matrix. The perspex cassette to hold the gel was prepared as follows:

Sponge or filter pads were soaked in buffer and placed in the cassette, and a piece of Whatman 3MM paper with dimensions slightly larger than the gel was soaked in the same buffer and placed on top of the pads. The gel and the nitrocellulose membrane were

equilibrated in this buffer also. The gel was placed on top of the Whatman 3MM paper and then the nitrocellulose was positioned on top of the gel. A second piece of wet 3MM paper was placed on top of the nitrocellulose along with another soaked sponge. The cassette was closed and inserted in an Electroblot transblotter tank filled with Blotting Buffer. The proteins were then subjected to electrophoresis for 2-4h at 400mA towards the anode at room temperature. After the transfer, the nitrocellulose filter was removed and placed face up in a glass vessel with a lid. The free protein binding sites on the nitrocellulose membrane were blocked using any of the methods described in the following procedures.

(i) [^{125}I] Protein A detection method

Blocking Buffer was prepared. This was essentially a Wash Buffer consisting of 0.9% (w/v) NaCl, 0.1% w/v NaN_3, 10mM Tris-HCl (pH7.4), which was diluted 10 times with water before use and supplemented with any combination of the following: 0.05% Tween 20, 3% Bovine serum albumin or 3% powdered milk. The blocking was performed overnight at 4°C or 37°C, or 1h at room temperature or 37°C on a mechanical shaker. After blocking, the nitrocellulose was washed with more Blocking Buffer and the antiserum applied. This was diluted 1:50 in Blocking Buffer containing 5% inactivated horse serum (v/v). The volume was the minimum necessary to cover the paper and incubation was for 2h at 37°C. In the case of blotting using strips of nitrocellulose and peptide antisera the following modifications were made. Apparatus consisting of a perspex block with 10 shallow slots (15cm x 0.6cm) was used. The nitrocellulose strips were placed in each compartment face up,

and a clean dry strip of Whatman Number 1 filter paper (15cm x 0.6cm) was placed on top of the nitrocellulose. 250µl of the appropriate antiserum was pipetted on top of the filter paper, wetting it along its length. The top of the block of strips was covered with a glass plate and placed (in a sealed container) at 37°C for 2h. After this time the filter strips were removed from the nitrocellulose. (If it was necessary to conserve antisera, the filter paper was sealed in plastic and stored at -20°C. It could be re-utilized without loss of signal with a supplement of only 100µl of antiserum). The nitrocellulose was then washed with Blocking Buffer.

For routine blots washing was for 30 min with 5 changes of buffer at room temperature. More rigorous washing was used in the case of the strip blotting with peptide antisera. Washing was for 2-24h at 37°C with several changes of Blocking Buffer. [^{125}I]Protein A was then applied in Wash Buffer (i.e. Blocking Buffer without Tween 20) and 3% serum albumin. 5 x 10^5 cpm of [^{125}I]Protein A per strip was used in 3ml of Wash Buffer, or 1-8 x 10^5 cpm/ml of [I^{125}]Protein A in Wash Buffer was used, to cover a large piece of nitrocellulose and incubation was for 2-3h. The nitrocellulose filters were then washed briefly in Blocking Buffer for 30 min with 5 changes of buffer, or more rigorously. This rigorous procedure involved washing for 2-24h at 37°C with several changes of buffer. Finally, two washes were performed in Blocking Buffer containing 1M KI for 1h each. Then the filters were washed twice in PBS, and dried at room temperature on blotting paper. The filters were then subjected to autoradiography.

(ii) Horseradish peroxidase detection method

After electrophoresis, the polypeptides were transferred electrically to a nitrocellulose sheet (as described above) and reacted with monoclonal antibodies. Briefly, the nitrocellulose sheet was incubated for 1h at 37°C in PBS containing 3% (w/v) serum albumin. The monoclonal antibody was then applied, (usually diluted 1:50-1:100 in PBS with 1% (w/v) serum albumin) and incubation with the antibody was for 2h at 37°C. The unbound antibodies were then removed by washing with PBS containing 1% serum albumin.

At least 3 washes, each with 100-200ml of solution, were performed. Then the nitrocellulose sheet was incubated in PBS containing 1% serum albumin and 5µl per 3ml of horseradish peroxidase-coupled rabbit anti-mouse immunoglobulin G (Miles Laboratories, Inc, Elkhart, Ind.) for 1-2h. After washing with PBS, the nitrocellulose sheet was transferred to a freshly made substrate solution containing 0.02% (w/v) ortho-dianisidine dihydrochloride and 0.06% hydrogen peroxide. When the antibody-decorated protein bands were visible at the desired intensity the nitrocellulose was washed with PBS and dried on blotting paper. The procedure described above was essentially from Ackermann et al. (1984).

4.4 Appendix

Labelling of Protein A with ^{125}I.

All reagents were prepared just before use; the key reagents were:

(i) PBS

(ii) Chloramine T (10mg in 20ml PBS)

(iii) Sodium metabisulphite (20mg in 20ml PBS)

(iv) 0.1% bovine serum albumin (0.1g in 100ml PBS)

(v) Protein A (Sigma P8143). The protein A was reconstituted in PBS (1mg in 2ml) and dispensed in 20µl (10µg) aliquots. These were stored in plastic vials at -80°C until required

(vi) ^{125}I[NaI] (Amersham IMS 30)

(vii) 1M potassium phosphate (pH7.6)

Approximately 10µl of potassium phosphate buffer was added to 10µg of protein A. 200µCi of ^{125}I [NaI] and 20µl of chloramine T solution were then added. The mixture was incubated at room temperature for 20 seconds. 20µl of the sodium metabisulphite solution was added and the mixture was incubated further for 20 seconds and 1ml of PBS was added. The free ^{125}I [NaI] was removed by desalting this mixture on a Sephadex G25 column. The column was pre-equilibrated with 25ml of PBS containing 0.1% serum albumin. 1ml of the iodonated protein A mixture was loaded and the column was eluted with the equilibration solution. Approximately 20 fractions of 0.5ml were collected. The radioactivity of 10µl of each fraction was determined in a gamma counter and the fractions containing the most highly labelled protein A were pooled and aliquoted.

5 PHOSPHORYLATION REACTION IN VITRO

5.1 Measurement of Protein Kinase Activity

A. Phosphorylation of protein substrates

Protein kinases were assayed by measuring the incorporation of ^{32}p from [γ^{32}P]ATP into various protein substrates. The procedure followed in principle, the method of Corbin and Reimann (1974). The standard assay mixture for protein kinase activity contained in a total volume of 0.12ml : 20mM Tris-HCl (pH7.6) 10mM $MgCl_2$, 10mM 2-mercaptoethanol, 0.1mM ATP containing 0.5-1µCi [γ^{32}P]ATP, KCl in the range 50-150mM and 96µg protamine sulphate. Protamine sulphate was routinely used as the substrate, however mixed histones (0.8mg/ml) or dephosphorylated casein (0.8mg/ml) were also used occasionally, and assays for cyclic AMP-dependent protein kinase contained 10µM cyclic AMP. The reaction was started by addition of the protein kinase (40µl in the case of column fractions, or 80µl in the case of experiments performed with the recombinant viruses to increase the sensitivity of the assay). Incubation was at 30°C for 30 min, except when initial velocities were being determined, in which case the reaction was terminated after 10 min. At the end of the incubation period, 100µl samples were applied to Whatman 3MM filter paper discs or P81 filters (2 x 2.5cm pieces) which were washed for 15 min periods, twice in 10% (w/w) trichloroacetic acid, and four times in 5% (w/w) trichloroacetic acid, and then rinsed in absolute ethanol, dried and their radioactivity measured either by scintillation spectrometry or as Cherenkov radiation in the ^3H channel of a scintillation spectrometer.

B. Phosphorylation of Synthetic Peptides

The reaction mixture for assay of protein kinase activity contained in a total volume of 60µl, 20mM Tris-HCl (pH7.5), 100mM, 500mM or 1M KCl, 10mM $MgCl_2$, 10mM 2-mercaptoethanol and 0.1mM ATP containing 1-2µCi [γ^{32}P]ATP, 0.05 units of viral protein kinase (or in some experiments cyclic AMP-dependent protein kinase with OM KCl and 10µM cyclic AMP) and peptides (at the concentrations indicated in the figure legends of Results, Section II, 2). Incubation was for 10min at 30°C, after which time the reaction was terminated by addition of 6µl of 20% trichloroacetic acid. 60µl of the sample was applied to phosphocellulose (P81) paper and washed for 20 min in 0.3% orthophosphoric acid and then twice in distilled water for a further 40 min. The filters were dried and their radioactivity determined by scintillation spectrometry. In control experiments the binding to phosphocellulose paper of many of the phosphopeptides used here (including one of the least basic ones, RPTVA) was checked by comparison with the recovery of phosphoserine and phosphothreonine after acid hydrolysis, and found to be greater than 90% in all peptides tested. For determination of kinetic constants, the concentrations of peptides were varied in the appropriate ranges, these being from 2-80µM to 0.1-4mM, and values were calculated from double reciprocal plots fitted to the Michaelis-Menten Equation.

C. Phosphorylation of Ribosomal 40S Subunits

Ribosomal 40S subunits were prepared as described in Methods, Section 2.4 and 2.5, and used as substrates for the viral protein kinases. The assay conditions for phosphorylation were as described in Methods, Section 5.1A, but with some modifications.

The assay volume was reduced to 40µl, 0.5A_{260} units of ribosomal 40S subunits in 2-5µl were used instead of protamine, and 10µl of enzyme fraction was added. After 30 min incubation at 30°C the reaction was stopped by adding 20µl of SDS sample buffer and the products of the phosphorylation reaction were analysed by one-dimensional gel electrophoresis as described in Methods, Section 4.1,A, employing the 15% Acrylamide, 0.02% bisacrylamide system. The gel was then stained, destained, dried and subjected to autoradiography, all as described in Methods (Section 4.2A and C).

5.2 Autophosphorylation of Viral Protein Kinases

For assay of autophosphorylation of the purified viral kinases the following modifications were made to the standard assay described in Methods, Section 5.1A: Protamine sulphate was omitted, the assay volume was decreased to 60µl and the enzyme volume to 20µl, the concentration of $MgCl_2$ was 20mM, and the specific radioactivity of the ATP was increased by using 0.01mM ATP containing 5µCi [γ^{32}P]ATP. The reaction was terminated by the addition of 5 volumes of ice-cold acetone, and maintained at -20°C for 24h to precipitate the protein, which, after centrifugation (for 10 min in an Eppendorf microfuge), was resuspended and concentrated in 10-20µl of sample buffer in preparation for gel electrophoresis under denaturing conditions or isoelectric focussing as described in Methods, Section 4.1A and B.

6 EXTRACTION OF PHOSPHORYLATED PEPTIDES FROM P81 PAPER

The phosphorylated peptide was precipitated on P81 filter paper as described in Methods Section 5.1B. The phosphopeptide was eluted from the paper as follows:

The filter paper was rolled and placed in a thin glass tube within a test tube. 300µl of 6M HCl was added and the tubes were centrifuged for 1-2 min in a bench centrifuge. The liquid (HCl and extracted phosphopeptide) was removed from the bottom of the tube and transferred to a bulb-shaped glass flask. The procedure was repeated 5-6 times using 100µl of 6M HCl each time 2.5ml of 6M HCl was added to the extracted phosphopeptide in the bulb shaped flask. The radioactivity of the phosphopeptide was monitored and if sufficient radiolabelled phosphopeptide was recovered (at least 10,000cpm recorded on a Geiger counter) the phosphopeptide was subjected to analysis by high-voltage paper electrophoresis as described in Methods, Section 7. Mono- and di-phosphorylated forms of the particular peptide could be detected by this method.

7 ANALYSIS OF PHOSPHOPEPTIDES BY HIGH VOLTAGE PAPER ELECTROPHORESIS

This was performed essentially as described by Donella-Deana et al. (1979). Phosphorylated peptides prepared as described in Methods, Sections 5.1B and 6 were separated from [γ^{32}P]ATP and, in some instances, their mono- and di-phosphorylated forms resolved by high-voltage paper electrophoresis. The sample was dried in a vacuum desiccator and the residue resuspended in 0.1ml of electrophoresis buffer (2.5% (v/v) formic acid and 7.8% (v/v) acetic acid, pH1.9). Aliquots of this sample were applied to Whatman 3MM paper. The paper was wetted with buffer and subjected to electrophoresis towards the anode at 3000V (150V/cm) for approximately 1-2h.

After electrophoresis, the paper was allowed to dry and analysed. The radioactivity in the electrophoretogram was sometimes quantified with a Packard radiochromatogram scanner, but was routinely subjected to autoradiography using Kodak X-omat XAR5 film and an intensifying screen at -80^{o}C. The areas corresponding to the phosphopeptides were cut out and their radioactivity was determined by scintillation spectrometry, or the phosphopeptide was eluted from the paper as described in Methods, Section 6, but using 10% acetic acid so that it could be further analysed.

8 ISOLATION AND ESTIMATION OF LABELLED PHOSPHOAMINO ACIDS

Phosphopeptides prepared as described in Methods, Sections 6 and 7 were further analysed as follows: Each phosphopeptide was hydrolysed in approximately 2.5ml of 6M HCl at 110°C for 4h in sealed flasks. The reaction was terminated by cooling in ice after the 4h period. The flask was opened and the solution containing the hydrolysed peptide was dried, washed once with 0.5ml of distilled water and re-dried in a vacuum desiccator. The dried sample was then resuspended in 0.1ml of electrophoresis buffer (described in Methods, Section 7). Aliquots of this sample (50µl) were applied to Whatman 3MM paper, separately and in a mixture with phosphoamino acid standards, o-phosphoserine (20µg) and o-phosphothreonine (20µg), and 5µCi of ^{32}Pi was also applied separately to the paper. The paper was wetted with buffer and subjected to high-voltage paper electrophoresis, as described in Methods, Section 7, but the duration of the run was increased to 3-4h. After this time the electrophoretogram was allowed to dry and analysed. The portion of the electrophoretogram containing phosphoamino acid markers was stained with a ninihydrin/cadmium acetate solution (Dreyer and Bynum, 1967). The portion containing ^{32}P-labelled samples was subjected to autoradiography using Kodak X-omat XAR5 film and an intensifying screen at -80°C.

After analysis of the autoradiograph of the electrophoretogram, the areas of the electrophoretogram that corresponded to [^{32}P]serine and [^{32}P]threonine were cut out of the paper. The amounts [^{32}P]serine and [^{32}P]threonine were determined by scintillation spectrometry. The values were corrected for hydrolytic losses of 57% and 18% for [^{32}P]serine and [^{32}P]threonine respectively (as determined by Bylung and Huang, 1976).

9 DETERMINATION OF THE CONCENTRATION OF PROTEIN

The concentration of protein was measured by the dye-binding method of Bradford (1976) as modified by Spector (1978). Coomassie Brilliant Blue G-250 solution (1ml) at a concentration of 0.01% (w/v) in 0.95% (v/v) ethanol and 8.5% (w/v) phosphoric acid, was mixed with an aliquot of the protein solution (containing up to 20µg of protein in Buffer A) and the absorbance at 595nm measured in a spectrophotometer. A standard curve was prepared using known concentrations of bovine serum albumin.

10 COMPUTER ANALYSIS

To aid in the identification of possible viral coded substrates for the viral protein kinases the whole VZV sequence and the complete Us region of HSV-1 and HSV-2 were analysed using the ASTL program (R. Eason, University of Glasgow, unpublished). The program allows the user to identify in amino acid sequences seryl and threonyl residues with either basic or acid environments on their N-terminal or C-terminal sides. Visual examination was made of all seryl and threonyl residues with at least one basic amino acid residue in the five adjacent N-terminal amino acid residues.

I THE GENETIC ORIGIN OF A NEW PROTEIN KINASE FOUND IN CELLS INFECTED WITH HERPESVIRUS

The new protein kinase activity detected in cells infected with the α-Herpesvirus, PRV, was initially designated ViPK: virus-induced protein kinase (Katan et al., 1985). This somewhat clumsy name had been deliberately chosen to indicate that the genomic origin of the enzyme was unknown. In an attempt to solve the question of the genetic origin of the enzyme in the present work several indirect approaches were undertaken, and these were subsequently followed by more direct genetic and immunochemical strategies.

1.1 Characteristics of induction of a new protein kinase in cells infected with herpesviruses

As a first step towards answering the question of genomic origin, the relationship of the induction of ViPK to the life cycle of the virus was examined. Studies were done to see if the characteristics of appearance of ViPK were consistent with those for virally coded enzymes.

Experiments were performed to see whether infection of BHK cells with another member of the herpesvirus family, HSV-1, caused the induction of a protein kinase similar to the ViPK induced by PRV. The rationale of this was to examine the two enzyme activities for possible structural or functional differences which might suggest that the enzymes were distinct entities. Such differences would be consistent with the two enzymes being encoded by viral DNA.

Induction of ViPK by infection with PRV or HSV-1

Because of the number of protein kinases in crude cellular extracts, it was necessary to fractionate these extracts by anion-exchange chromatography to identify and assay ViPK. This fractionation is shown in Fig. 1.1(A) for uninfected cells and in Fig. 1.1(B) for cells infected with PRV where, as observed by Katan et al. (1985), an additional peak of protein kinase activity was found. This enzyme was found to elute at a higher ionic strength (approximately 220mM on DEAE-cellulose) than the other protamine kinases. These other enzymes were identified as protein kinase C, which eluted at 50-80mM KCl, and the so-called protein kinase M, its presumed proteolytic derivative. This latter activity eluted at approximately 150mM KCl (Fig. 1.1). It has been shown previously that viral infection does not influence the yield or relative proportions of these latter enzymes (Katan et al. 1985). When cells were infected with HSV-1 rather than PRV, a new protamine kinase activity was also observed. This routinely eluted at a somewhat lower ionic strength than the enzyme found in cells infected with PRV, and was sometimes incompletely resolved from the kinase M activity (Fig. 1.1D). This different chromatographic behaviour was the first indication that the enzymes induced by PRV and HSV-1 were different.

Correlation of induction of ViPK with viral infection

Infection of cells with herpesviruses results in the controlled sequential appearance of virus-induced mRNA and proteins (Honess and Roizman, 1974; 1975). Experiments were performed to see if it was possible to correlate the appearance of ViPK with the immediate-early (α), early (β) or late (γ) viral proteins; and whether

factors affecting the induction of virally coded proteins had similar effects on the induction of ViPK.

The time at which ViPK appeared in cells infected with PRV or HSV-1 was examined (Fig. 1.2A and B). There was a steady increase in activity during the growth cycle of the virus, and this roughly paralleled that of the early (β) protein, viral DNA polymerase, and preceeded the release of virus from the cells (Fig. 1.2A).

The amount of ViPK detected depended on the multiplicity of infection with either PRV or HSV-1 at multiplicities of up to 20 p.f.u./cell (Fig. 1.3). A decreased yield with PRV was obtained at high multiplicities, but this was similar to effects observed for other enzymes induced by HSV-1 (Perera, 1970). These findings were consistent with the possibility that the enzymes induced by PRV and HSV-1 were products of early viral genes.

Initial characterization of the catalytic and physical properties of ViPKs

In view of the slight differences observed in elution from DEAE-cellulose and DEAE-Sephacel chromedia it was necessary to determine the extent to which these enzymes had similar catalytic properties. In addition to their preference for protamine as an artificial substrate, two features were initially observed which argued their similarity. Firstly they were both active at concentrations of KCl up to 1M, at least (Fig. 1.4); and secondly they were both able to phosphorylate ribosomal protein S7 on 40S ribosomal subunits *in vitro* (Fig. 1.5), a characteristic of ViPK previously reported for the enzyme from cells infected with PRV (Katan *et al.*, 1985). Other protein kinases (notably protein

kinase C) do not phosphorylate this protein (Parker et al., 1985). This characteristic was useful in subsequent experiments for confirming a particular enzyme was ViPK. The preparation of ViPK from cells infected with HSV-1 was also capable of phosphorylating ribosomal protein S6 at low ionic strength (Fig. 1.5), whereas the enzyme induced by PRV could only phosphorylate this protein at high ionic strength. However the ability of the HSV-1 enzyme preparation to phosphorylate ribosomal protein S6 at low ionic strength may be a consequence of cross contamination with protein kinase M rather than an intrinsic difference from the enzyme isolated from cells infected with PRV.

The different chromatographic properties observed on DEAE-Sephacel of the ViPKs from cells infected with the different viruses (Fig. 1.1) could indicate that the two enzymes are distinct, or it might merely be an artefact caused by non-specific association of an identical cellular protein kinase with different viral proteins. To investigate this further, preparations of ViPK isolated by DEAE-Sephacel chromatography of extracts of cells infected with PRV or HSV-1 were mixed and subjected to re-chromatography on the high performance Mono Q anion exchange column as described in Methods, Section 3.6. This resolved the mixture into two peaks of activity (Fig. 1.6). Separate chromatography of the two enzymes revealed that the first peak represented ViPK from cells infected with HSV-1 and the second from cells infected with PRV. This order of elution is similar to that from DEAE-cellulose and DEAE-Sephacel. Further evidence that the first peak of activity was HSV-1 ViPK was its ability to phosphorylate both ribosomal proteins S6 and S7 at moderate ionic strength, as had been found for cruder preparations of

ViPK from cells infected with HSV-1 (Fig. 1.5). Under the same conditions the second peak of activity only catalysed the phosphorylation of ribosomal protein S7 (inset Fig. 1.6). Because the enzymes from the two sources showed apparently genuine differences in behaviour in anion-exchange chromatography they were subjected separately to another type of fractionation, gel-permeation chromatography on Sephacryl S-200 (Fig. 1.7). It was found that the apparent relative molecular mass (approximately 200,000) for ViPK from cells infected with HSV-1 was much greater than that (approximately 90,000) from cells infected with PRV. The fact that the chromatography was conducted at 0.5M KCl would argue against the differences being due to non-specific aggregation, but would rather imply that two distinct enzymes were involved.

One possible trivial explanation of the different physical properties of the ViPK preparations was partial proteolytic cleavage of one of the enzymes during extraction from the cells and subsequent chromatography. However, when isolation was performed in the presence of protease inhibitors (2mM EGTA, 40µg/ml PMSF, 100µg/ml leupeptin) no difference in the position of elution from DEAE-Sephacel was observed. Specific intracellular partial proteolysis could not, however be excluded at this stage.

Requirement for viral gene expression in the induction of ViPK

Experiments were performed to investigate what role, if any, the viral genome played in the induction of ViPK in cells infected with HSV-1 or PRV. If ViPK were virally coded or if it required for its induction, activation by a virally specified protein; disruption of viral gene expression would be expected to affect the appearance of ViPK in infected cells.

It was found that the induction of ViPK was prevented if the virus was inactivated by ultra-violet light (Fig. 1.8). The result of this experiment is consistent with there being a need for viral gene expression for the induction of ViPK. Inactivation of the virus by ultra-violet light however, has some limitations. The dose of ultra-violet radiation necessary to inactivate viral DNA may also inactivate proteins of the virion. Thus it could not be completely excluded from this experiment that induction of the enzyme only requires interaction of virion proteins with the host cell (cf. Kemp et al., 1986).

In the case of HSV-1 a less equivocal approach was possible because of the availability of temperature-sensitive mutants. The mutant, tsK (Preston, 1979) was used, in which a mutation in the 175,000 molecular weight immediate early protein, $\alpha 4$, prevents the synthesis of mRNA for the early and late proteins at the restrictive temperature (Watson and Clements, 1980). It was found that ViPK was induced at the permissive temperature ($31^{o}C$) but not at the restrictive temperature ($38.5^{o}C$), even though the enzyme was induced by infection with the parent virus at the latter temperature (Fig. 1.9). It should be noted that the amount of kinase detected in this experiment was less than in other experiments because circumstances dictated the use of only 5×10^{8} cells (rather than the 5×10^{9} cells of the experiment illustrated in Fig. 1.1). The DNA polymerase activity of a portion of the post-ribosomal supernatants was also determined in each case, and the results confirmed that there was no escape into the synthesis of early viral proteins at the restrictive temperature ($38.5^{o}C$) (Fig. 1.10).

Therefore the appearance of ViPK in cells infected with PRV or HSV-1 both required and correlated with the expression of the viral genome. However, although viral gene expression was required, a necessary condition if ViPK were to be a viral enzyme, the results did not exclude the possibility of a newly synthesised viral protein activating or stimulating the synthesis of a cellular protein kinase.

1.2 Relation of the protein kinase induced by HSV-1 to the US3 gene

As already mentioned in the Introduction, strong evidence that α-herpesviruses do indeed encode a protein kinase came from the finding that the amino-acid sequences of the predicted products of the US3 gene of HSV-1 and the US2 gene of VZV contain most of the sequence motifs of protein kinases. It was now pertinent to investigate whether the knowledge of the structure of the gene product of US3 could be used to relate it to ViPK.

Two different strategies were followed for this investigation. One entailed using a mutant virus with a deleted US3 gene. The other involved the use of an antiserum raised against the predicted US3 gene product.

1. Studies with a Mutant Virus with a Specific Deletion in the US3 Gene

To determine whether the protein kinase induced by HSV-1 was encoded by the open reading frame US3, experiments were performed with a mutant virus with a genetically engineered deletion in the US3 gene. This mutant was generated by Richard Longnecker (University of Chicago), and the construction and

restoration of the deletion (the latter being conducted during a collaboration between RL and the author of this thesis) is described briefly below.

Construction and Properties of the HSV-1 Mutants

Four mutants, R7035, R7040, R7041 and R7050 were constructed, starting with HSV-1(F)Δ305 which is a TK$^-$ strain derived by recombination replacement of the wild-type thymidine kinase (TK) gene with a fragment from which 700 bp within the domain of the TK gene had been deleted (Post and Roizman, 1981).

(i) R7035 contains a chimeric TK gene inserted into the US3 gene of HSV-1(F)Δ305. It was constructed by co-transfection of HSV-1(F)Δ305 intact TK$^-$ viral DNA with a DNA fragment in which a chimeric TK gene replaced a small NcoI fragment within the coding region for US3 (Fig. 1.11, lines 3,8). The chimeric TK gene was constructed by fusion of the promoter-regulatory domain of the α27 gene to the transcribed non-coding and coding sequences of the TK gene. Analysis of plaque-purified progeny selected for TK activity verified the site of insertion of the chimeric TK gene. (Longnecker and Roizman, 1987).

(ii) R7040 is a TK$^-$ derivative of R7035 containing a deletion in the US3 gene. It was constructed by co-transfection of intact R7035 viral DNA with plasmid pRB3696 DNA. The HSV-1(F) DNA fragment cloned in plasmid pRB3696 contained a deletion within the US3 coding region from the Pst I site located at the predicted amino acid 69 of

the 5' end of US3 to the Bam HI site located at amino acid 357 of US3 (Fig. 1.11, line 9) (Longnecker and Roizman, 1987; McGeoch et al., 1985). As a result of the deletion, the US3 coding sequences downstream of the deletion have been frameshifted (Fig. 1.11, line 9). The domains of the coding sequence deleted from the open reading frame included the motifs common to protein kinases cited in the Introduction and located in positions shown in Fig. 1.11, line 10 A and B. Plasmid pRB3696 was then co-transfected with intact R7035 DNA into rabbit skin cells. TK^- progeny was selected in 143 TK^- cells in the presence of bromodeoxyuridine and the plaque purified R7040 DNA was shown to lack the sequences deleted from pRB3696 DNA (Longnecker and Roizman, 1986; 1987).

(iii) R7041 is a US3 deletion strain identical to R7040 except that the 700 bp deletion in the TK gene was restored by co-transfection of intact R7040 DNA and Bam HI Q DNA cloned as pRB103 into rabbit skin cells. The progeny of the transfection were plated on 143 TK^- cells and TK^+ progeny was selected, plaque purified and tested for the presence of an intact Bam HI Q fragment (Fig. 1.11 D, line 3) with restriction endonucleases (Longnecker and Roizman, 1986; 1987). R7041 would be expected to differ from the wild-type HSV-1(F) solely with respect to the deletion in the US3 open reading frame (Fig. 1.11, line 5.)

(iv) Recombinant R7050 is a derivative of R7035 in which the US3 gene has been rescued. It was constructed by co-transfecting on rabbit skin cells intact R7035 DNA with a cloned HSV-1(F) Sac I fragment (pRB3446) containing the entire US3 coding region and flanking DNA. The progeny of the transfection was plated on 143 TK^- cells and TK^- virus was selected, plaque purified, and tested for the presence of an intact Bam HI N DNA fragment containing an intact US3 open reading frame.

(v) Recombinant R7051 is a derivative of R7041 in which the US3 gene has been rescued. It was constructed by co-transfecting on rabbit skin cells intact R7041 DNA with an excess of cloned HSV-1(F) Sac I fragment (pRB3446) containing the entire US3 coding region and flanking DNA. Plaques from the progeny of the transfection were selected and viral DNA was isolated from these and tested for the presence of an intact Bam HI fragment containing an intact US3 open reading frame.

To test whether the HSV-1 induced protein kinase was encoded by the open-reading frame US3, BHK (TK^+), BHK (TK^-) and Vero (TK^-) cells were infected with the specially constructed viral mutants. Cellular cytoplasmic extracts were then prepared and fractionated by anion-exchange chromatography, modified to improve the resolution of ViPK from the other protein kinases in the extract (Methods, Section 3.1B). Because of the instability of the protein kinase C peak of activity in BHK cells, which eluted at 0.1M KCl (Fig. 1.12), Vero

cells, in which this enzyme activity proved more stable, were used for the major experiments. HSV-1(F) could induce ViPK well in these cells and highly reproducible relative yields of protein kinases C and M obtained.

The results of infection with the mutant viral constructs are shown in Fig. 1.13 C and D. It can be seen that the elution profile of the protein kinases from extracts of Vero cells infected with the US3 deletion strain R7041 (Fig. 1.11) lacks the protein kinase activity eluting at 0.22M KCl in the extracts of cells infected with wild-type virus. The profile obtained with this mutant is almost identical to that of the mock-infected cells (Fig. 1.13). Similar differences in the elution patterns were obtained in two independent experiments with cytoplasmic extracts of infected Vero cells and in two experiments with cellular cytoplasmic extracts of infected BHK cells (Fig. 1.12 A and B).

It was necessary to establish that the lack of the virus-induced protein kinase activity in cells infected with these viral mutants is due to the lack of a functional US3 gene, and not to a fortuitous mutation introduced into the domain of the TK gene during its rescue, and concurrently in the genes which partially overlap the TK gene. The US3 open reading frame of recombinant virus R7035 was therefore repaired and this repaired recombinant virus designated R7050 (Fig. 1.11). Infection of cells with R7050 resulted in the re-appearance of the protein kinase activity induced by the wild-type virus (Fig. 1.13 D).

The rescue of R7035 to R7050, although a persuasive result, required further controls. It was necessary to demonstrate that R7035, like R7041, did not induce viral kinase activity. Further a more direct control would be a rescued strain generated from R7041 in a single step. This latter strain, R7051, was constructed by Dr. Richard Longnecker, as already noted above. Strain R7051 and R7035, together with HSV-1(F) and R7041, were used in further experiments to infect BHK cells (Fig. 1.12 B). It can be seen that R7035 did not induce the viral protein kinase whereas the strain R7051, directly rescued from R7041, did. In this experiment there was loss of protein kinase C in the case of R7041. However, the inability of R7041 to induce viral protein kinase had already been demonstrated in BHK and Vero cells under conditions when there was no loss of protein kinase C (Fig. 1.12 A and 1.13). To control for the possibility that the inability of R7041 to induce the protein kinase reflected poor growth of this strain, two additional experiments were done. The results of the first, summarized in Table 1.1, shows that similar yields of virus were obtained from duplicate Vero roller bottle cultures 18h after infection with R7041, R7050 and HSV-1(F) under conditions similar to those used for the preparation of extracts tested for protein kinase activity. In the second experiment, samples of the cytoplasmic cellular extracts previously used for the determination of the protein kinase activity were tested for the presence of glycoprotein B. In these experiments, identical amounts of total protein from the cell extracts were

electrophoretically separated in denaturing polyacrylamide gels, transferred to nitrocellulose and reacted with monoclonal antibody H1163, specific for HSV-1(F) glycoprotein B (all as described in Methods, Section 4.3(ii)). The results shown in Fig. 1.14 indicate that the cell extracts of R7041, R7050 and HSV-1(F) contained similar amounts of glycoprotein B, and confirm that the infection with the viral mutants was as good as that with the wild-type.

2. Studies with an Antiserum raised against a Synthetic Oligopeptide from the predicted product of the HSV-1 Gene US3

Experiments were performed with an antiserum raised by Margaret Frame (Virology Dept., University of Glasgow) against an oligopeptide in the US3 gene. The peptide used was:

NH_2-Tyr-Cys-Leu-Pro-Leu-Phe-Gln-Gln-Lys-COOH

which represents the 8 carboxyl amino acids (474-481) together with an additional tyrosine residue (not specified by the US3 sequence) at the N-terminus, to allow coupling to bovine serum albumin. Rabbits were immunised with the peptide-serum albumin conjugate and the anti-serum albumin antibodies were removed from the immune sera generated by adsorption with serum albumin. The resulting antiserum was designated anti-US3 serum.

This antiserum was used in immunoblotting experiments to detect the protein product of the US3 gene. Proteins extracted from BHK cells infected with 20 p.f.u./cell HSV-1(G) for 18h and labelled with 100μCi/ml[^{35}S] methionine were electrophoretically separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose strips all as described in Methods, Section 4.1 and 4.3. Antigens immobilised on the nitrocellulose were detected by the antibody and [^{125}I] protein A (Methods, Section 4.3(i)).

The product of the US3 gene was detected as a 68,000 molecular weight protein (Fig. 1.15, lanes 2 and 3). It can be seen that the anti-US3 serum also weakly immunoblotted a lower molecular

weight species of 50,000 molecular weight (Fig. 1.15, lane 2). This may be related to the 68,000 molecular weight polypeptide, perhaps as a cleavage product of this. No reaction was seen when the antiserum was blotted against an extract from mock-infected cells. To determine whether or not the 68,000 molecular weight protein recognised by the anti-US3 serum was the protein kinase induced by HSV-1, the enzyme was purified from cellular cytoplasmic extracts of BHK cells infected for 18h with HSV-1(G) at a multiplicity of infection of 10 p.f.u./cell as described in Methods, Sections 1.3 and 2.3. This entailed successive chromatography on DEAE-cellulose, TSK phenyl-5PW, threonine-sepharose and protamine-agarose. This is described in Methods, Sections 3.1C-3.4 and illustrated in Results, Section II.

Although the final preparation of the protein kinase was not homogeneous, it contained only two major bands when analysed by polyacrylamide gel electrophoresis under denaturing conditions (Results, Section II, Fig. 2.8). The anti-US3 serum was used in immunoblotting experiments with material from various stages of the purification and with a purified preparation of protein kinase C as a control. After the threonine-sepharose stage of the purification a 68,000 molecular weight protein was detected by the anti-US3 serum at a low level. (Fig. 1.16 lanes 5 and 6). After the protamine-agarose stage the amount of the 68,000 molecular weight species detected was increased considerably (Fig. 1.16, lanes 2 and 3). The US3-antiserum showed no reaction with homogeneous protein kinase C, another protein kinase capable of phosphorylating protamine (Fig. 1.16, lanes 8

and 9). A number of unrelated anti-peptide sera, including that shown in Fig. 1.16 against the immediate-early polypeptide product of gene αO(1E 110) of HSV-1 (Perry et al., 1986) were used and showed no reaction with any of the protein kinase preparations. These served as controls against the possibility that a component of the anti-US3 serum, other than the US3 antibodies, might be binding non-specifically or specifically to the 68,000 molecular weight protein. Although there may not appear to be much difference in intensity of the reaction of crude extracts and enzyme purified to the protamine-agarose stage, it should be pointed out that approximately 2000 times more crude extract protein than protamine-agarose purified protein was loaded on the gel (Fig. 1.15). Thus the amount of the 68,000 molecular weight US3 product detected in the purified material after affinity chromatography on protamine-agarose correlated well with the increase in the specific activity of the viral kinase (Results, Section II, Table 2.2(A)).

Further evidence consistent with the 68,000 molecular weight polypeptide detected by immunoblotting being a protein kinase was obtained from autophosphorylation of the preparation (Fig. 1.16). This is discussed in more detail in Results, Section II, where the purification of the protein kinase is presented. However, at this juncture what is pertinent to mention is the fact that autophosphorylation resulted in the labelling of a protein of Mr 68,000, the same value obtained for the immunoblotting.

Considered together, all the results presented in Section I provide overwhelming evidence that the ViPK is the product of the HSV-1 US3 gene. This will be argued in detail in the discussion. However, it would appear justified, from this point in the thesis onwards, to refer to the protein kinase as the protein kinase specified by the particular herpesvirus, namely HSV-1 PK and PRV PK.

Fig. 1.1 DEAE-Sephacel Column Chromatography of Protein Kinases from BHK Cells Infected with Herpesviruses

Mock-infected BHK cells (A,C), or (B) cells infected for 8h with PRV, or (D) for 18h with HSV-1(G), were processed as described in Methods, Section 2.3, and their post-ribosomal supernatants were subjected to anion-exchange chromatography on DEAE-Sephacel as described in Methods, Section 3.1.

The protein kinase activity (●-●) of a 40µl aliquot of each column fraction was assayed with protamine as a substrate employing the standard protein kinase assay conditions described in Methods, Section 4.1A.

The KCl concentration (---) of the column fractions was also determined from conductivity measurements.

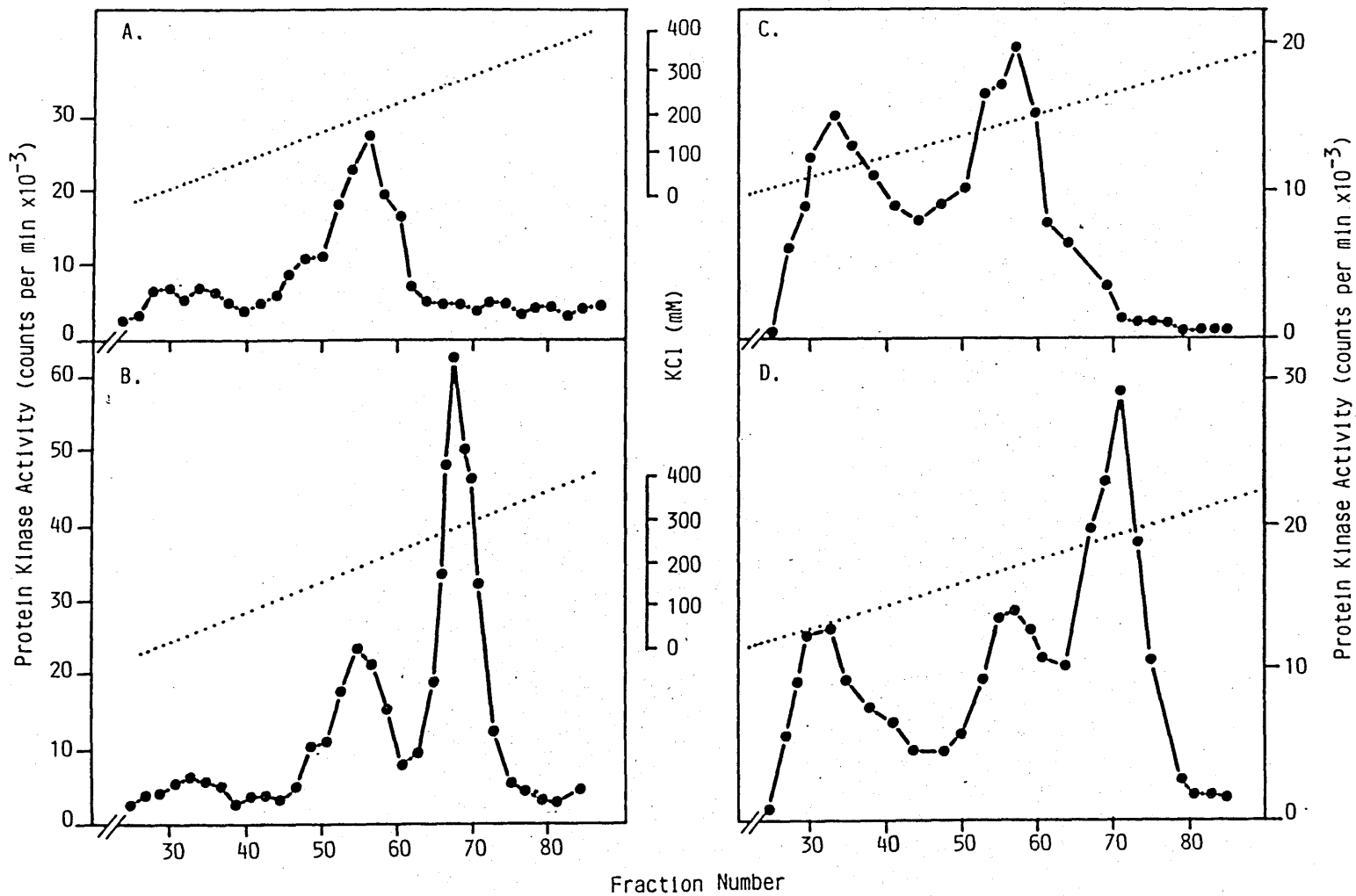

A.
B.
C.
D.
Protein Kinase Activity (counts per min x10^{-3})
KCl (mM)
Fraction Number
0
10
20
30
40
50
60
100
200
300
400
30
40
50
60
70
80

Fig. 1.2 Temporal Relationship of Appearance of ViPK to other Events During the Infection of BHK Cells with the Two Alpha-Herpesviruses PRV and HSV-1

BHK cells were infected (A) with PRV, or (B) with HSV-1(G), as described in Methods, Sections 1.2 and 1.3, and the post-ribosomal supernatants isolated at the times indicated. The major portions of these were subjected to chromatography on DEAE-Sephacel as in Fig. 1.1, and a 40µl aliquot of each column fraction assayed for protein kinase activity. The total ViPK activity (●-●) was estimated and expressed as a percentage of the maximum value, as was the DNA polymerase activity (o-o) assayed on a small portion of the original post-ribosomal supernatant.

(The initial DNA polymerase activity is that of the host enzyme). The number of p.f.u. of virus in the growth medium was also determined(■--■).

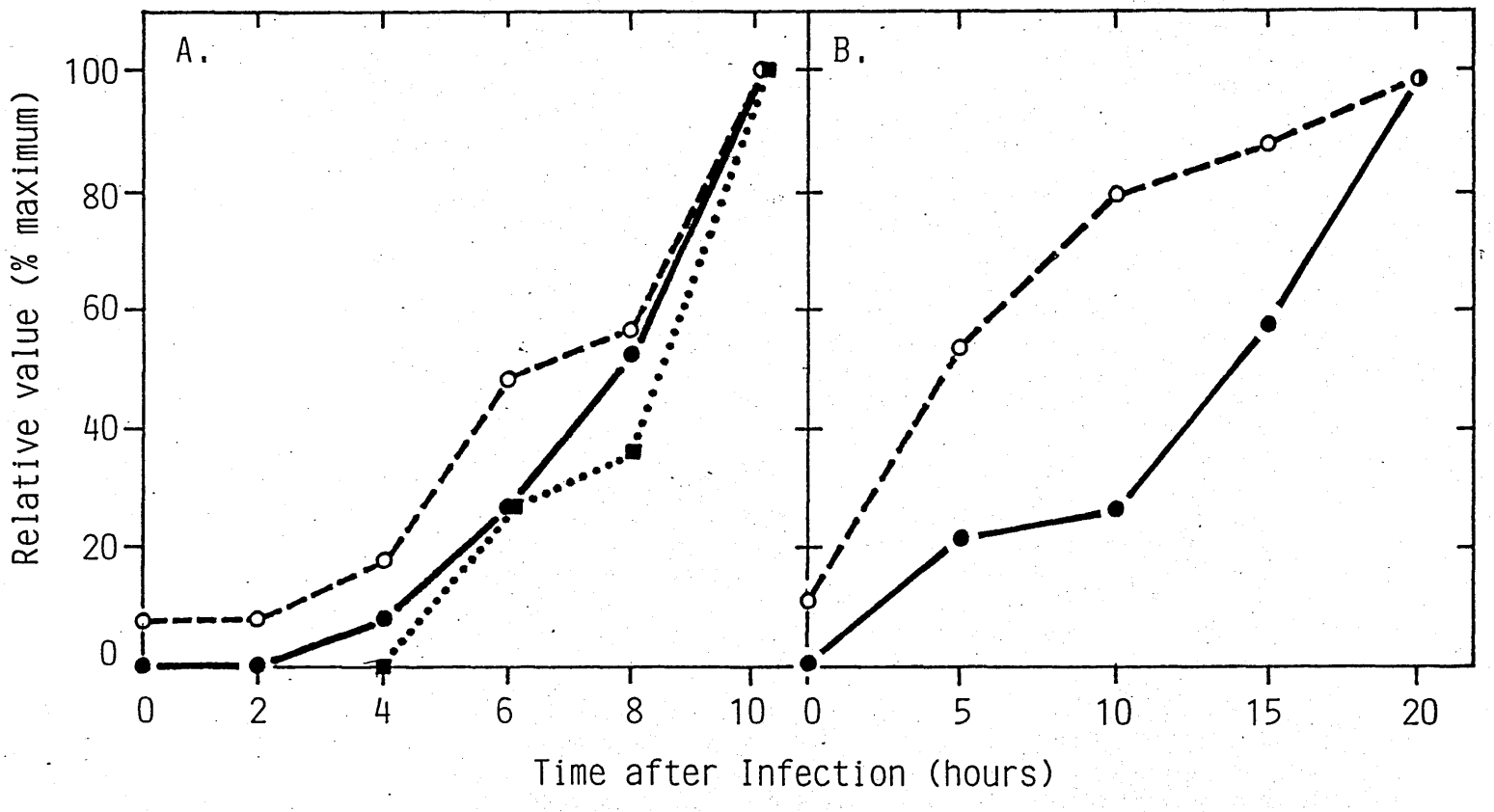

A.
B.
Relative value (% maximum)
100
80
60
40
20
0
0
2
4
6
8
10
0
5
10
15
20
Time after Infection (hours)

Fig. 1.3 Effect of Multiplicity of Infection with PRV or HSV-1 on the Yield of ViPK

BHK cells were infected for 8h with PRV with input multiplicities of 1, 5, 20, 40 and 100 p.f.u./cell, or for 18h with HSV-1(G) at input multiplicities of 1, 5 and 20 p.f.u./cell. Post-ribosomal supernatants were prepared from each condition as described in Methods, Section 2.3.

These were subjected to DEAE-Sephacel chromatography and column fractions were assayed for protein kinase activity. The total PRV ViPK (●-●) and HSV-1 ViPK (o-o) activity was estimated in each condition.

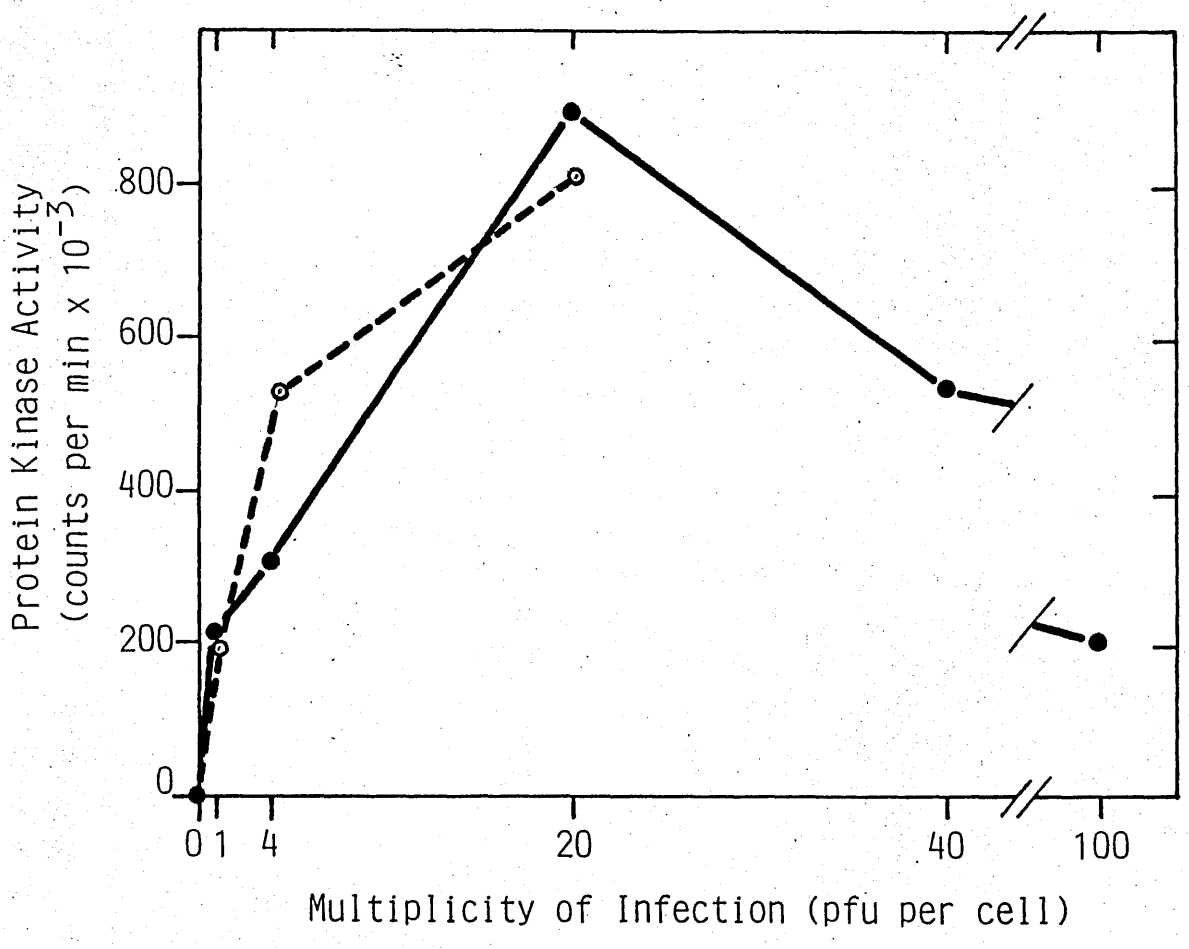

Protein Kinase Activity
(counts per min x 10^{-3})
800
600
400
200
0
0 1 4
20
40
100
Multiplicity of Infection (pfu per cell)

Fig. 1.4 Effect of KCl on the Activity of PRV ViPK and HSV-1 ViPK

Partially purified ViPK preparations (40µl) obtained by chromatography on DEAE-Sephacel (Fig. 1.1) were used to phosphorylate protamine at various concentrations of KCl at 10mM $MgCl_2$. The enzyme prepared from cells infected with PRV (●-●) was compared with that obtained from cells infected with HSV-1(G) (■-■).

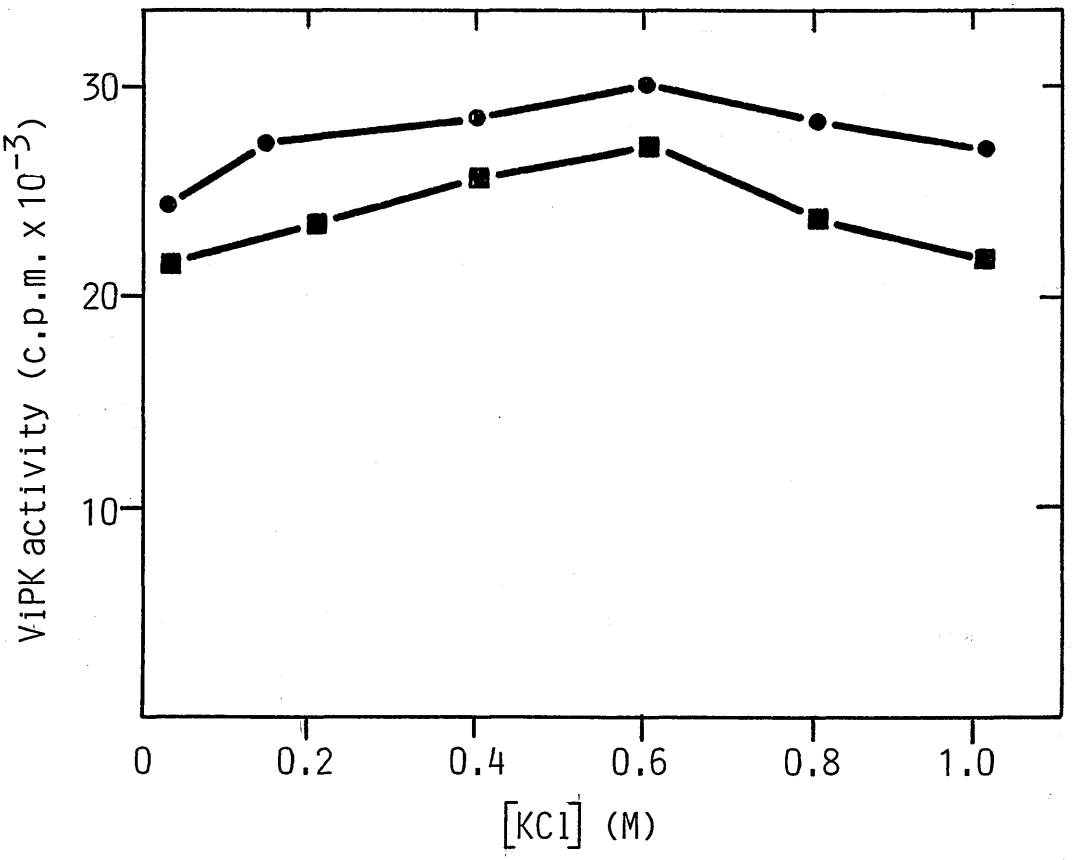

ViPK activity (c.p.m. x 10⁻³)
30
20
10
0
0.2
0.4
0.6
0.8
1.0
[KCl] (M)

Fig. 1.5 Phosphorylation of Ribosomal Proteins by ViPK from BHK Cells Infected with Herpesviruses

Partially purified ViPK preparations obtained by chromatography on DEAE-Sephacel (Fig. 1.1) were used to phosphorylate BHK 40S ribosomal subunits *in vitro* at 150mM KCl and these were subjected to SDS-polyacrylamide gel electrophoresis, all as described in Methods, Sections 5.1C and 4.1A.

An autoradiograph of the dried gel is presented.

(A) ViPK from BHK cells infected with PRV.

(B) ViPK from BHK cells infected with HSV-1(G).

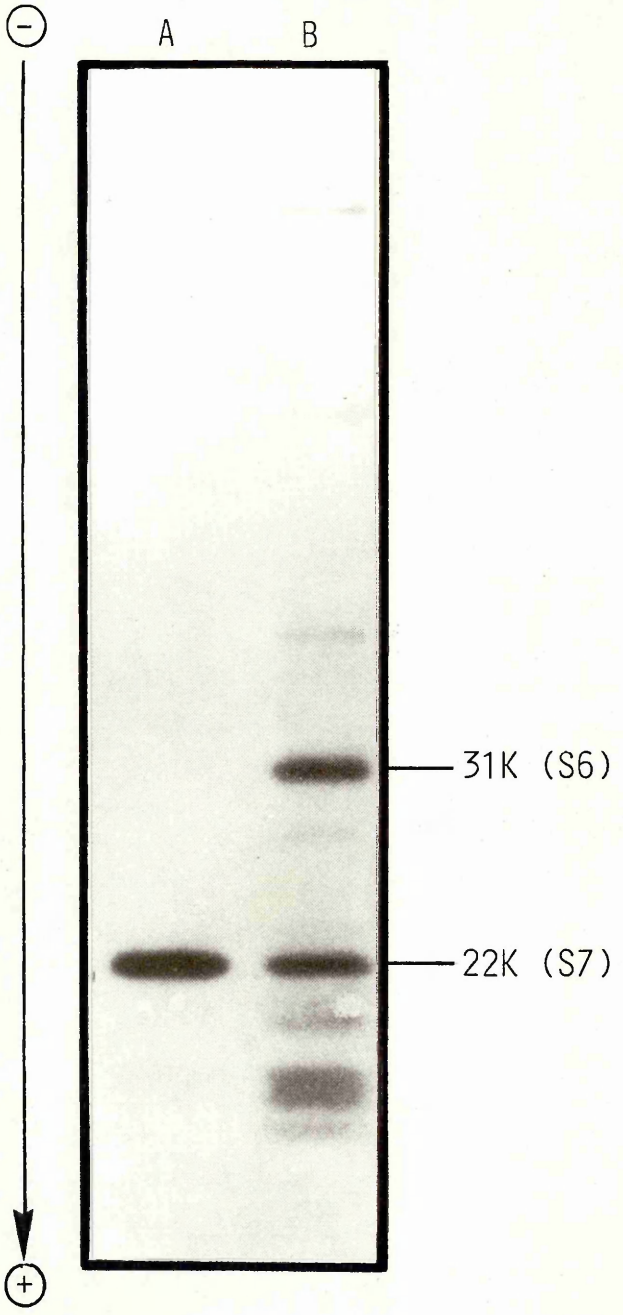

A
B
31K (S6)
22K (S7)

Fig. 1.6 Resolution of ViPK Activities by High Performance Anion-Exchange Chromatography on Mono Q

Equal activities of ViPK, partially purified from BHK cells infected with PRV or HSV-1(G) by chromatography of post-ribosomal supernatants on DEAE-Sephacel, were combined and subjected to chromatography on Mono Q (Methods, Section 3.6). Fractions, the NaCl concentrations of which are indicated (---), were assayed for protein kinase activity (●-●). Standard assay conditions were used, i.e. 40µl aliquots of the column fractions were used to phosphorylate protamine sulphate as described in Methods, Section 5.1A.

The absorbance at 280nm (---) is also shown. The inset shows the result of phosphorylation of 40S ribosomal subunits by the ViPK peaks 1 and 2, performed as described in Methods, Section 5.1C at 50mM KCl and 80mM NaCl and 10mM $MgCl_2$.

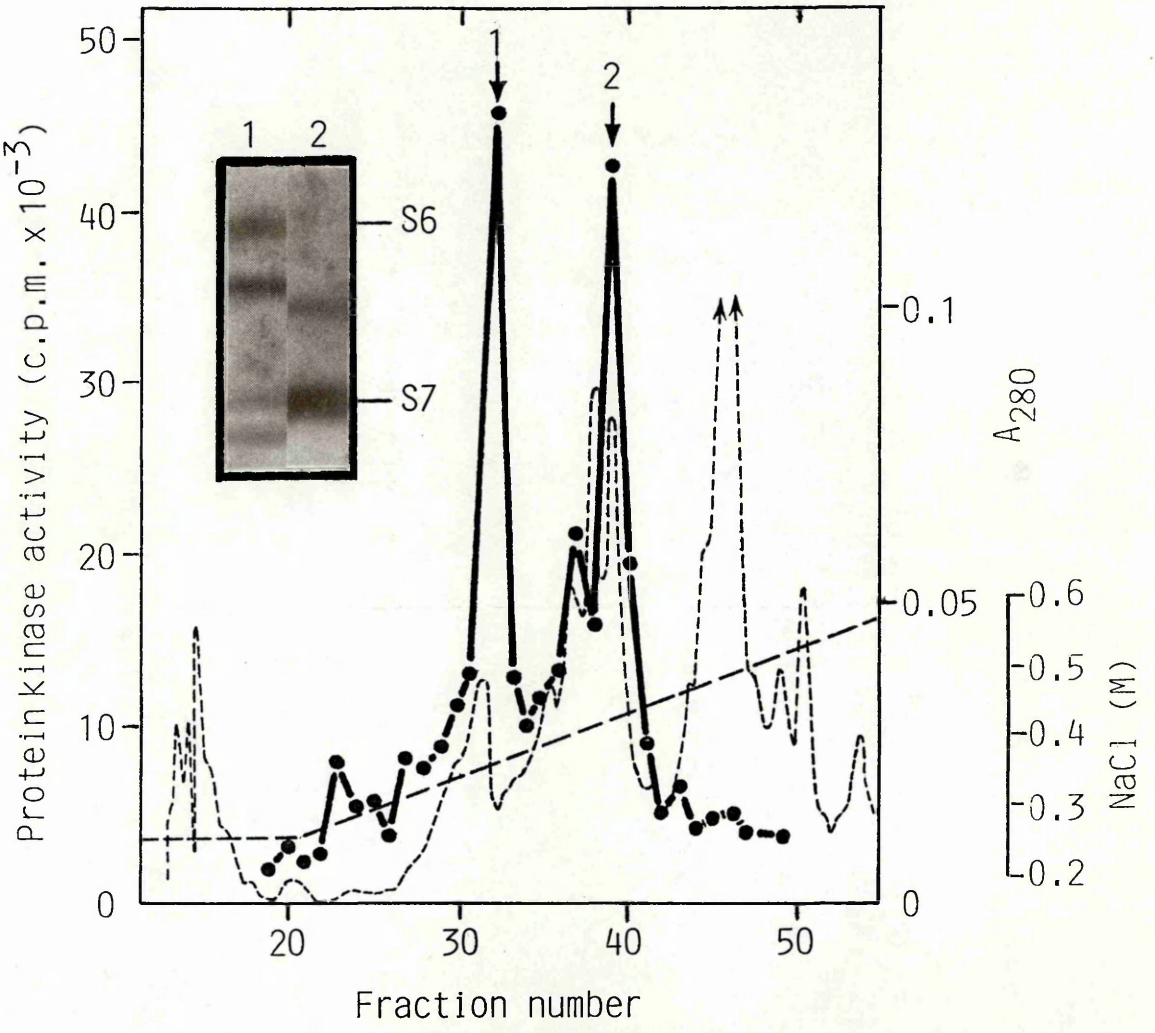

Protein kinase activity (c.p.m. x 10^{-3})
50
40
30
20
10
0
1
2
1 2
S6
S7
0.1
0.05
0
A_{280}
0.6
0.5
0.4
0.3
0.2
NaCl (M)
20
30
40
50
Fraction number

Fig. 1.7 Gel-Permeation Chromatography on Sephacryl S-200

Preparations of ViPK, partially purified by DEAE-Sephacel anion-exchange chromatography from BHK cells infected with PRV (●-●) or HSV-1(G) (o-o), were concentrated. These preparations (each containing approximately 1mg of protein) were separately subjected to chromatography on sephacryl S-200 in a buffer containing 500mM KCl, 1mM EDTA, 10mM 2-mercaptoethanol, 20mM Tris-HCl (pH7.6) as described in Methods, Section 3.8. Aliquots (40µl) of the column fractions were assayed for protein kinase activity using protamine as substrate.

The positions of elution of proteins of known relative molecular mass (Mr) are indicated and the inset shows a semilogarithmic plot of Mr X 10^{-3} against K_{av} $[(V_e-V_o)/(V_t-V_o)]$, where V_e is the elution volume, V_o the void volume and V_t the column volume.

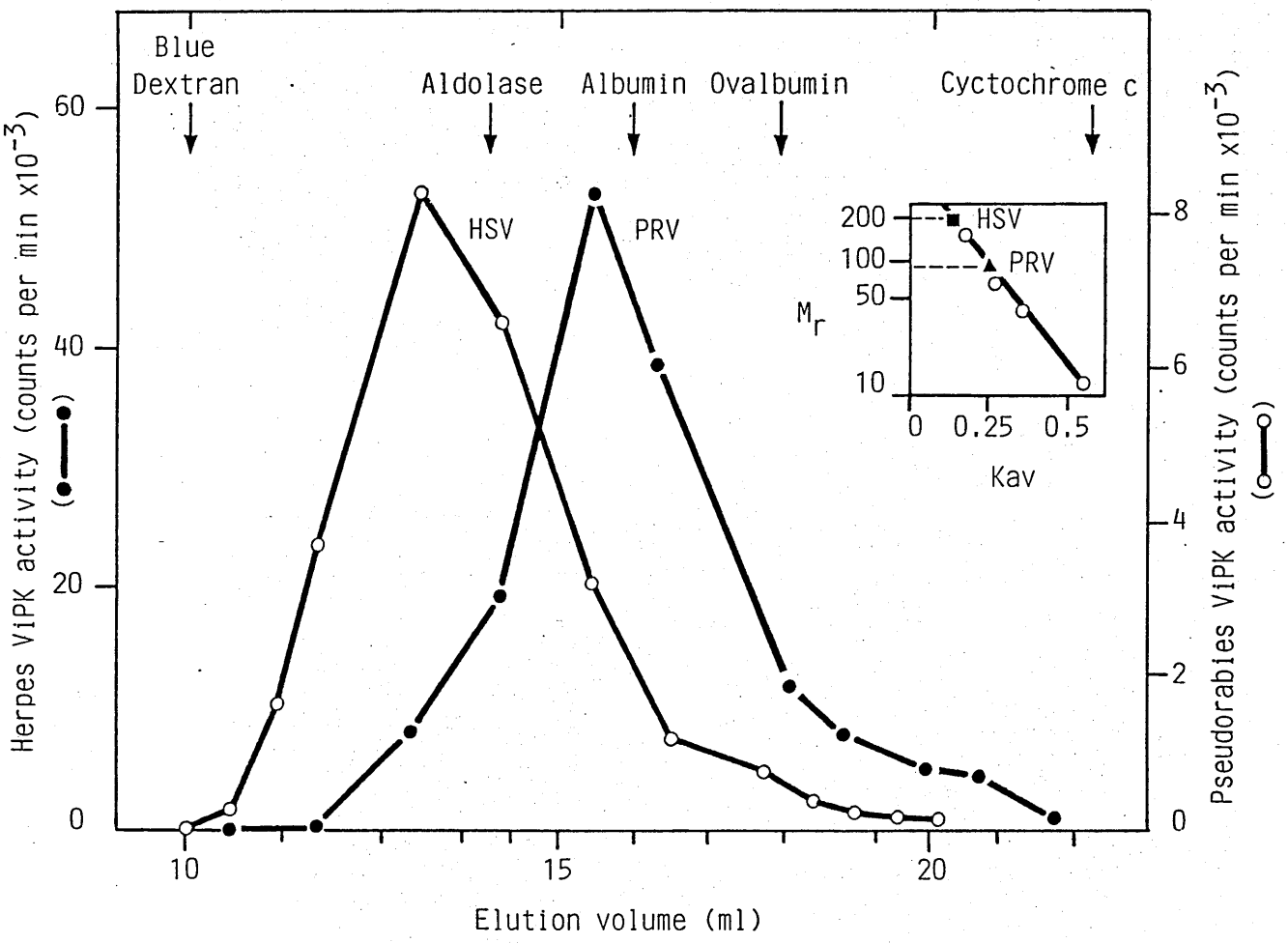

Blue
Dextran
Aldolase
Albumin
Ovalbumin
Cyctochrome c
HSV
PRV
Herpes ViPK activity (counts per min x10⁻³) (●—●)
Pseudorabies ViPK activity (counts per min x10⁻³) (○—○)
60
40
20
0
8
6
4
2
0
10
15
20
Elution volume (ml)
200
100
50
10
Mr
HSV
PRV
0
0.25
0.5
Kav

Fig. 1.8 Effect of Irradiation with Ultra-Violet Light on the Ability of PRV to Cause the Induction of ViPK in BHK Cells

BHK Cells were infected for 8h with 20 p.f.u./cell PRV (A), or with an equivalent amount of virus that had been subjected to $5kJ/m^2$ ultra-violet radiation (B), as described in Methods, Sections 1.2 and 1.12.

The post-ribosomal supernatants were isolated, subjected to chromatography on DEAE-Sephacel and fractions, the KCl concentrations of which are indicated (---), were assayed for protein kinase activity (●-●).

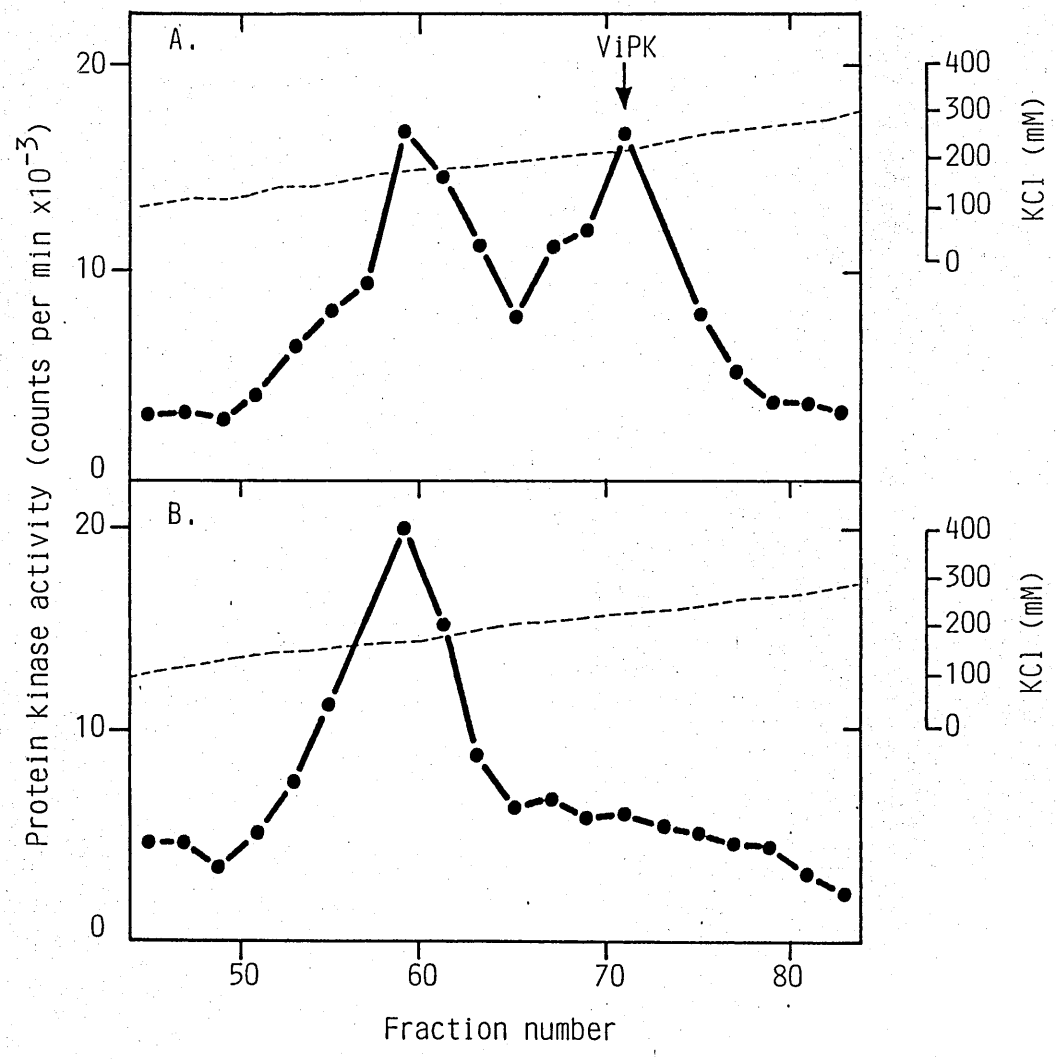

A.
ViPK
B.
20
10
0
400
300
200
100
0
KCl (mM)
Protein kinase activity (counts per min x10^{-3})
50
60
70
80
Fraction number

Fig. 1.9 Induction of ViPK in BHK Cells Infected with the tsK Mutant of HSV-1(G)

BHK cells were infected with tsK (10 p.f.u./cell) at either 31°C (A), or 38.5°C (B), or with the parent wild-type virus at 38.5°C (C), and harvested 18h later. The post-ribosomal supernatants were isolated, subjected to chromatography on DEAE-Sephacel using a somewhat shallower gradient than elsewhere (Fig. 1.1).

40µl aliquots of the column fractions were assayed for protein kinase activity with the substrate protamine (●-●). (Methods, Section 5.1A). The KCl concentrations of the fractions are indicated (---).

ViPK is indicted by the arrows, eluting at approximately 220mM KCl.

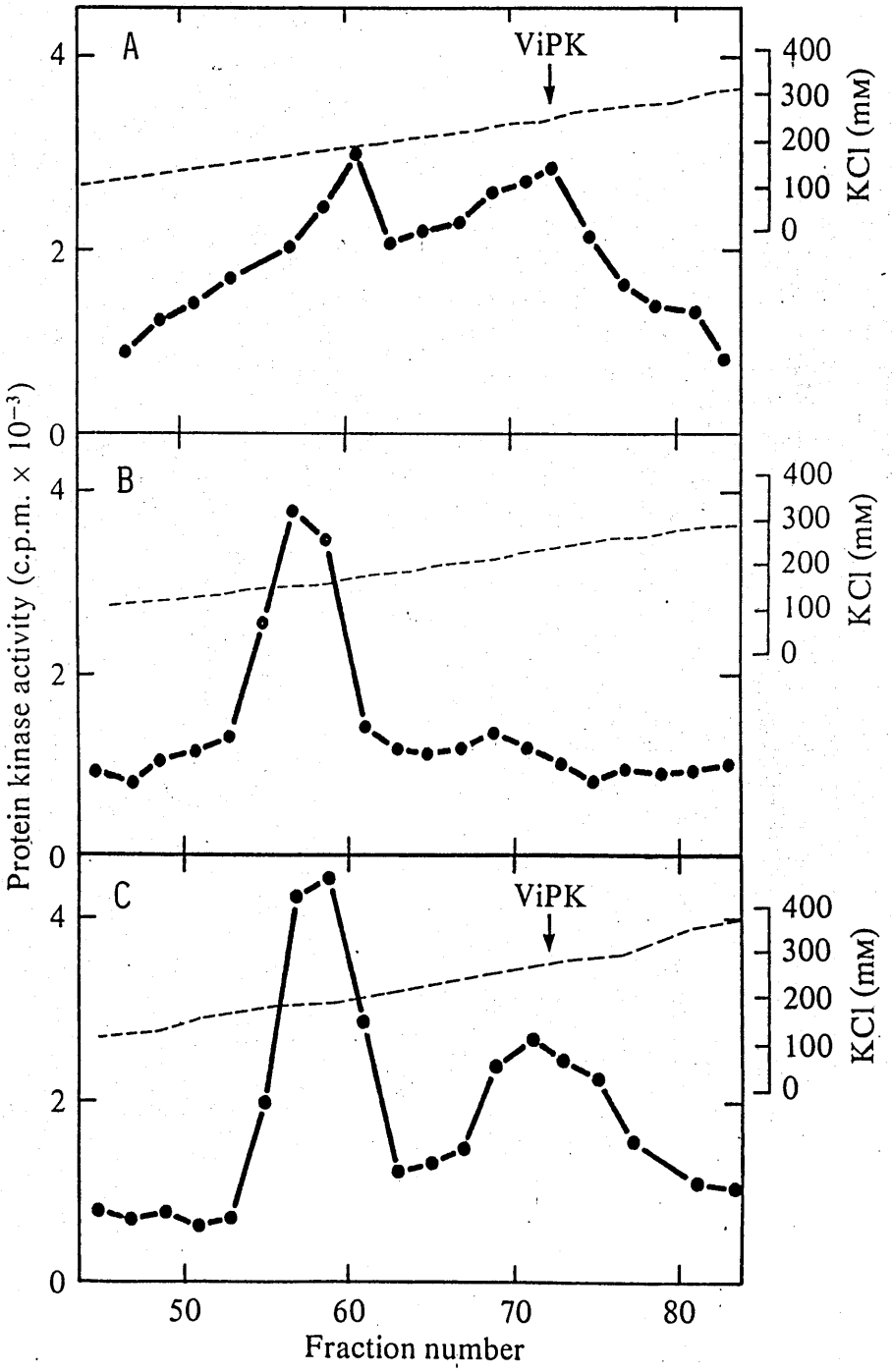

A
ViPK
B
C
ViPK
Protein kinase activity (c.p.m. × 10^{-3})
KCl (mM)
400
300
200
100
0
4
2
0
50
60
70
80
Fraction number

Fig. 1.10 DNA Polymerase Activity in BHK Cells Infected with the tsK Mutant of HSV-1(G) at either 31°C or 38.5°C, or with the Parent Wild-Type Virus at 38.5°C.

Time courses of appearance of viral DNA polymerase were performed over a 60 minute incubation period.

The early or β viral protein, DNA polymerase, was assayed (Methods, Section 1.13) in a sample of the post-ribosomal supernatant prepared from cells infected for 18h with the various viruses at the appropriate temperatures.

(●-●) tsK at the permissive temperature (31°C)

(▲-▲) tsK at the restrictive temperature (38.5°C)

(■-■) HSV-1(G) at the restrictive temperature (38.5°C)

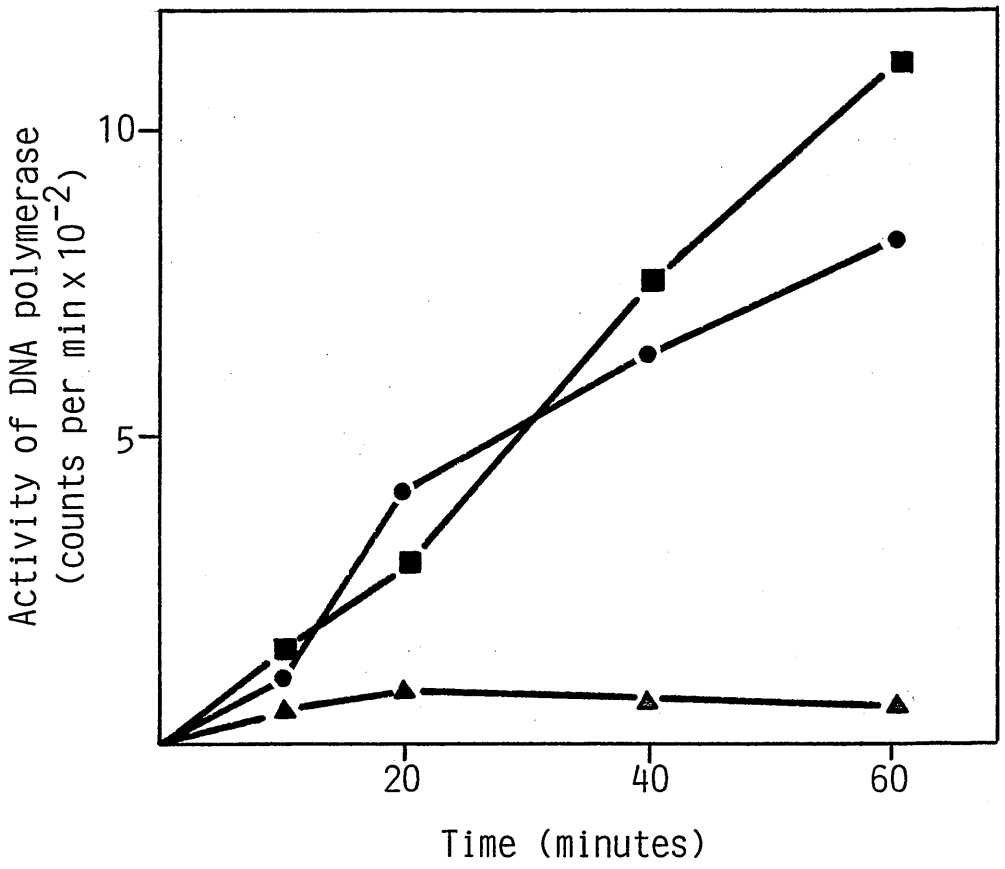

Activity of DNA polymerase
(counts per min x 10^{-2})
10
5
20
40
60
Time (minutes)

Fig. 1.11 Schematic Representations in the DNA Sequence Arrangements in HSV-1(F) and the HSV-1(F) Recombinants R7035, R7040, R7041 and R7050

Panel A: The boxes in lines 1-5 represent the terminal sequences that are repeated internally and divide the HSV-1(F) genome into the long (L) and short (S) components.

Line 1: Sequence arrangement in the HSV-1(F) genome. TK and US3 indicate the locations of the thymidine kinase gene and of US3 open reading frame, respectively.

Lines 2-5: Sequence arrangement of the various viral recombinants. The genotype of each of the viral recombinants is indicated.

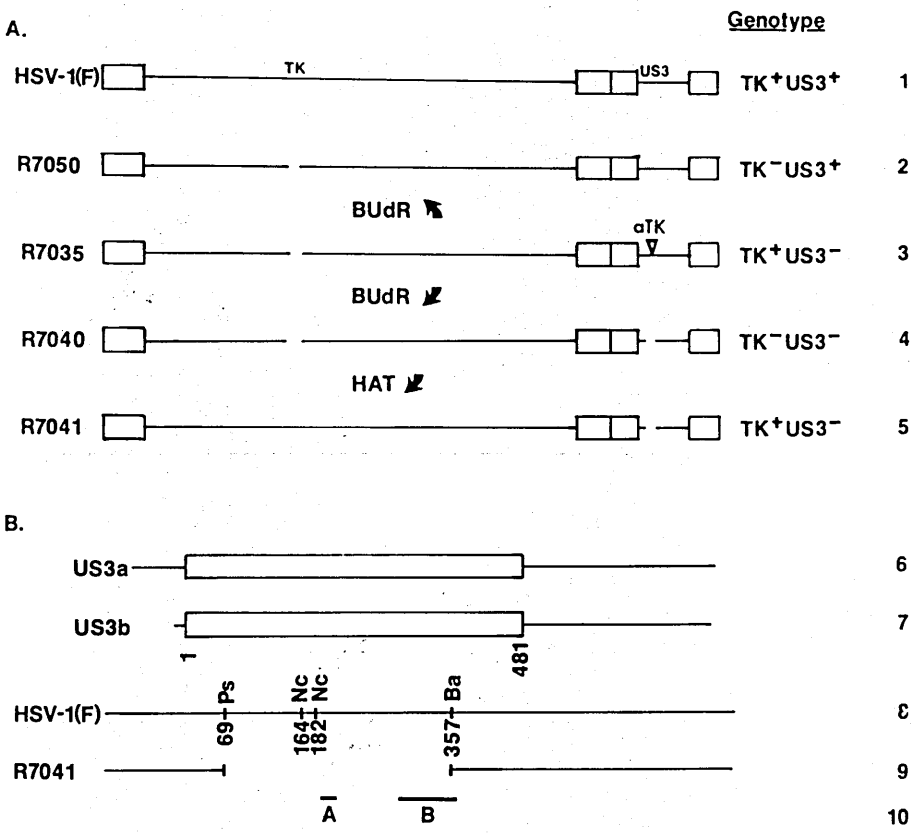
Panel B: Lines 6 and 7 show the two species of mRNA that are encoded by the US3 open reading frame (McGeoch et al., 1985; McGeoch and Davison, 1986). The boxes indicate the translated portion of the mRNA.

Line 8: Pertinent restriction endonuclease sites located within the US3 coding region (Ps = Pst I, Nc = Nco I, and Ba = Bam HI). The Nco I sites indicate the insertion site of the α27-TK. The sequences intervening between the Pst I site and Bam HI site were deleted in the R7040 and R7041 viral genomes. As a result of the deletion the protein encoded by the US3 mRNA is out of frame downstream (3') to the Bam HI site. Numbers in lines 7 and 8 correspond to the predicted amino acid number in the predicted translation product of the US3 open reading frame.

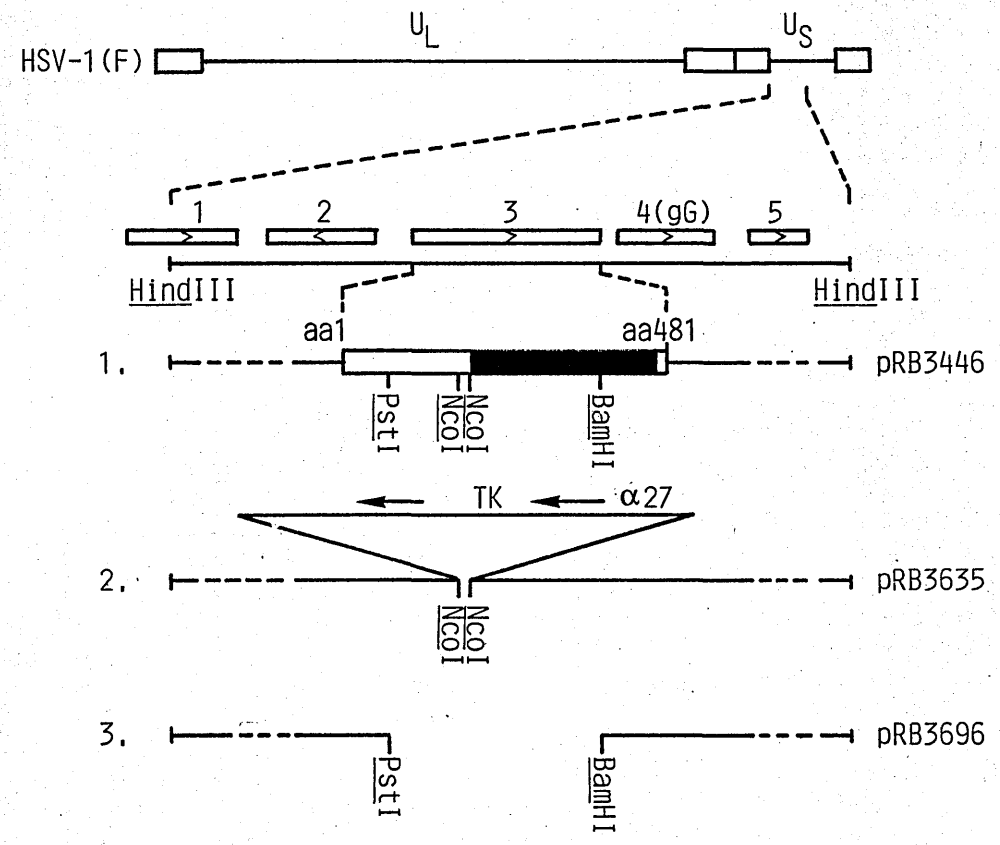
Line 10: The short line designated as A indicates the position of the motifs thought to constitute the nucleotide binding site. The longer line designated as B indicates the position of the domain containing the other motifs shared by known protein kinases (Hunter and Cooper; 1985, McGeoch and Davison; 1986).

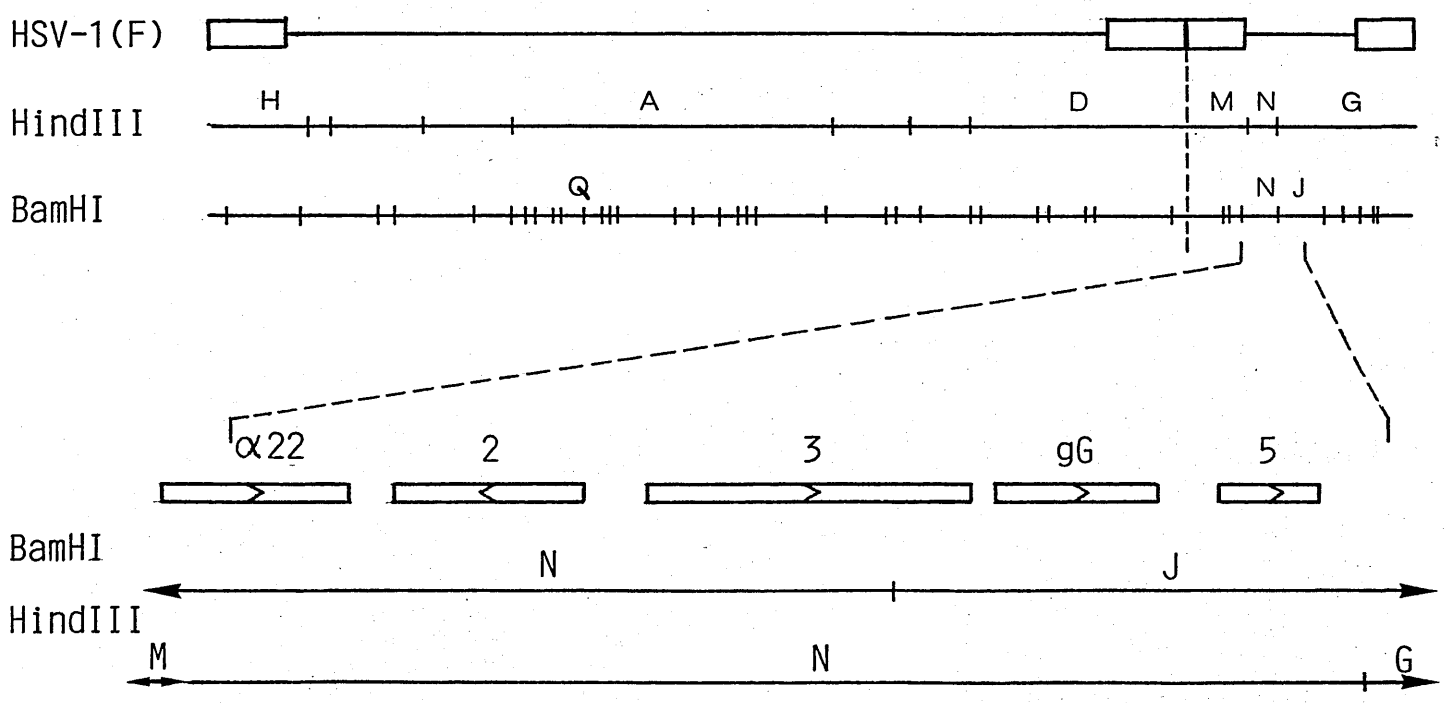
Panel C: Shows the structure of plasmid inserts containing intact and deleted versions of the US3 gene of HSV-1(F).

Panel D: Shows a restriction endonuclease map of the HSV-1(F) genome showing the position of the Bam HI Q and N fragments.


A.
Genotype
HSV-1(F)
TK
US3
TK+US3+
1
R7050
TK−US3+
2
BUdR
αTK
R7035
TK+US3−
3
BUdR
R7040
TK−US3−
4
HAT
R7041
TK+US3−
5
B.
US3a
6
US3b
7
1
481
HSV-1(F)
69 Ps
164 Nc
182 Nc
357 Ba
8
R7041
9
A
B
10

C Structure of plasmid inserts containing intact and deleted versions of the US3 gene of HSV-1




HSV-1(F)
HindIII
H
A
D
M
N
G
BamHI
Q
N
J
α22
2
3
gG
5
BamHI
N
J
HindIII
M
N
G

D

Fig. 1.12A DEAE-Cellulose Chromatography of Protein Kinases from Cytoplasmic Extracts of BHK Cells Infected with Different Strains of HSV-1(F)

BHK cells (15 roller bottles containing a total of approximately 3 x 10^9 cells) were infected with either HSV-1(F) or R7041 at 10 p.f.u./cell, and harvested 17h after infection all as described in Methods, Section 1.3. A cellular cytoplasmic extract was prepared for each condition and subjected to DEAE-cellulose chromatography (Methods, Section 3.1B). The column was eluted with a linear gradient of KCl (---). 80µl aliquots of each column fraction was assayed for protein kinase activity using protamine as a substrate all as described in Methods, Section (5.1A): (●-●) (HSV-1(F) and (o-o) R7041. Arrow indicates the protein kinase activity found only in cells infected with HSV-1(F).

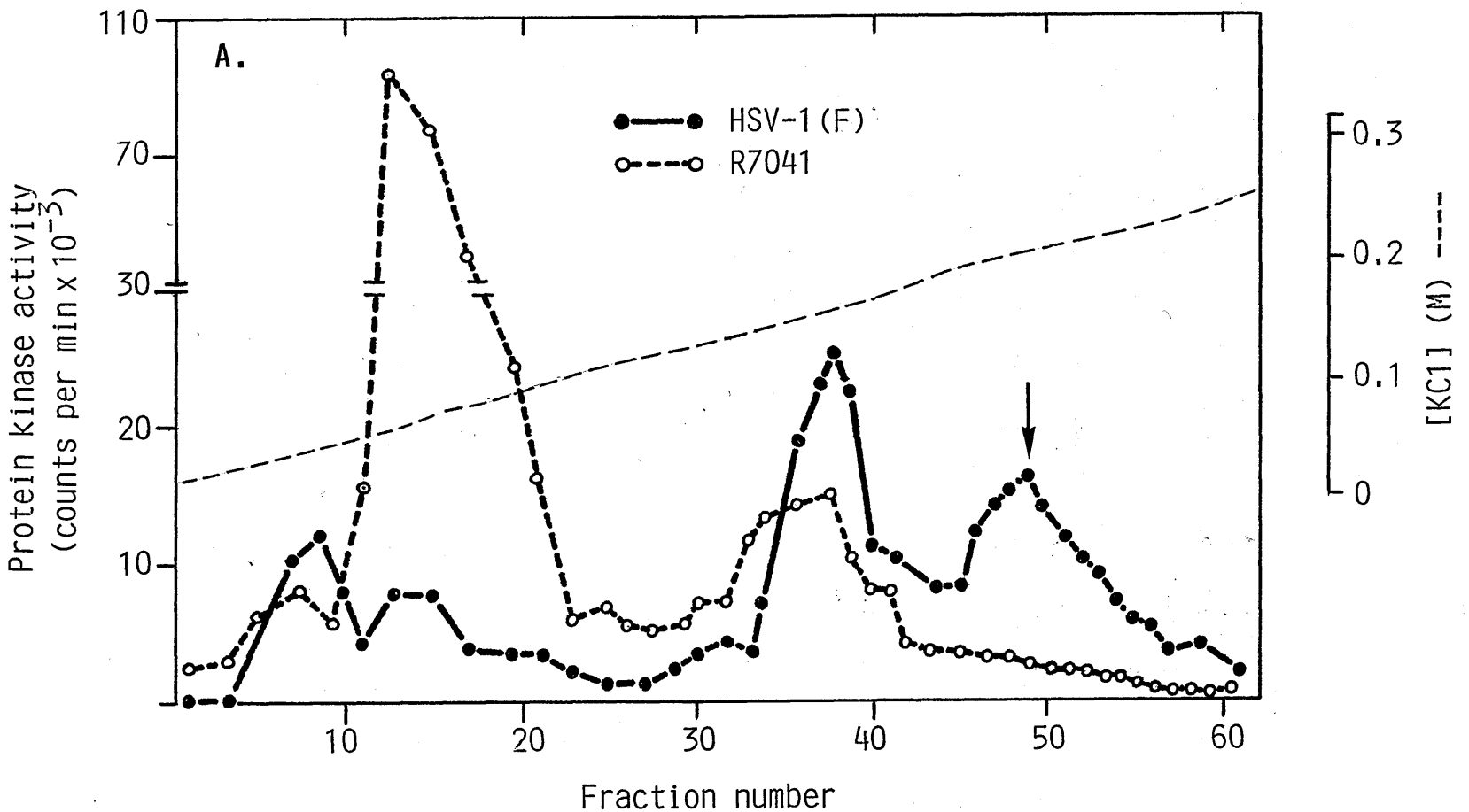

A.
HSV-1 (F)
R7041
Protein kinase activity
(counts per min x 10^{-3})
110
70
30
20
10
10
20
30
40
50
60
Fraction number
0.3
0.2
0.1
0
[KCl] (M) ----

Fig. 1.12B DEAE-Cellulose Chromatography of Protein Kinases from Cytoplasmic Extracts of BHK Cells Infected with Different Strains of HSV-1(F)

BHK cells (15 roller bottles containing a total of approximately 3×10^9 cells) were infected with either HSV-1(F), R7041, R7035 or R7051, with a total of 5.5×10^{10} p.f.u. virus/condition, and harvested 17h after infection. A cellular cytoplasmic extract was prepared for each condition and subjected to DEAE-cellulose chromatography on a 5 x 1.6 cm (ht x diameter) column at a flow rate of 60ml/hr, 5 ml fractions being collected. The column was eluted with a linear gradient of KCl.

80µl aliquots of each column fraction were assayed for protein kinase activity using protamine as a substrate (●-●) (Methods, Section (5.1A)).

The arrows indicate the protein kinase activity found only in cells infected with wild-type F or the rescued US3 R7051 mutant virus of HSV-1(F).

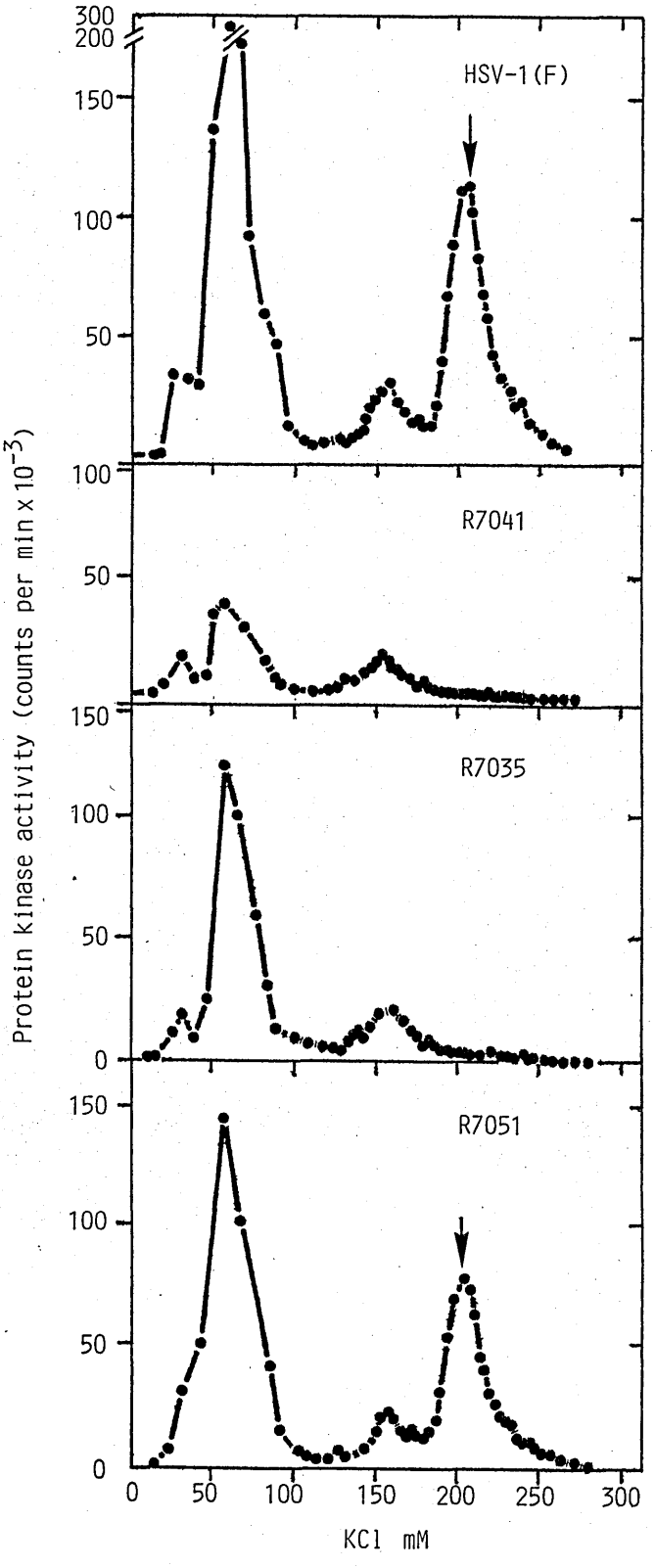

HSV-1(F)
R7041
R7035
R7051
Protein kinase activity (counts per min x 10^{-3})
KCl mM
300
200
150
100
50
0
0
50
100
150
200
250
300

Fig. 1.13 DEAE-Cellulose Chromatography of Protein Kinases from Cytoplasmic Extracts of Vero Cells Infected with Different Strains of HSV-1(F)

Vero cells (15 roller bottles containing a total of approximately 3 x 10^9 cells) were infected with either HSV-1(F), R7041, R7050, or mock-infected at 10-15 p.f.u./cell, and harvested 17h after infection, all as described in Methods, Section 1.3.

A cellular cytoplasmic extract of Vero cells was prepared for each condition and subjected to DEAE-cellulose chromatography by adsorbing the extract onto DE-52 and eluted with a gradient of KCl (---). 80µl aliquots of each column fraction was assayed for protein kinase activity using protamine as a substrate (•-•), all as described in Methods, Sections 2.3, 3.1B and 5.1A. The elution profiles illustrated are:

A. Mock-infected

B. HSV-1(F)

C. R7041

D. R7050

The structures of the recombinants are shown in Fig. 1.11.

The arrows indicate the protein kinase activity found only in cells infected with wild-type F or the rescued US3 R7050 mutant virus of HSV-1(F).

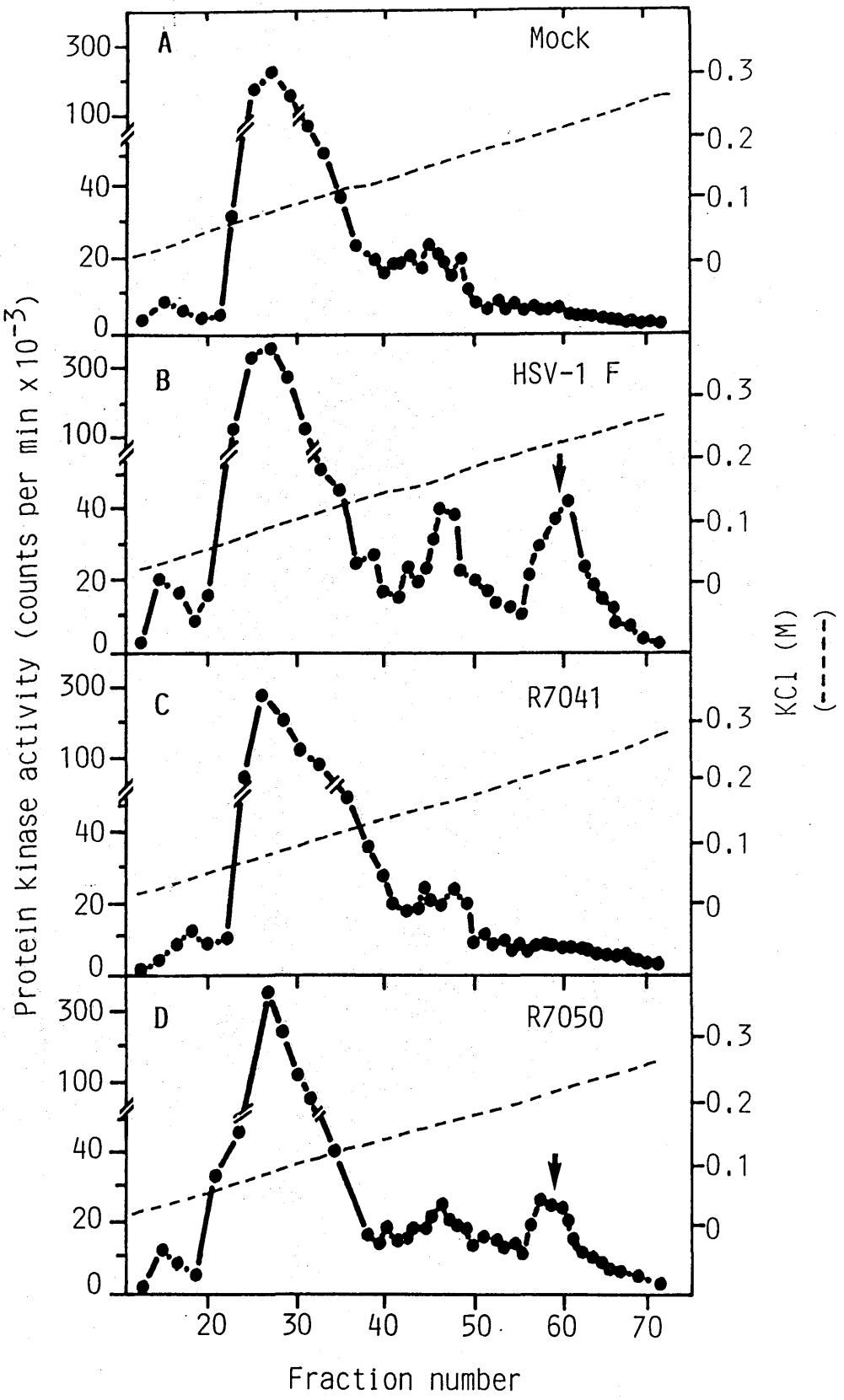

A
Mock
B
HSV-1 F
C
R7041
D
R7050
300
100
40
20
0
0.3
0.2
0.1
0
20
30
40
50
60
70
Protein kinase activity (counts per min x 10^{-3})
KCl (M)
(- - - -)
Fraction number

Fig. 1.14 Titration of the Glycoprotein B Synthesised in Vero Cells Mock-Infected or Infected with the Wild-Type Parent Strain HSV-1(F) and the Recombinant Viruses R7041 and R7050

Mock-infected or infected cell proteins were solubilized, dissolved in sample buffer and subjected to polyacrylamide gel electrophoresis under denaturing conditions as described in Methods, Section 4.1A.

The separated proteins were then electrically transferred to nitrocellulose, and reacted with monoclonal antibody H1163 to glycoprotein B in an immunoperoxidase-coupled reaction, as described in Methods, Section 4.3(ii).

Protein concentrations were determined by the method of Bradford (1976) and the amount loaded in each lane is indicated in the figure.

Expression of Glycoprotein B during infection of cells with HSV-1 mutant

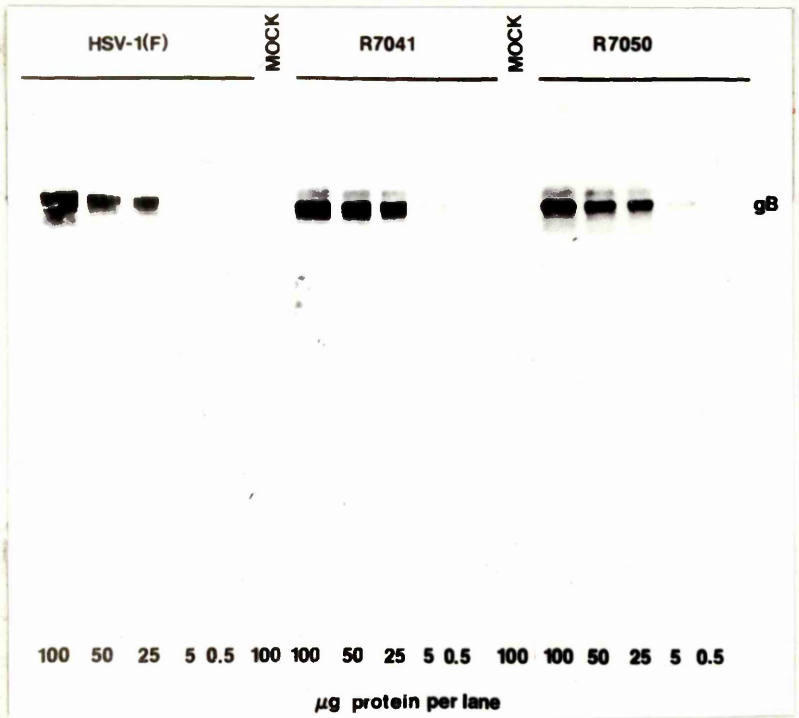


Fig. 1.15 Identification of HSV-1 US3 Gene Product by Immunoblotting

Extracts of BHK cells infected with HSV-1 at a multiplicity of 20 p.f.u./cell for 18h and labelled with [^{35}S]methionine were prepared (Methods, Sections 1.3 and 2.1). The proteins were solubilised, dissolved in sample buffer and separated on a 10% SDS polyacrylamide gel as described in Methods, Section 4.1A. (lane 1) from unlabelled cells The separated proteins were blotted onto nitrocellulose (650μg protein per strip) probed with anti-US3 serum (A/US3) at 1:2 dilution (lane 2), 1:10 dilution (lane 3), and normal rabbit serum (NRS) at a 1:2 dilution (lane 4), all as described in Methods, Section 4.3. Lane 1 shows the blotted infected [^{35}S]-labelle cell polypeptide profile. The molecular weights of known viral polypeptides are shown on the left-hand side.

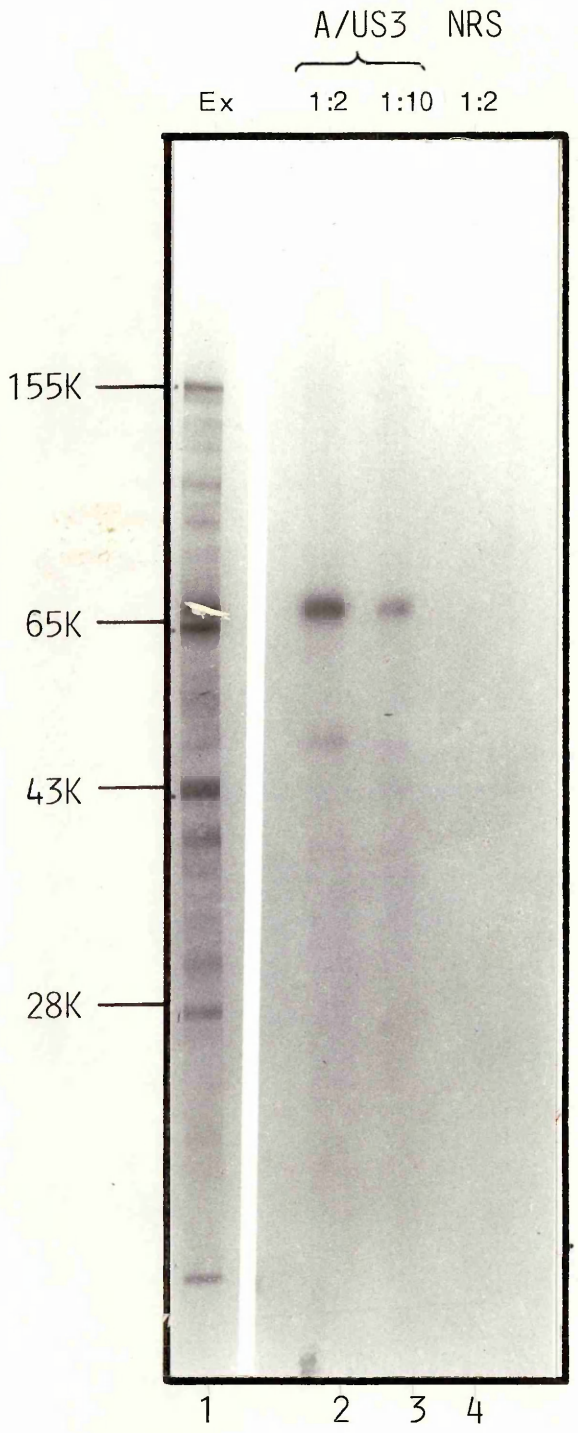

A/US3
NRS
Ex
1:2
1:10
1:2
155K
65K
43K
28K
1
2
3
4

Fig. 1.16 Detection of the US3 Gene Product in Purified Preparations of HSV-1 PK

HSV-1 PK preparations after the threonine-sepharose (5µg protein/strip) and after the protamine-agarose stage (1µg protein/strip) of the purification scheme (described in Methods, Section 3) were blotted and probed with anti-US3 serum at a 1:2 dilution (lanes 5 and 2), or a 1:10 dilution (lanes 6 and 3); or with a 1:2 dilution (lanes 4 and 7) of control anti-oligo peptide serum against the viral immediate-early polypeptide IE 110, (α0) (C).

A purified protein kinase C preparation was included as an antigen control and probed with 1:2 and 1:10 dilutions of the anti-US3 antiserum (lanes 8 and 9), or the control antiserum (lane 10). Lane 1 shown an infected cell polypeptide profile blotted from the same polyacrylamide gel of ^{35}S-labelled cells.

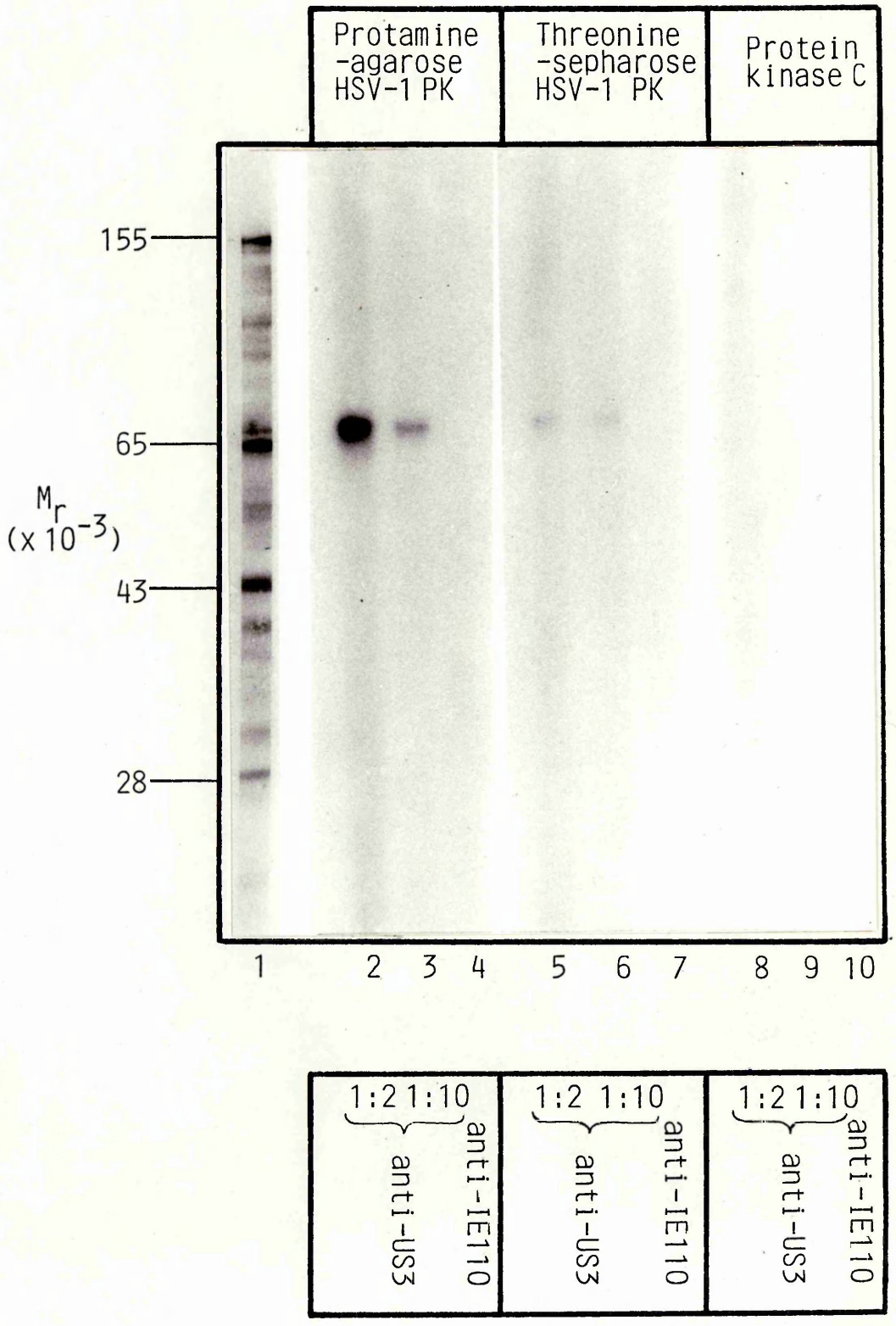

Protamine -agarose HSV-1 PK
Threonine -sepharose HSV-1 PK
Protein kinase C
155
65
43
28
M_r (x 10^{-3})
1
2
3
4
5
6
7
8
9
10
1:2 1:10
anti-US3
anti-IE110
1:2 1:10
anti-US3
anti-IE110
1:2 1:10
anti-US3
anti-IE110

Table 1.1 Titres of HSV-1(F) and Recombinant Viruses R7041 and R7050 obtained in replicate Roller Bottle Cultures of Vero Cells.

Vero cells were harvested 18h after infection with 10-15 p.f.u./cell of the appropriate virus and centrifuged for 10 min at 2000 rpm (500g) to sediment the cells and virus. The cells and virus were resuspended in growth medium, sonicated and the virus was titred on Vero cell monolayer cultures, as described in Methods, Section 1.9C.

Virus	Titre (p.f.u. per ml x 10^{-7})
HSV-1(F)	4.8
R7041	7.9
R7050	3.6

II PURIFICATION AND SUBSTRATE-SPECIFICITY OF HSV-1 PK AND PRV PK

1 PURIFICATION

1.1 Complete purification of the pseudorabies virus protein kinase; PRV PK

A partial purification of PRV PK has been described previously (Katan et al., 1985). This employed DEAE-cellulose, high performance gel-permeation and high performance anion-exchange chromatography, and resulted in a preparation with a specific activity of approximately 30 units/mg. Although this was a satisfactory method for resolving PRV PK from other known protein kinases in order to obtain an initial characterization, the specific activity it yielded fell far short of the 1000-5000 units/mg of most homogeneous protein kinases (e.g. Beavo et al., 1974: Parker et al., 1984). To attempt to achieve complete purification from the limited amounts of material provided by infected tissue culture cells it was necessary to devise an alternative strategy employing a relatively small number of more powerful steps. These latter were, in fact, suggested by the characteristics of partially purified preparations of the enzyme. The exact details of the complete purification of PRV PK are presented in Methods, Section 3.

The first steps of the purification entailed the preparation of a cellular cytoplasmic extract (also referred to as a post-ribosomal supernatant), as previously described in Methods, Section 2.3, followed by anion-exchange chromatography (Fig. 2.1). This was followed by hydrophobic-interaction chromatography, a step suggested by the peculiar stability of the enzyme at high ionic strength and its activity in the presence of non-ionic detergent NP40 at 10%.

Initially phenyl-sepharose CL-4B (Pharmacia) was used; but this was subsequently replaced by a rapid HPLC system employing a TSK phenyl-5PW column, the high capacity of which allows the material for one preparation to be subjected to chromatography in a single run (Fig. 2.2A). The next step, the threonine-sepharose affinity chromatography (Fig. 2.2B), was employed after it emerged that PRV PK could catalyse the transfer of phosphoryl groups to threonyl residues (Results, Section II, 2, Fig. 2.15). The final purification step, protamine-agarose chromatography (Fig. 2.2C), was also an affinity method. However, this capitalised on a different facet of the substrate specificity of the enzyme, its preference for a highly basic environment at the target seryl or threonyl residue. Table 2.1 shows quantitive data for the purification. It can be seen that one of the most significant features of the purification was the high specific activity (1000 units/mg) achieved. The other salient feature was the single 38,000 Mr band stained with Coomassie Brilliant Blue, obtained when the preparation from the last stage of purification was subjected to gel-electrophoresis under denaturing conditions (Fig. 2.3). Although in some preparations silver staining revealed a slight contamination with the 55,000 Mr band from the threonine-sepharose stage, it seems most likely that the 38,000 Mr band (Fig. 2.3, lanes 5 and 6) corresponds to PRV PK.

The overall 446-fold purification given in Table 2.1 is almost certainly an underestimation, perhaps by a factor of 10. This is because, although accurate estimations of PRV PK in the crude post-ribosomal supernatant fraction could not be done, because of the other contaminating kinases present at this stage, it seems unlikely that the DEAE-cellulose step would have not resulted in any increase

in specific activity. The relatively good 23% overall recovery may, likewise, be a slight overestimation if there were incomplete recovery at the DEAE-cellulose stage.

To obtain a further perspective on the relationship of the 38,000 Mr band of Fig. 2.3 to the enzymic activity, the ability of PRV PK to undergo the so-called "autophosphorylation" reaction was investigated. This phenomenon appears to be a feature of many, if not all protein kinases (Introduction, Section 1.1). In the absence of added substrates the 38,000 Mr band in the most purified preparation of PRV PK did become phosphorylated (Fig. 2.4). The 38,000 Mr band was also the major phosphorylated protein when a preparation from the previous step was used, even though other stained bands predominated in this latter case. The results were completely consistent with the conclusion that the 38,000 Mr band was PRV PK.

The Mr 38,000 obtained for the stainable protein of purified PRV PK when subjected to gel-electrophoresis under denaturing conditions (Fig. 2.3) constrasted markedly with a value of 68,000 for that of the enzyme activity in a partially purified preparation obtained by gel-permeation chromatography and, consistent with this, a sedimentation value on sucrose density gradient centrifugation similar to that of serum albumin (Katan et al., 1985). It was therefore necessary to re-examine the native Mr value using the most purified enzyme preparation. A Superose 12 high performance gel-exclusion column was used for this purpose (Methods, Section 3.5A). All the enzyme activity eluted at a position which corresponded to a Mr of approximately 70,000, as did the protein

(Fig. 2.5). Analysis of this latter by denaturing gel-electrophoresis again showed a Mr of 38,000 and an ability to undergo autophosphorylation (inset, Fig. 2.5). It therefore seems most likely that the native form of PRV PK comprises two identical subunits.

Another physical characteristic of PRV PK which was determined was its isoelectric point. The O'Farrell two-dimension electrophoresis system was employed (Methods, Section 4.1B) and autophosphorylated enzyme was used for ease of detection. The value obtained (Fig. 2.6) was approximately pH 4.9 for the phosphorylated form of the enzyme. That of the unphosphorylated form is presumably somewhat higher. This rather low value is consistent with the relatively high concentration of KCl required to elute PRV PK from DEAE-Sephacel (Fig. 1.1) and DEAE-cellulose (Fig. 2.1).

1.2 Partial purification of the Herpes Simplex Type-1 protein kinase, HSV-1 PK

HSV-1 PK was purified from cellular cytoplasmic extracts of BHK cells infected for 18h with HSV-1 at a multiplicity of infection of 10 p.f.u./cell. The same chromatographic methods used for the purification of PRV PK were applied to purify HSV-1 PK, with and without the threonine-sepharose stage (Fig. 2.7). The combined separation techniques were not as effective for the purification of HSV-1 PK as they had been for PRV PK. This is not unreasonable, as HSV-1 PK eluted differently from DEAE-cellulose and at each stage of its purification the proteins present in the mixture would be different from those present during the purification of PRV PK.

Although the final preparation of purified HSV-1 PK was not homogeneous, it nevertheless contained only two major bands of apparent molecular weights 68,000 and 61,000 when analysed by gel-electrophoresis under denaturing conditions (Fig. 2.8). Because the power of the chromatography methods employed has already been demonstrated for the purification of PRV PK, it is reasonable to consider that one of the two major bands may be HSV-1 PK, even though the highest specific activity obtained so far was only 200 units/mg. This is because both this final specific activity and the apparently modest 94-fold increase from the DEAE-cellulose stage (Table 2.2(A)) are almost certainly underestimates of the protein purification because of the poor overall recovery of activity compared with PRV PK. This can be attributed to a very variable initial specific activity: routinely five to ten-fold lower yields of HSV-1 PK were obtained at the outset. Consistent with this interpretation was the fact that loss of activity was less (Table 2.2B) when the threonine-sepharose stage was omitted and the protamine-agarose column eluted with a linear gradient of KCl (Fig. 2.7).

Further circumstancial evidence that the protein kinase was one of the two major components of the most purified preparation of HSV-1 PK was provided by autophosphorylation experiments. The most purified preparation of HSV-1 PK could autophosphorylate, and a single major phosphorylated species with Mr 68,000 was detected after analysis by gel-electrophoresis under denaturing conditions (Fig. 2.9). It can be seen that autophosphorylation could not be detected in the previous stage of the purification, where the protein complexity is

much greater. It has already been mentioned that this 68,000 Mr protein corresponded to the polypeptide which reacted with the anti-US3 serum (Results, Section I and Fig. 1.16).

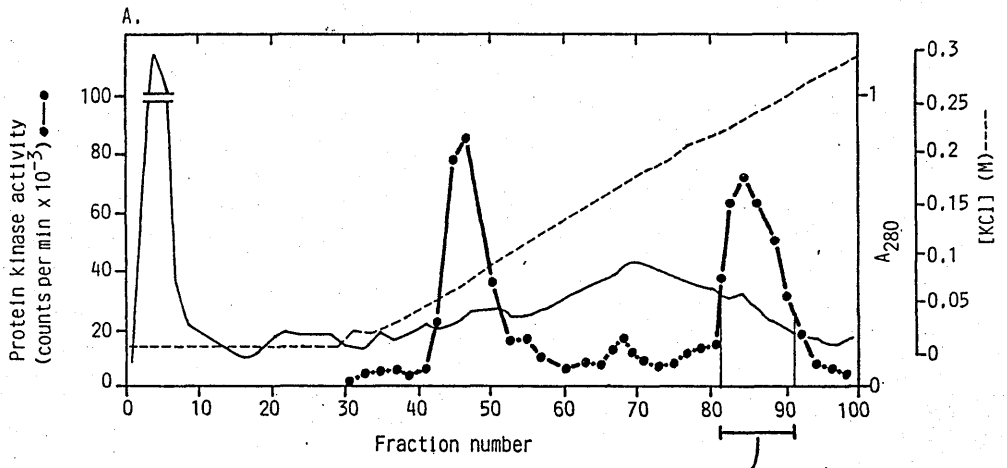
The isoelectric point of the autophosphorylated form of HSV-1 PK was also determined. A value of pH 5.6 (Fig. 2.10) was obtained, which was somewhat greater than the pH 4.9 previously found for PRV PK, but is consistent with the respective positions of elution of HSV-1 PK and PRV PK from DEAE-cellulose.

High performance gel-exclusion chromatography using a Superose 6 column (Methods, Section 3.5B) revealed that HSV-1 PK eluted at approximately the same position as aldolase, a protein of Mr 158,000 (Fig. 2.11). Because of the poor resolution of proteins above Mr 100,000 on this column, it was not possible to determine a more accurate molecular weight. However the result obtained suggested that HSV-1 PK was not a monomer but possesses a multimeric structure, most probably as a homodimer, which would be consistent with the indications for PRV PK.

Fig. 2.1 Scheme for the Complete Purification of PRV PK and the Partial Purification of HSV-1 PK

(A) The post-ribosomal supernatant from BHK cells (approximately 10^{10} cells) was subjected to chromatography on a DEAE-cellulose column (Methods, Section 3.1C). Column fractions were assayed for protein kinase activity with the substrate, protamine (●-●). The KCl gradient (----) was determined by conductivity measurements and the absorbance of column fractions was measured at 280nm (——).

(B) Fractions containing ViPK activity were pooled and 5-6 such preparations were concentrated by Aquacide II and subjected to further purification by the schemes shown.


A.
Protein kinase activity
(counts per min x 10^{-3})
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
Fraction number
1
0
A_{280}
0.3
0.25
0.2
0.15
0.1
0.05
0
[KCl] (M)

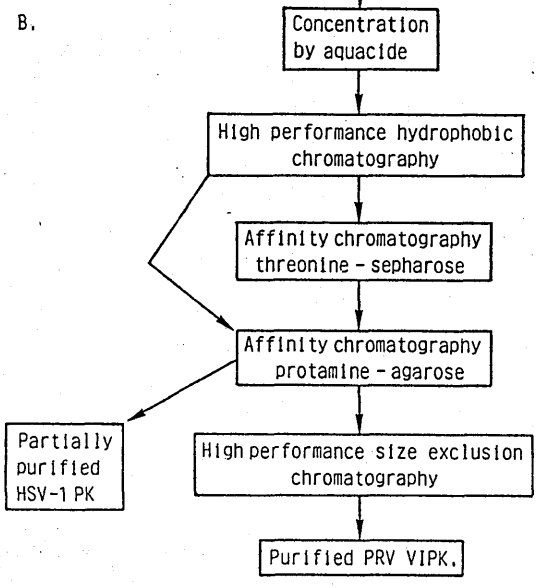

B.
Concentration by aquacide
High performance hydrophobic chromatography
Affinity chromatography threonine - sepharose
Affinity chromatography protamine - agarose
Partially purified HSV-1 PK
High performance size exclusion chromatography
Purified PRV VIPK.

Fig. 2.2 Chromatographic Purification of Pseudorabies Virus Protein Kinase, PRV PK

Post-ribosomal supernatant from approximately 10^{10} cells infected with PRV (20 p.f.u./cell) was subjected to DEAE-cellulose chromatography and active fractions were combined.

4-5 such preparations were concentrated and then subjected to successive chromatography on (A) TSK phenyl-5PW, (B) threonine-sepharose, and (C) protamine-agarose, as described in Methods, Section 3.

Aliquots (40µl) of the fractions indicated were assayed for protein kinase activity using protamine as substrate (●-●). The protein concentration or A_{280} of fractions (——), as indicated, and the concentration of the salt used to develop each particular column (---), are also presented.

The arrows in A indicate the multiple injections of sample.

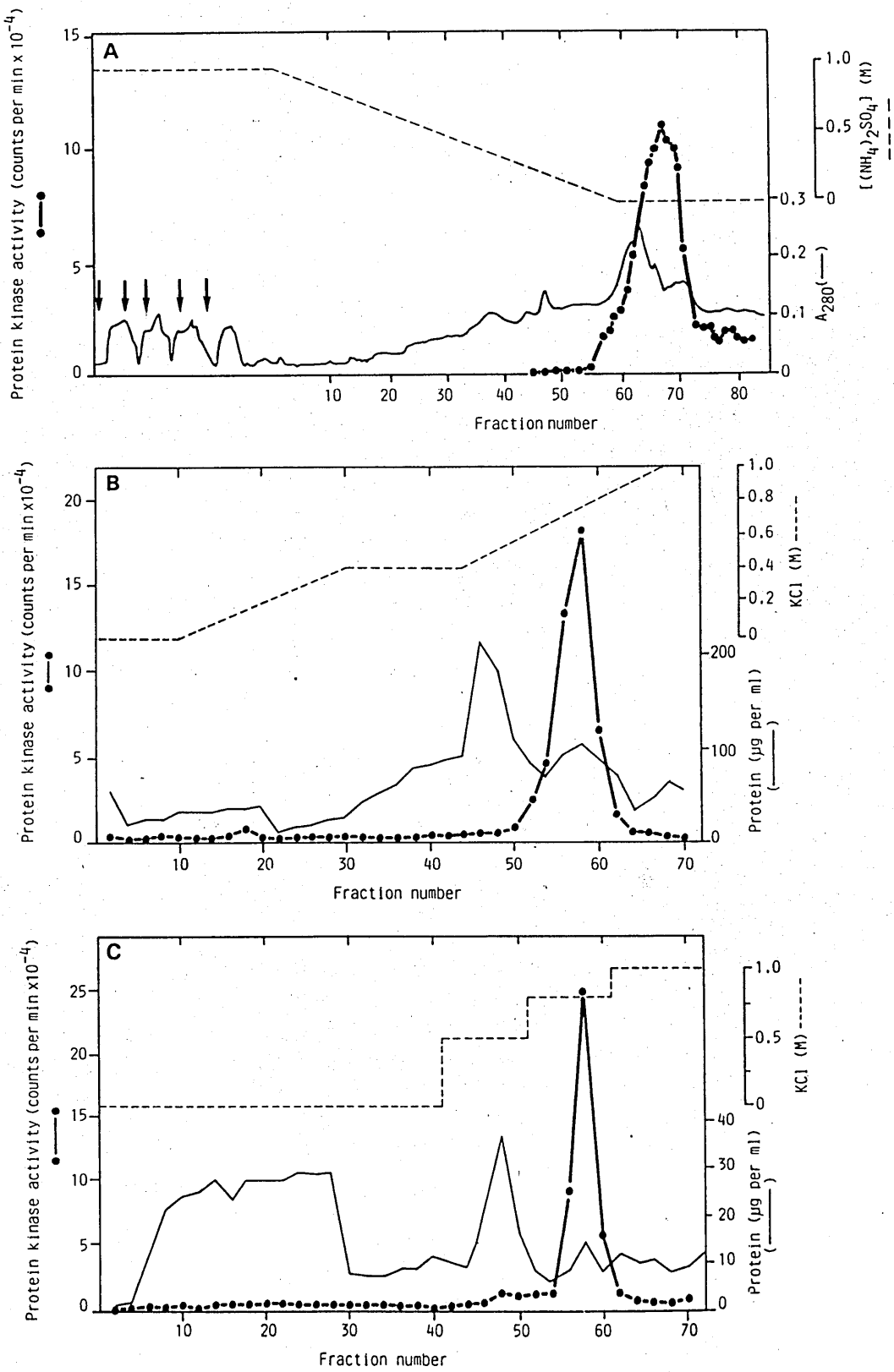

A
Protein kinase activity (counts per min x 10^{-4})
A_{280} (——)
$[(NH_4)_2SO_4]$ (M)
Fraction number
B
Protein kinase activity (counts per min x 10^{-4})
KCl (M) ----
Protein (µg per ml) (——)
Fraction number
C
Protein kinase activity (counts per min x 10^{-4})
KCl (M) ----
Protein (µg per ml) (——)
Fraction number

Fig. 2.3 Polyacrylamide Gel Analysis of PRV PK at Different Stages of Purification

One-dimensional gel electrophoresis under denaturing conditions was performed as described in Methods, Section 4.1A on protein from the different stages of purification listed in Table 2.1

lane 1 - post-ribosomal supernatant (25µg)

lane 2 - DEAE-cellulose stage (25µg)

lane 3 - TSK phenyl-5PW stage (25µg)

lane 4 - threonine-sepharose stage (25µg)

lane 5 - protamine-agarose stage (5µg)

lane 6 - protamine-agarose stage (7µg)

The electrophoretic separation from which lane 6 is taken employed narrower wells and a thinner gel to facilitate visualisation. This was a different gel with greater electrical resistance, and the protein migrated a shorter distance compared with lane 5, but still at the 38,000 Mr position as determined from standard proteins.

Photographs of gels stained with Coomassie Brilliant Blue are presented, and the positions of migration of proteins of known Mr are indicated.

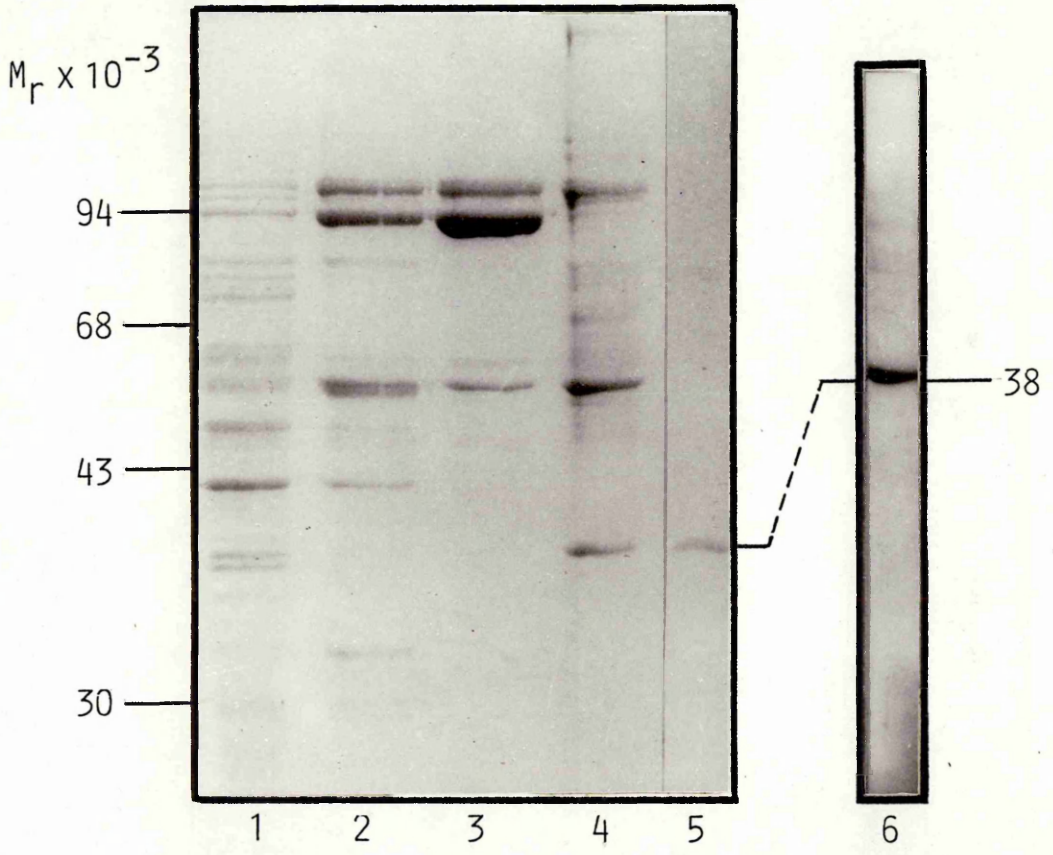

M_r x 10^{-3}
94
68
43
30
38
1
2
3
4
5
6

Fig. 2.4 Autophosphorylation of PRV PK

Enzyme (containing 1.5µg protein purified to either: 1, 2, the threonine-sepharose stage or, 3, 4, the protamine-agarose stage) was subjected to phosphorylation in the absence of exogenous substrate and prepared for gel electrophoresis, as described in Methods, Section 4.1A and 5.2.

Polyacrylamide gel electrophoresis was performed under denaturing conditions, the gel stained with Silver (1, 3), dried, and subjected to autoradiography (2, 4), all as described in Methods, Sections 4.1 and 4.2B.

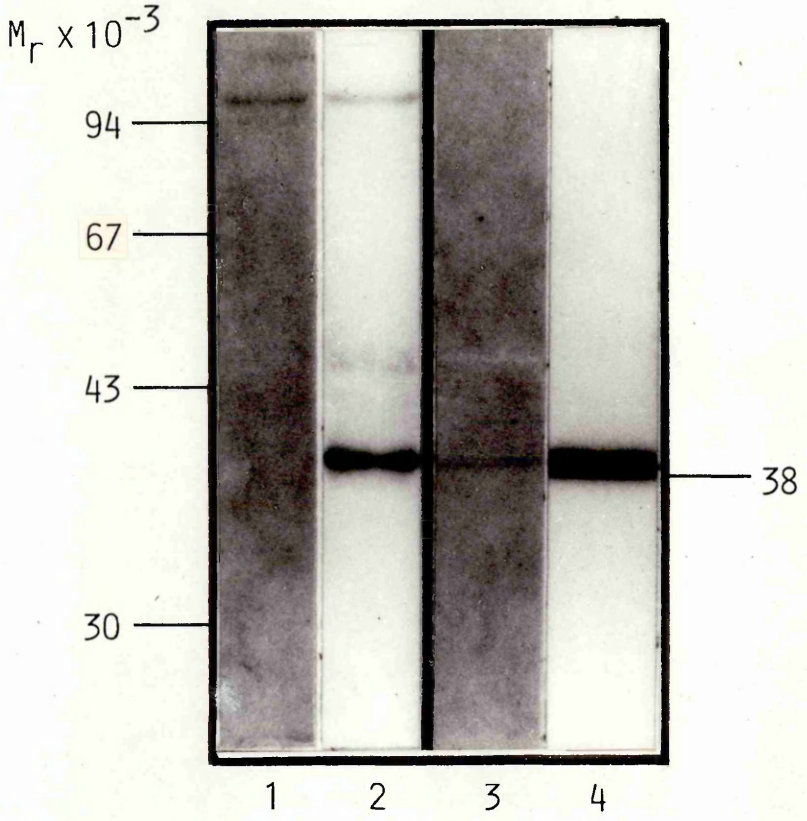

$M_r \times 10^{-3}$
94
67
43
30
38
1
2
3
4

Fig. 2.5 High-Performance Gel-Exclusion Chromatography of PRV PK on Superose 12

Purified enzyme was concentrated and subjected to chromatography on Superose 12 as described in Methods, Section 3.5A, and the protein kinase activity (o-o) of fractions determined. The A_{280} (——) of fractions is also indicated, the increase after fraction 20 being non-protein in origin. The positions of elution of aldolase, serum albumin, ovalbumin and myoglobin are indicated by arrows surmounted by the Mr ($\times 10^{-3}$) of each individual protein. The inset shown the results of two separate gel electrophoretic analyses of aliquots of fractions 13-16:

A. photograph of silver stained gel (80µl applied),

B. autoradiograph of gel lane of sample (100µl) subjected to autophosphorylation, the Mr of the band being indicated.

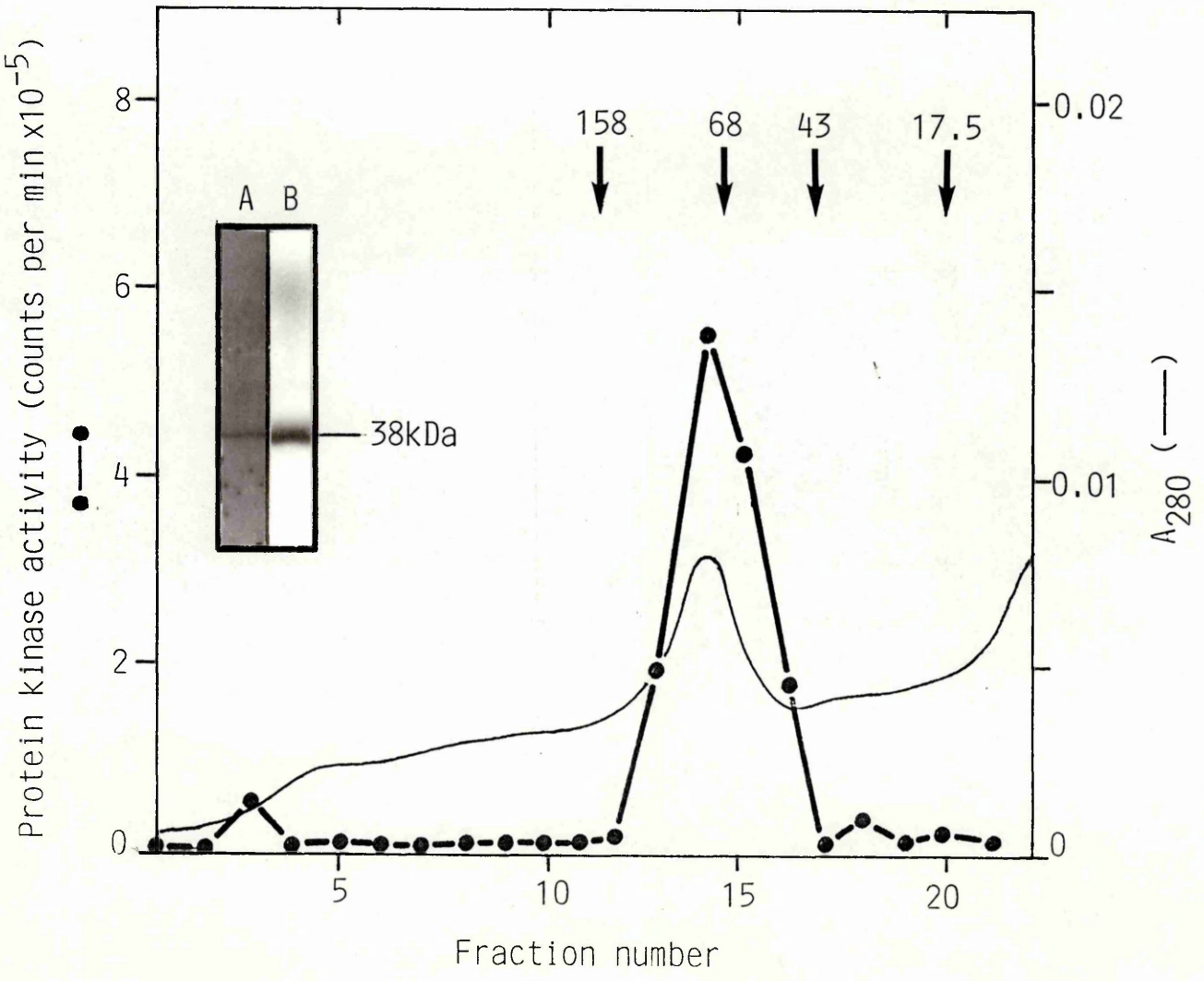

Protein kinase activity (counts per min x10^{-5})
8
6
4
2
0
158
68
43
17.5
A
B
38kDa
0.02
0.01
0
A_{280} (——)
5
10
15
20
Fraction number

Fig. 2.6 Determination of the Isoelectric Point of Phosphorylated PRV PK

Purified protein kinase (1μg) was subjected to autophosphorylation followed by two-dimensional polyacrylamide gel separation, as described in Methods, Sections 5.2 and 4.1B. The gel was dried and subjected to autoradiography. The scale above the autoradiograph indicates the pH gradient in the first (isoelectric focussing) dimension, as deduced from measurements of ampholines eluted into degassed water as described in Methods, Section 4.2.

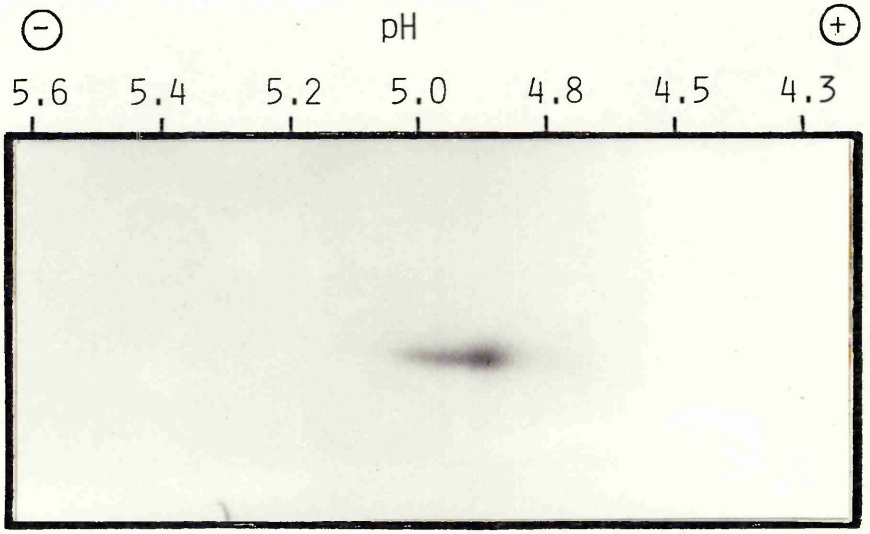
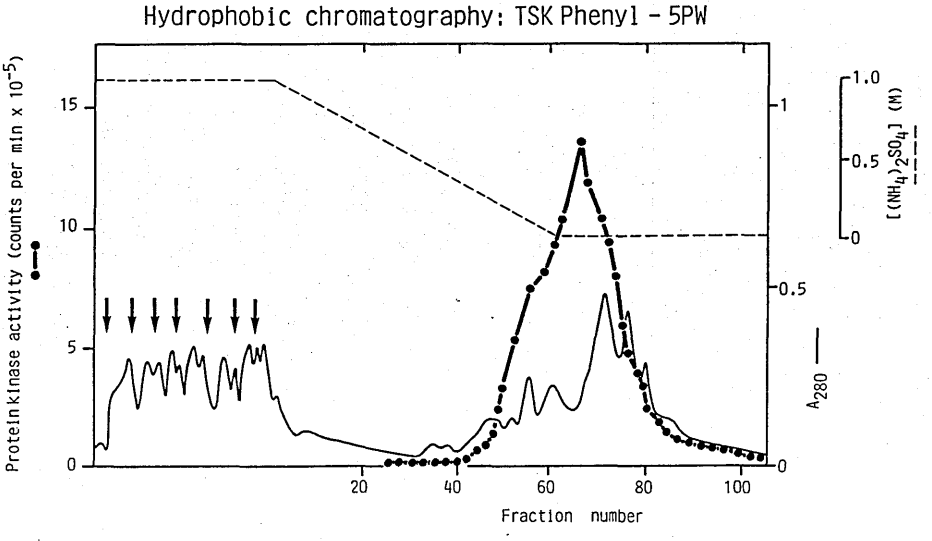

⊖
pH
⊕
5.6
5.4
5.2
5.0
4.8
4.5
4.3

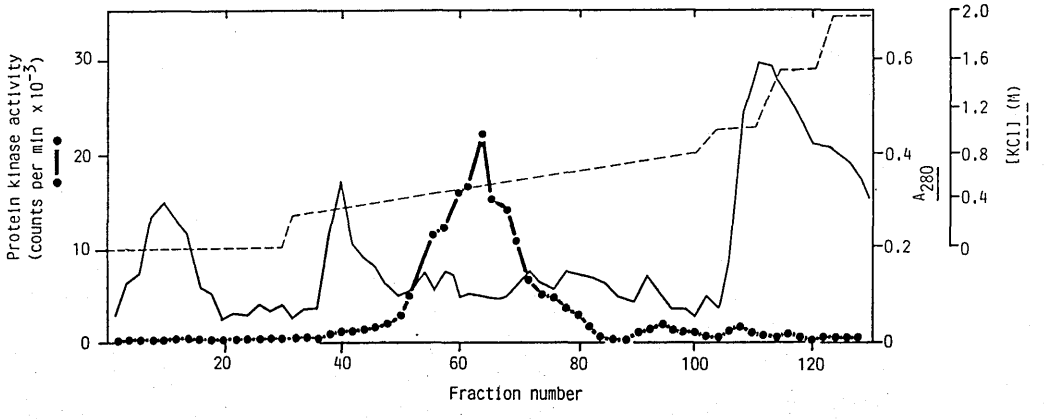
Fig. 2.7 Chromatographic Purification of HSV-1 PK

Post-ribosomal supernatant from approximately 10^{10} BHK cells infected with HSV-1 at 10 p.f.u./cell was subjected to DEAE-cellulose chromatography and active fractions were combined. 5-8 such preparations were concentrated and then subjected to successive chromatography on 1(A) TSK phenyl 5PW and 1(B) protamine-agarose or TSK phenyl-5PW followed by 2(A) threonine-sepharose and 2(B) protamine-agarose, as described in Methods, Section 3. Aliquots (40µl) of the fractions indicated were assayed for protein kinase activity (●-●). The protein concentration or A_{280} of fractions (——), as indicated, and the concentration of the salt used to develop each particular column (---), are also presented. The arrows in 1(A) represent multiple injections of sample via a 5ml sample loop.

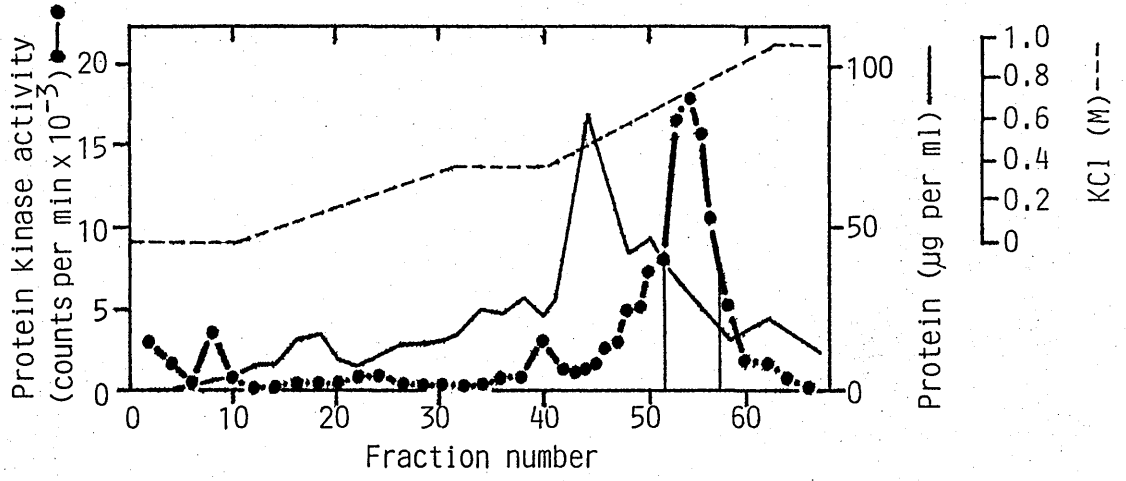
1A


Hydrophobic chromatography: TSK Phenyl - 5PW
Protein kinase activity (counts per min x 10^{-5})
A_{280}
$[(NH_4)_2SO_4]$ (M)
Fraction number

1B


Affinity chromatography: Protamine Agarose
Protein kinase activity (counts per min x 10^{-5})
A_{280}
[KCl] (M)
Fraction number

2A


Affinity chromatography: Threonine– sepharose
Protein kinase activity (counts per min x 10^{-3})
Protein (µg per ml)
KCl (M)
Fraction number

2B

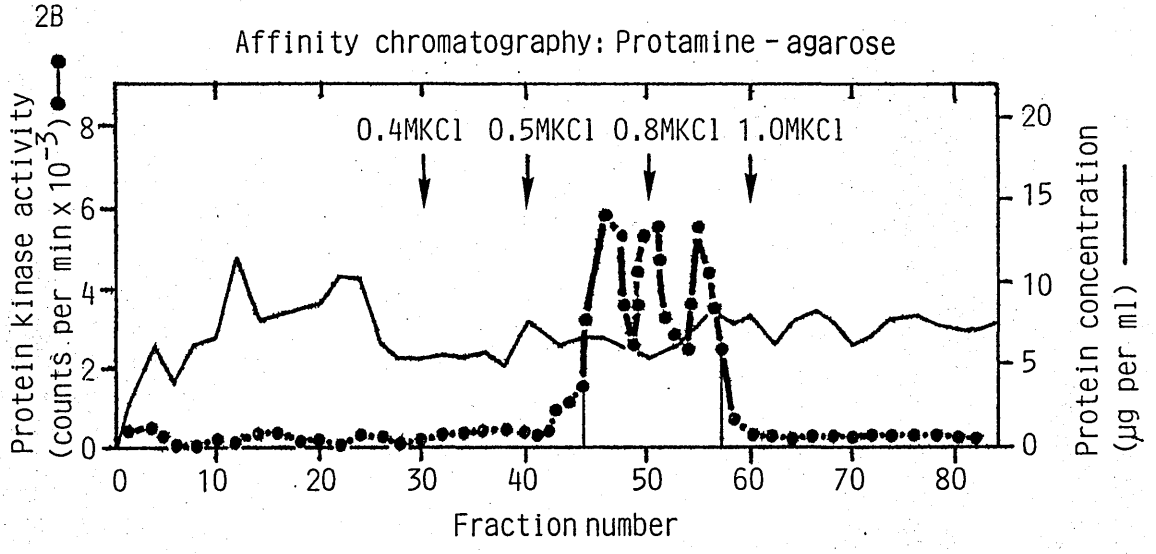

Affinity chromatography: Protamine - agarose
0.4MKCl
0.5MKCl
0.8MKCl
1.0MKCl
Protein kinase activity (counts per min x 10^{-3})
Protein concentration (µg per ml)
Fraction number

Fig. 2.8 Polyacrylamide Gel Analysis of HSV-1 PK at Different Stages of Purification

One-dimensional gel electrophoresis under denaturing conditions was performed on protein from various stages of the purification of HSV-1 PK. A represents a gel showing the progression of the purification of HSV-1 PK with threonine-sepharose chromatography before protamine-agarose. B represents a similar gel but with the threonine-sepharose chromatography performed after protamine-agarose.

Gel A

lane 1	post-ribosomal supernatant	(50µg)
lane 2	DEAE-cellulose	(25µg)
lane 3	TSK phenyl-5PW	(25µg)
lane 4	threonine-sepharose	(25µg)
lane 5	protamine-agarose	(5µg)

The gel was stained with Coomassie Brilliant Blue, and the position of proteins of known molecular weights are indicated.

Autophosphorylation of 1µg protein obtained after the threonine-sepharose stage, and 0.25µg of protein obtained after protamine-agarose, were incubated with [γ^{32}P]ATP in the absence of exogenous substrate according to Methods, Section 5.2, and subjected to electrophoresis on the same gel. The arrows indicate that the phosphorylated species corresponded to one of the major components in the purified fraction in lane 5.

Gel B

lane 1	post-ribosomal supernatant	(50µg)
lane 2	DEAE-cellulose	(25µg)
lane 3	TSK Phenyl-5PW	(25µg)
lane 4	protamine-agarose	(5µg)
lane 5	threonine-sepharose	(5µg)

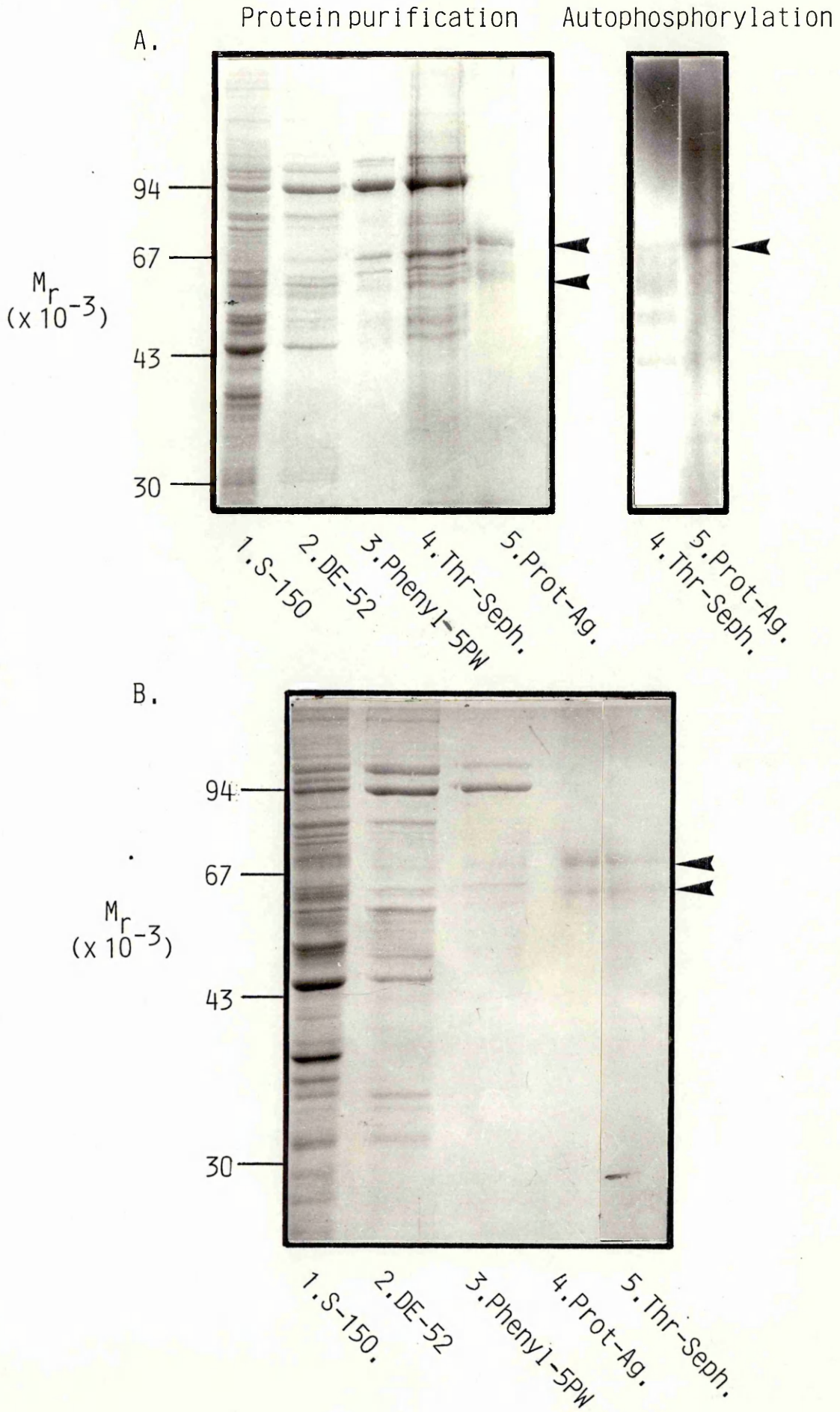

Protein purification
Autophosphorylation
A.
94
67
43
30
M_r ($\times 10^{-3}$)
1.S-150
2.DE-52
3.Phenyl-5PW
4.Thr-Seph.
5.Prot-Ag.
4.Thr-Seph.
5.Prot-Ag.
B.
94
67
43
30
M_r ($\times 10^{-3}$)
1.S-150.
2.DE-52
3.Phenyl-5PW
4.Prot-Ag.
5.Thr-Seph.

Fig. 2.9 Autophosphorylation of HSV-1 PK

The final partially-purified preparation of HSV-1 PK (0.25µg) was subjected to autophosphorylation, followed by one-dimensional polyacrylamide gel electrophoresis under denaturing conditions, as described in Methods, Sections 5.2 and 4.1A.

The gel was fixed, dried and subjected to autoradiography. Positions of standard molecular weight marker proteins are indicated. The Arrow indicates the 68 kDa phosphorylated protein.

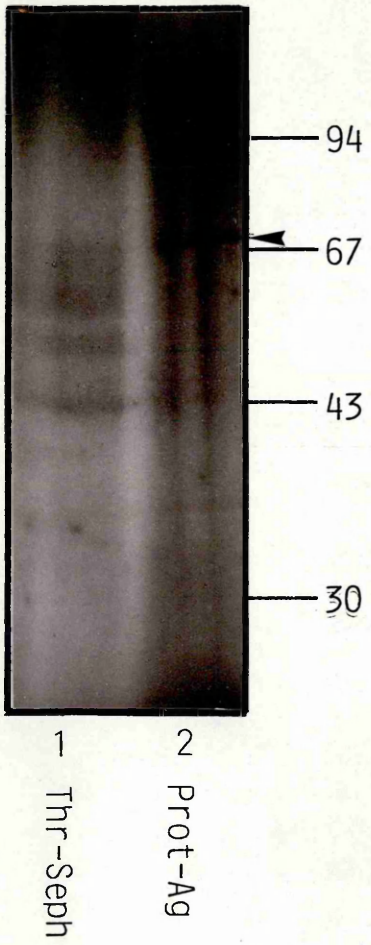

94
67
43
30
1
Thr-Seph
2
Prot-Ag

Fig. 2.10 Determination of the Isoelectric Point of Phosphorylated HSV-1 PK

Partially-purified HSV-1 PK from the protamine-agarose stage (0.5-1µg) was subjected to autophosphorylation followed by two-dimensional O'Farrell polyacrylamide gel electrophoresis as described in Methods, Section 4.1B. The gel was dried and subjected to autoradiography. The horizontal scale above the autoradiograph indicates the pH gradient in the first (isoelectric focussing) dimension, and the vertical scale indicates the positions of migration of proteins of known Mr in the second dimensions.

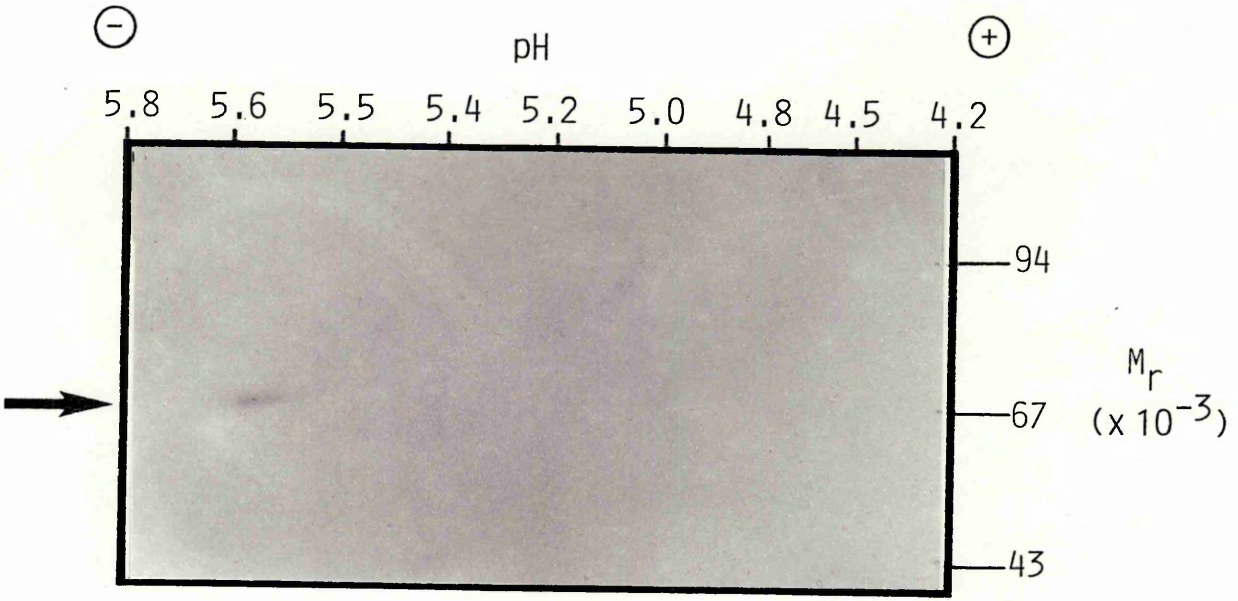

⊖
pH
⊕
5.8
5.6
5.5
5.4
5.2
5.0
4.8
4.5
4.2
94
67
43
M_r
(x 10^{-3})

Fig. 2.11 High-Performance Gel-Exclusion Chromatography of HSV-1 PK on Superose 6

A preparation of HSV-1 PK was purified by chromatography on DEAE-cellulose and then TSK Phenyl-5PW. The enzyme (0.5mg) was concentrated and subjected to chromatography of Superose 6 as described in Methods, Section 3.5B.

40µl aliquots of the column fractions were assayed for protein kinase activity (●-●) using protamine as the substrate. The A_{280} (——) of fractions is also indicated.

The positions of elution of the standard proteins, aldolase (158,000), serum albumin (68,000), ovalbumin (43,000) and chymotrypsinogen (25,000) are indicated by arrows surmounted by the Mr ($x10^{-3}$) of each individual protein.

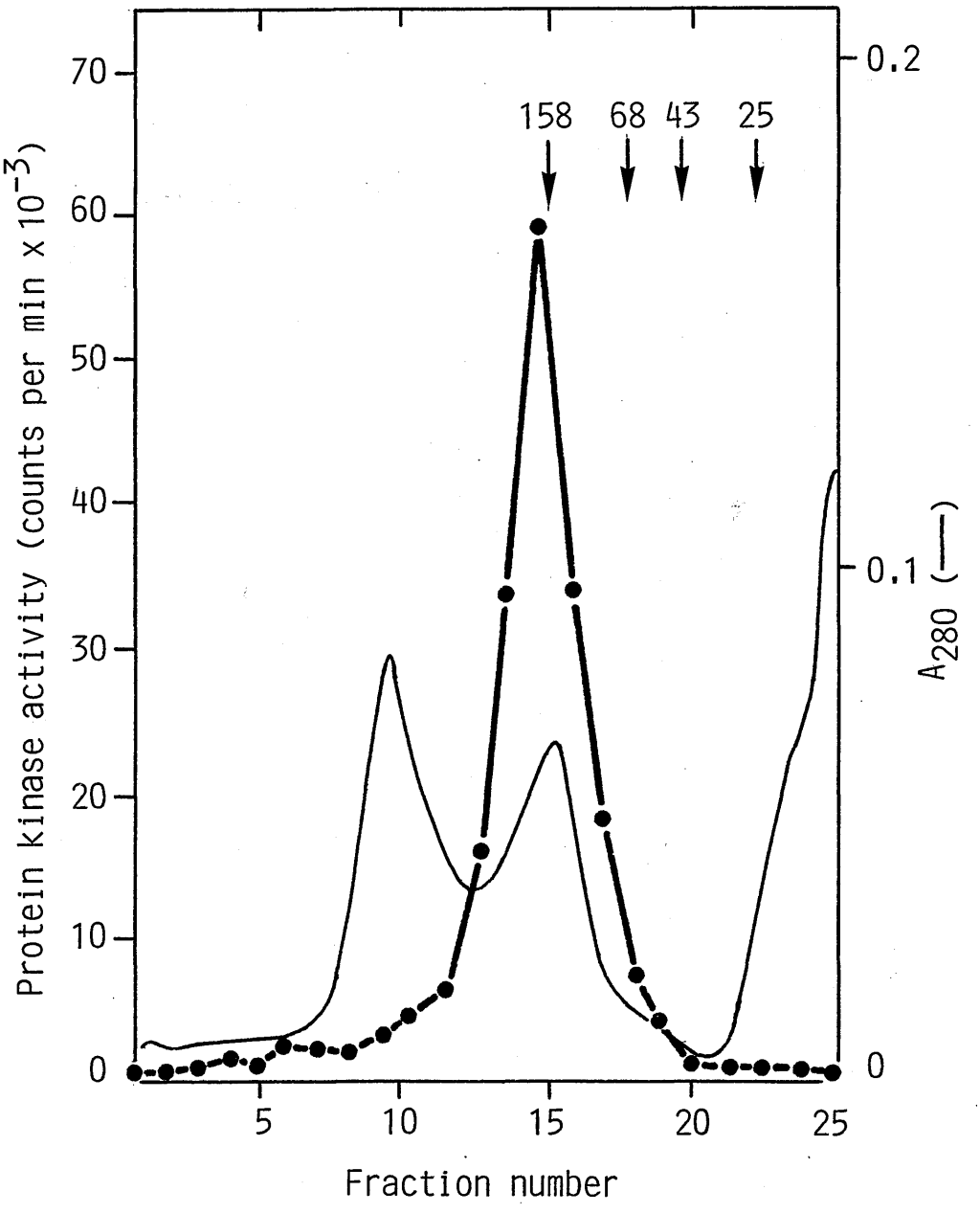

158
68
43
25
70
60
50
40
30
20
10
0
0.2
0.1
0
5
10
15
20
25
Protein kinase activity (counts per min x 10^{-3})
A_{280} (——)
Fraction number

TABLE 2.1 PURIFICATION OF PSEUDORABIES VIRUS PROTEIN KINASE

The protein kinase was purified and assayed as described in Materials and Methods.

Purification step	Volume (ml)	Protein (mg)	Enzyme activity * (units)	Recovery (%)	Specific activity *(units/mg)	Purification (fold)
1. Post-ribosomal supernatant	80	393	-	-	-	-
2. DEAE-cellulose	37	98	238	(100)	2.4	(1)
3. TSK Phenyl-5PW	20	5.1	192	81	37.7	16
4. Threonine-sepharose	17.5	0.875	146	61	167	70
5. Protamine-agarose	5	0.050	54	23	1070	446

* 1 unit PRV PK incorporates 1nmol phosphate/min into protamine under standard assay conditions

TABLE 2.2 (A) PARTIAL PURIFICATION OF HERPES SIMPLEX-1 VIRUS PROTEIN KINASE

Purification step	Enzyme activity (units*)	Recovery (%)	Specific activity (units/mg)	Purification (-fold)
1. Post-ribosomal supernatant	-	-	-	-
2. DEAE-cellulose	29	(100)	0.33	(1)
3. TSK phenyl-5PW	5	37	1.3	4
4. Threonine-sepharose	3	22	14	42
5. Protamine-agarose	0.7	5	31	94

* 1 unit HSV-1 PK incorporates 1 nmol phosphate/min into protamine under standard assay conditions.

TABLE 2.2 (B) PARTIAL PURIFICATION OF HERPES SIMPLEX-1 VIRUS PROTEIN KINASE

Purification step	Enzyme activity (units*)	Recovery (%)	Specific activity (units/mg)	Purification (-fold)
1. Post-ribosomal supernatant	-	-	-	-
2. DEAE-cellulose	726	100	11	(1)
3. TSK phenyl-5PW	239	34	77	7
4. Protamine-agarose	113	16	187	17

* 1 unit HSV-1 PK incorporates 1 nmol phosphate/min into protamine under standard assay conditions.

2 THE SUBSTRATE-SPECIFICITY OF THE PROTEIN KINASES SPECIFIED BY THE ALPHA-HERPESVIRUSES

Synthetic peptides synthesised by Prof. F. Marchiori were used to investigate the site specificity of highly purified PRV PK and HSV-1 PK in experiments performed by the author in the laboratory of Prof. L.A. Pinna (University of Padua, Italy). PRV PK and HSV-1 PK were purified as described in the previous section except that the threonine-sepharose stage was not included. (This work was initiated before the final purification of PRV PK). Nevertheless the PRV PK was purified 1000-fold at least, had a specific activity of approximately 200 units per mg, and exhibited two major stained bands on polyacrylamide gel-electrophoresis under denaturing conditions. The HSV-1 PK was of similar purity (Fig. 2.8).

The conditions for the assay of protein kinase activity with the synthetic peptide substrates are described in Methods, Section 5.1(B), and in the individual figure legends.

The peptides used in this study are referred to by a logical nomenclature to provide unambiguous reference to definite fragments of naturally occurring polypeptides. In this nomenclature, such peptides were designated by the initials of the parent protein followed, in parenthesis, by the original sequence numbers of the first and last residues of the peptide. Where a modified derivative was synthesised in which a new amino acid residue has been substituted for one of the original ones, this is also indicated. Thus, I-1(32-37) indicates the hexapeptide RRPTPA, corresponding to fragment 32-37 of protein phosphatase inhibitor-1, and its derivative with Pro^{34} replaced by an Ala termed Ala^{34} I-1(32-37).

2.1 The site-specificity of PRV PK

Because of the preference of PRV PK for protamine over histones (Katan et al., 1985), many of the peptide substrates used in this study were based in whole or in part on three potential phosphorylation sites within the protamines, thynnine and galline: TH(1-12), PRRRRRSSRPVR; TH(13-33), RRRRYRRSTVARRRRRVRRRR and GA(52-65), RRRRYGSRRRRRRY. Others were similarly based on preferred sites for the cyclic AMP-dependent protein kinase in protein phosphatase inhibitor-1 and pyruvate kinase (Type L): I-1(30-37), RRRRPTPA, and PK(8-13), RRASVA.

The results of these studies allowed the following main conclusions regarding the site specificity of PRV PK.

(i) In order to be able to act as positive determinants for substrates of PRV PK, basic residues must apparently be on the N-terminal side of the amino acid residue that is the target of phosphorylation.

This is indicated by the results in Fig 2.12 and Table 2.3 (in which the peptides were assayed at a concentration of 50μM and 1mM respectively). Thus, TH(1-12) is the best substrate for the viral protein kinase among those tested, and the conclusion that this is due to the five N-terminal Arg residues may be drawn from the inability of the peptide lacking these, TH(7-12), to serve as a substrate for PRV PK. It is true that TH(7-12) has only two Arg residues on the C-terminal side of the Ser residues; however GA(57-65) with six C-terminal Arg residues is also not a substrate for PRV PK even though

GA(52-65), with four additional N-terminal Arg residues is (Fig. 2.12, Table 2.3). Other peptides that reinforce this conclusion are TH(12-23) and I-1(30-37), which can act as substrates, and TH(18-33), which cannot.

(ii) At least two basic N-terminal residues are required, and additional basic residues had a further positive modulating effect. This is evident from Table 2.3 from a comparison of Val^{36} I-1(33-37) and Val^{36} I-1(32-37), containing one and two Arg residues, respectively; and I-1(32-37) and I-1(30-37), containing two and four Arg residues, respectively. In Table 2.4, it can be seen that the peptides with the lowest Km values contain between four and six Arg residues on the N-terminal side of the amino acid residues which become phosphorylated.

(iii) Both Thr and Ser can be phosphorylated (Fig 2.15) but Tyr most probably cannot.

It was not previously known whether Thr residues could be phosphorylated by PRV PK, as salmine, the protamine used to assay the protein kinases routinely, lacks Thr. The phosphorylation by PRV PK of I-1 peptides made it clear that Thr could act as a phosphate acceptor. Comparison of the phosphorylation of PK(8-13) and Thr^{11} PK(8-13) indicated that Thr was not such a good phosphate acceptor as Ser; the difference in Vmax between these peptides was considerable (Table 2.4). Although directly comparable peptides in which a Tyr residue replaced a Ser residue were not available, it seemed most unlikely that the viral kinases could phosphorylate

Tyr residues. Thus after incubation with viral kinase no phosphotyrosine was detected in TH(12-23), GA(52-65) (Table 2.3), or in the peptide RRRRY, all of which have a Tyr residue flanked on its N-terminal side by multiple Arg residues. Nor did the viral kinase phosphorylate angiotensin II (DRVYIHPF), a good artificial substrate for many tyrosine kinases (Casnellie et al., 1982; Hunter, 1982; Swarup et al.; 1983; House et al., 1984).

(iv) The N-terminal Arg residues that are positive modulators of the ability of sites to serve as substrates for PRV PK may need to be displaced from the target Ser or Thr residue for optimal effect.

This is illustrated by the results of Table 2.5 for the phosphorylation of thynnine peptides containing adjacent Ser and Thr residues. In TH(12-23), which has a long run of Arg residues on its N-terminal side, Ser^{20} is preferred to Thr^{21}, consistent with the results for PK(8-13) mentioned above. However in TH(18-23), a truncated form of the peptide with only two Arg residues on the N-terminal side of the SerThr sequence, the phosphorylation of Ser^{20} markedly decreased relative to that of Thr^{21}. The simplest interpretation of this result is that an Arg has a much greater positive modulating influence on the phosphorylation of a target amino acid when located three, rather than one or two, positions to its N-terminal side. It should also be pointed out that GA(52-65) is a tolerably good substrate for PRV PK although it lacks basic residues in both positions -1 and -2.

(v) Other amino acids, on both the N-terminal and C-terminal sides of the phosphate acceptor, exerted a modulating influence on substrates for PRV PK.

Although more extensive studies are required, there were indications that hydrophobic amino acids in the position immediately adjacent to the target residue on its N-terminal side cause a decrease in phosphorylation by PRV PK. Thus the replacement of a Ser residue by an Ala residue in this position, illustrated in the comparison of the peptides TH(18-23) and Thr^{11} PK(8-13), caused a decrease of approximately 50% in the phosphorylation of the Thr residue. (This was calculated from the relative rates of phosphorylation of the two peptides given in Table 2.3; 26% and 7% maximum, the former figure was then adjusted to 14% because of the contribution of Ser to the phosphorylation of TH(18-23) as indicated in Table 2.5). A Pro residue adjacent to the target residue exerted a marked effect, depending on whether it was N-terminal or C-terminal. When it was on the N-terminal side phosphorylation was enhanced, as illustrated by comparison of Thr^{11} PK(8-13) or TH(18-23) with Val^{36} I-1(32-37), in which an Ala or Ser residue, respectively, was replaced by a Pro residue (Table 2.3). When a Pro residue is on the C-terminal side phosphorylation was decreased, as illustrated by comparison of Thr^{11} PK(8-13) with Ala^{34}(32-37), in which a Val residue was replaced by a Pro residue (Table 2.3). This behaviour recalls that of the cyclic AMP-dependent protein kinase (Chessa et al., 1983). When two additional Arg were

present on the N-terminal side of the target residue, the negative modulating effect of Pro in I-1(32-37) was relieved (Fig. 2.16).

Other amino acid residues on the C-terminal side of the target residue appeared capable of negative modulation of the phosphorylation of peptides by the viral kinase (Table 2.3). These included acidic residues, illustrated by the effect of a Glu residue as a replacement for Val in Glu^{22} TH(18-23). One might also be tempted to conclude from a comparison of TH(18-23) and TH (18-33) that Arg residues on the C-terminal side of the target could exert a similar effect. However the fact that GA(52-65), with six such Arg residues, could still be phosphorylated quite well, and TH(1-12), with two such residues, was an extremely good substrate (Tables 2.3 and 2.4), suggested that any effect of a single Arg residue on the C-terminal side of the target may be quite small.

(vi) Basic residues other than Arg were much inferior as positive determinants for substrates of PRV PK. It can be seen from Table 2.3 that the replacement of the Arg residues in TH(18-23) by Lys residues resulted in an 80% decrease in the rate of phosphorylation. The replacement of Arg residues by Orn residues in this and several other peptides (Table 2.3) caused an even more striking decrease in the ability of these to serve as substrates for PRV PK.

Although these studies were done with an enzyme preparation that was not homogeneous, their main features were subsequently confirmed with the homogeneous enzyme. Thus homogeneous PRV PK was equally active at 0.15M, 0.5M and 1.0M KCl. Synthetic peptides lacking serine but containing threonine could still serve as substrates for the enzyme. The relative substrate preference remained consistent with that already presented. Also the results with the synthetic peptides were consistent whether assays were performed at 150mM or 500mM KCl.

2.2 The site-specificity of HSV-1 PK

More limited studies were also performed with HSV-1 PK. The results of some of these are shown in Table 2.6, from which it can be seen that the site specificity of the HSV-1 enzyme was broadly similar to that of PRV PK. Thus, there was a similar preference for Arg residues on the N-terminal side of the target residue, a similar ability for Thr residues as well as Ser residues to act as phosphoryl acceptors and a similar influence of Pro and Glu on the site-specificity of the viral kinase. HSV-1 PK and PRV PK also had similar apparent Km and Vmax values for the protamine, salmine (Fig 2.13 and Fig 2.14).

2.3 Comparison of the site-specificity of herpesvirus protein kinases and other protein kinases

Because most of the peptides used in this study had previously been used as model substrates for the cyclic AMP-dependent protein kinase (Meggio et al., 1981; Chessa et al., 1983) and protein kinase C

(Ferrari et al., 1985), it was possible to make a direct comparison of the structural requirements of these enzymes with those of the viral enzymes.

Although both viral enzymes and protein kinase C prefer very basic peptides as substrates, their site-specificities may be distinguished by the fact that protein kinase C can readily phosphorylate peptides like GA(57-65), with Arg on their C-terminal side, whereas the viral kinases cannot. Furthermore unlike the viral kinases, protein kinase C will employ substrates in which Orn replaced Arg. Other differences than these must exist to explain why the viral kinases, unlike protein kinase C, could not phosphorylate Ser^{35} in the sequence RRKASGPP in bovine histone H1 (Iwasa et al., 1980).

There were several apparent similarities between the viral kinases and the cyclic AMP-dependent protein kinases. Both required basic residues on the N-terminal side of the phosphorylated residue and in both cases Arg could not be replaced by Orn (Meggio et al., 1981). An acidic group on the C-terminal side of potential target residues exerted a negative influence in both cases and both enzymes responded similarly to the insertion of a Pro residue immediately adjacent to the target residue on the N-terminal or C-terminal side. Nevertheless the difference between the site-specificities of these two types of enzyme was very clear when the relative Km values of some of their common substrates were compared (Table 2.7), Of these, PK(8-13), with only two Arg residues on the N-terminal side of the target residue, was the peptide with the lowest Km for the cyclic AMP-dependent protein kinase, but the one with the highest Km for PRV PK. The converse was true for the peptides TH(1-12) and TH(12-23),

with five and six Arg residues, respectively. Furthermore the much higher Km of the cyclic AMP-dependent protein kinase for TH(18-23) compared with PK(8-13), recalled the well-established importance of an N-terminal hydrophobic residue for this enzyme, a feature that appeared if anything to have a negative modulating influence on the substrates for the viral kinase.

A further point of difference was that Thr residues could substitute for Ser residues much more effectively in the case of the viral kinase than in the cyclic AMP-dependent protein kinase. This is illustrated in Table 2.5 in which are compared the relative extents of phosphorylation by the two types of enzymes of Thr^{21} in TH(18-23). It has previously been shown that substitution of Thr for Ser^{11} in PK(8-13) caused a dramatic rise in Km for the cyclic AMP-dependent protein kinase, in addition to a decrease in Vmax (Chessa et al., 1983). However with the viral protein kinase the Km is unaffected, only the Vmax being decreased (Table 2.4).

Fig. 2.12 Rates of Phosphorylation of Salmine and Synthetic Peptides by PRV PK

Peptide substrates employed were:-

PEPTIDE	STRUCTURE
(●) TH(1-12)	PRRRRRSSRPVR
(o) Salmine	PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR
(■) TH(12-23)	RRRRYRRSTVA
(□) GA(52-65)	RRRRYGSRRRRRRY
(Δ) PK(8-13)	RRASVA
Thr^{11}PK(8-13)	RRATVA
TH(7-12)	SSRPVR
TH(18-23)	RRSTVA
TH(18-33)	RRSTVARRRRRYRRRR
Val^{36}I-1(32-37)	RRPTVA
GA(57-65)	GSRRRRRRY

The peptides were used at a final concentration of 50μM and phosphorylated according to Methods, Section 5.1B.

The reactions were stopped by addition of 2% ice-cold trichloroacetic acid after incubation for 0 min, 5 min, 15 min, and 30 min at 30°C.

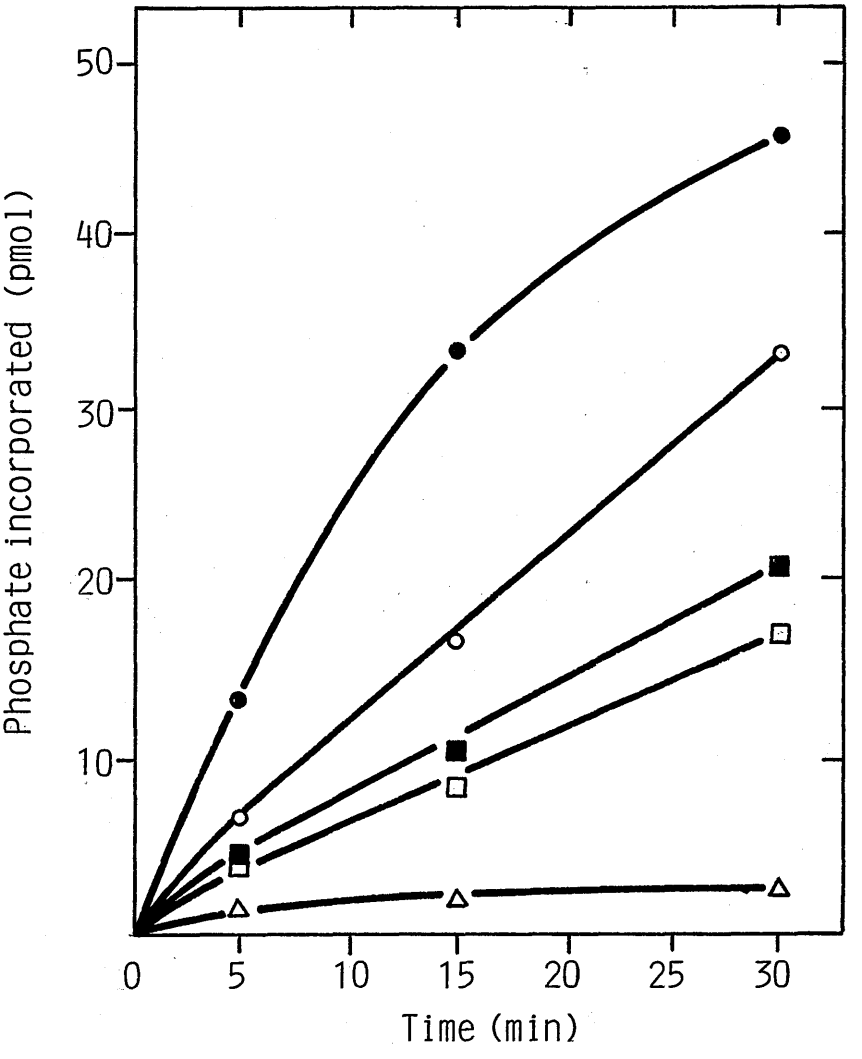

Phosphate incorporated (pmol)
50
40
30
20
10
0
5
10
15
20
25
30
Time (min)

Fig. 2.13 The Apparent Km of PRV PK for Salmine and Peptide TH(1-12)

A preparation of purified PRV PK was assayed in standard reaction mixtures (Methods, Section 5.1B) containing concentrations of salmine (●-●) or TH(1-12) (o-o) from 2-80μM. The initial velocity was measured from the values obtained by terminating the reaction after 0, 2, 10, 15 and 30 min at each of the concentrations tested. The apparent Km was calculated from the double reciprocal plot fitted to the Michaelis-Menten equation.

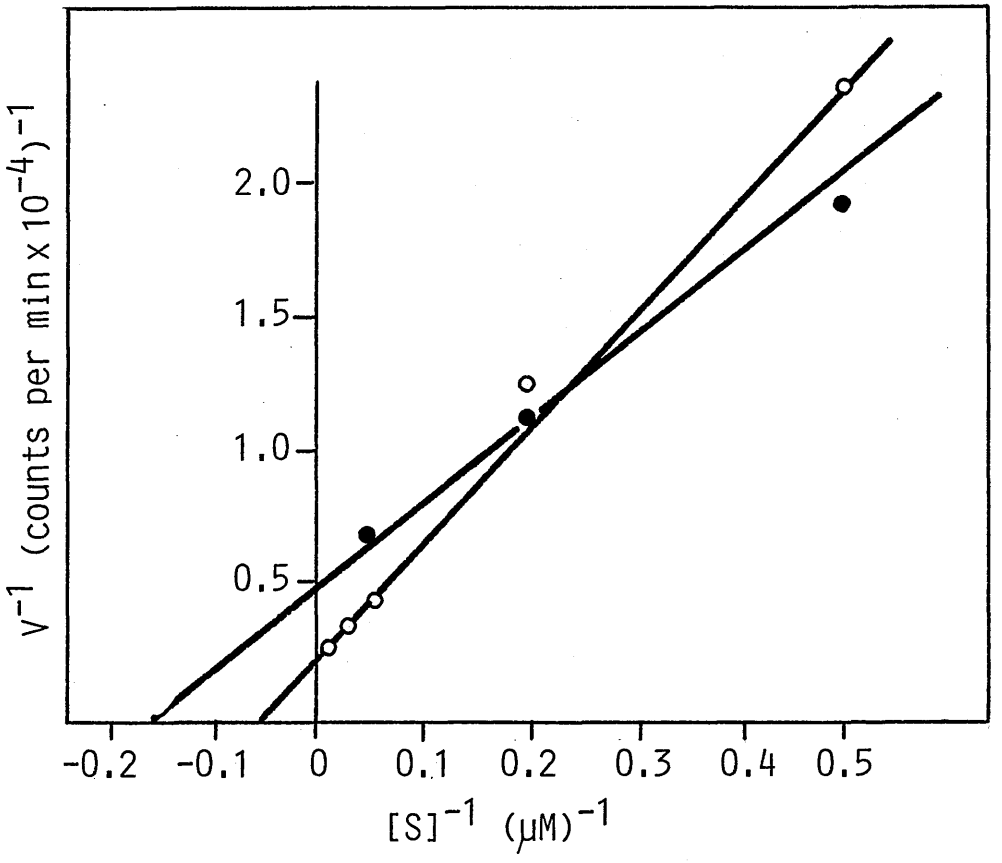

V^{-1} (counts per min x 10^{-4})$^{-1}$
2.0
1.5
1.0
0.5
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
$[S]^{-1}$ $(\mu M)^{-1}$

Fig. 2.14 The Apparent Km of HSV-1 PK for Salmine

A preparation of purified HSV-1 PK was assayed in a standard reaction mixture containing salmine in the concentration range 2-80μM. The initial velocity was measured from the values obtained by terminating the reaction after 0, 2, 5, 10, 15 and 30 min, at each of the concentrations tested.

The apparent Km was calculated from the double reciprocal plot fitted to the Michaelis-Menten equation.

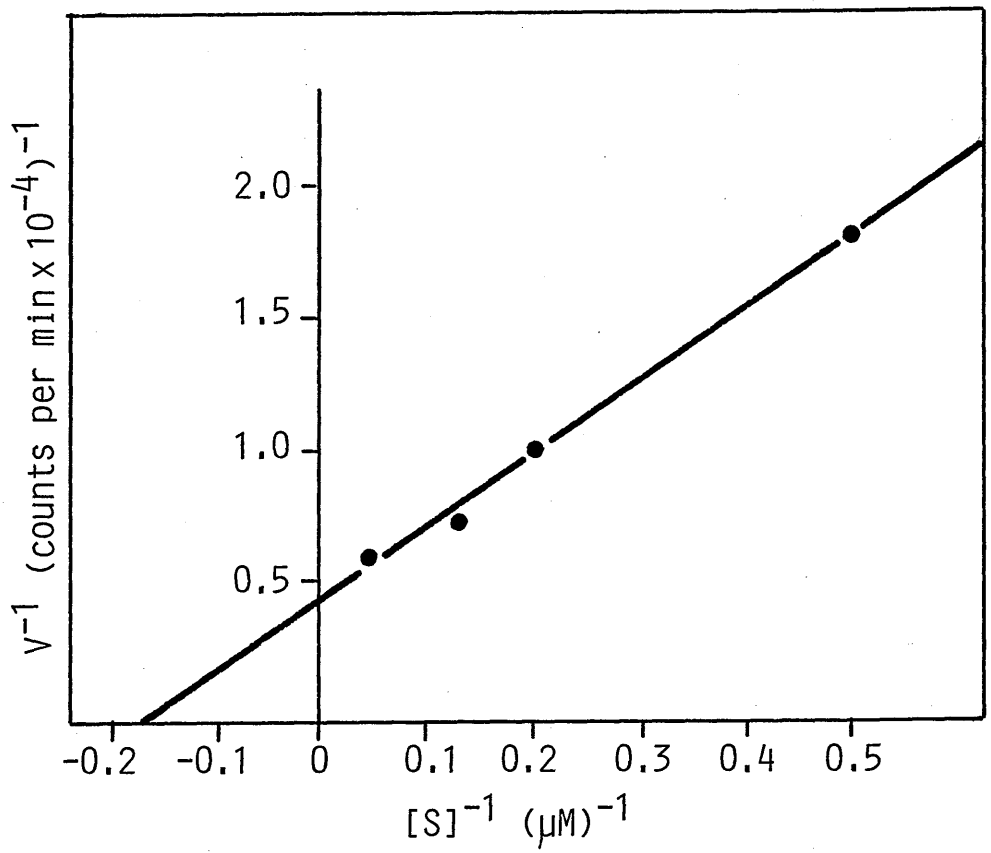

V^{-1} (counts per min x 10^{-4})$^{-1}$
2.0
1.5
1.0
0.5
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
$[S]^{-1}$ $(\mu M)^{-1}$

Fig. 2.15 The Phosphorylation of Both Seryl and Threonyl Residues in Synthetic Peptides by PRV PK

Autoradiograms of analyses of [^{32}P]phosphoamino acids in synthetic peptides. (A) TH(18-23), RRSTVA, and (B) TH(12-23), RRRRYRRSTVA. Both peptides were phosphorylated by PRV PK for 1h with a supplement of an additional 0.05 units of enzyme after 30 mins. The phosphorylated peptide was precipitated on P81 paper to separate it from ATP. The phosphopeptide was then eluted from the paper and subjected to acid hydrolysis followed by high voltage paper electrophoresis, all as described previously in Methods, Sections 6, 7 and 8.

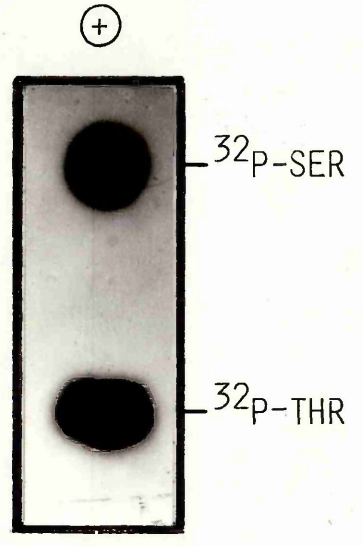
(A) Peptide TH(18-23), RRSTVA

Separation of [^{32}P]Ser and [^{32}P]Thr are illustrated.

(B) Peptide TH(12-23), RRRRYRRSTVA

The autoradiogram was scanned and the relative proportions of [^{32}P]Ser and [^{32}P]Thr are illustrated. The number of counts of radioactivity incorporated are also indicated.

A



Peptide: TH (18-23) RRSTVA

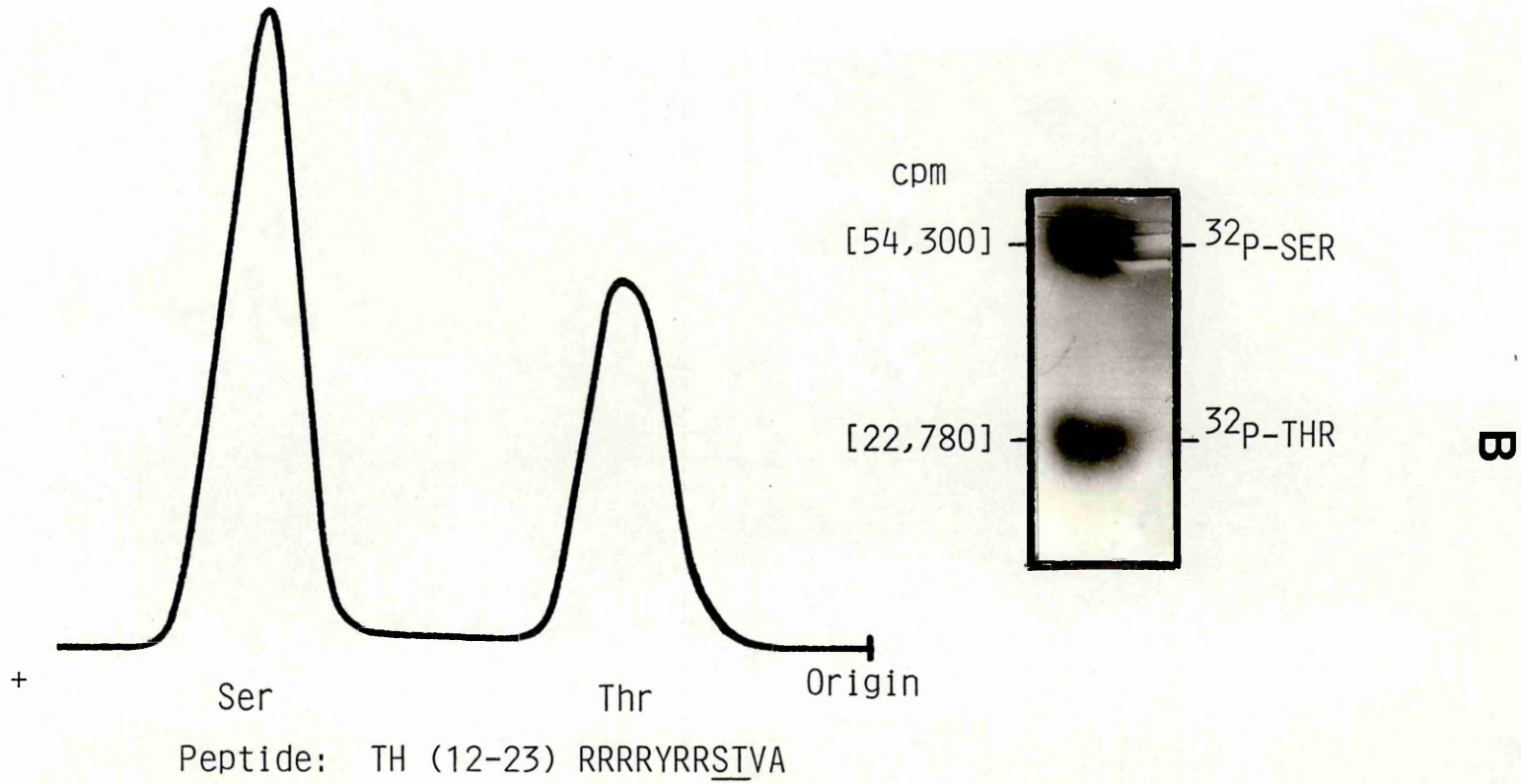

cpm
[54,300]
[22,780]
32P-SER
32P-THR
B
+
Ser
Thr
Origin
Peptide: TH (12-23) RRRRYRRSTVA

Fig. 2.16 The Effect of Proline Residues on both N-terminal and C-terminal Sides of the Phosphate Acceptor

Purified preparations of PRV PK were assayed with peptide substrates Val^{36} I-1(32-37), RRPTVA; I-1(32-37), RRPTPA; and I-1(30-37), RRRRPTPA, at concentrations in the range of 0.1-2.5mM, as described in Methods, Section 5.1B.

Incubation was for 15 min each case.

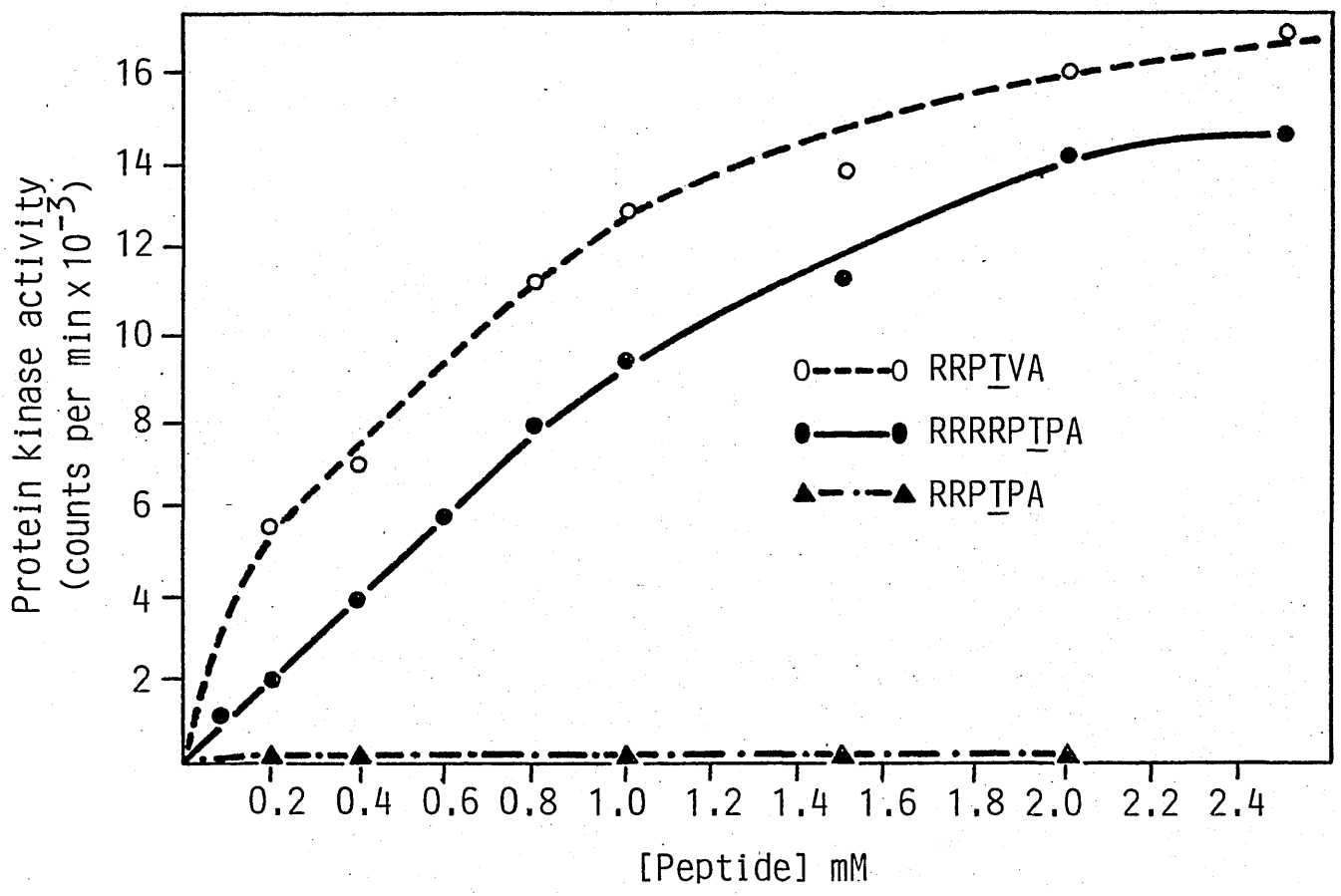

Protein kinase activity
(counts per min x 10^{-3})
16
14
12
10
8
6
4
2
0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4
[Peptide] mM
RRPTVA
RRRRPTPA
RRPTPA

Table 2.3 Rates of Phosphorylation of Synthetic Peptides by Pseudorabies Virus Protein Kinase (PRV PK)

Peptides were at a concentration of 1mM and the initial rates of their phosphorylation were determined by incubation for 10 min at 30°C with 0.05 units of PRV PK, as described in Methods, Section 5.1B.

Ser and Thr residues are underlined and basic residues are indicated in bold type. The standard one-letter code for amino acids if employed, with O representing ornithine.

Peptide	Structure	Rate of phosphorylation (% of that with TH(1-12))
TH(1-12)	PRRRRRSSRPVR	100
$Orn^{2-6,9,12}$TH(1-12)	POOOOOSSOPVO	<1
TH(7-12)	SSRPVR	<1
TH(18-23)	RRSTVA	26
$Lys^{18}Lys^{19}$TH(18-23)	KKSTVA	5
$Orn^{18}Lys^{19}$TH(18-23)	OKSTVA	<1
$Orn^{18,19}$TH(18-23)	OOSTVA	<1
TH(12-23)	RRRRYRRSTVA	26
TH(18-33)	RRSTVARRRRRVRRRR	<1
Glu^{22}TH(18-23)	RRSTEA	<1
PK(8-13)	RRASVA	25
Thr^{11}PK(8-13)	RRATVA	7
Val^{36}I-1(32-37)	RRPTVA	33
Val^{36}I-1(33-37)	RPTVA	<1
Ala^{34}I-1(32-37)	RRATPA	<1
I-1(32-37)	RRPTPA	<1
I-1(30-37)	RRRRPTPA	24
$Val^{39}Ala^{40}$I-1(32-40)	RRPTPATVA	<1
Ser^{35}I-1(32-37)	RRPSPA	<1
GA(52-65)	RRRRYGSRRRRRRY	18
$Orn^{52-55,59-64}$GA(52-65)	OOOOYGSOOOOOOY	<1
GA(57-65)	GSRRRRRRY	<1

Table 2.4 Kinetic Constants for Pseudorabies Virus Protein Kinase (PRV PK) with Synthetic Peptide Substrates

The concentrations of peptides were varied in the appropriate ranges these being from 2-80μM to 0.1-4mM, 0.05 units of PRV PK being used in each assay.

Apparent Km and Vmax values were calculated from double reciprocal plots fitted to the Michaelis-Menton Equation and are tabulated in the figure.

The structures of the peptides are indicated by the standard one-letter code.

Substrate	Structure	Apparent K_m (μM)	V_{max} (pmol.min^{-1})	$\frac{V_{max}.10^{-3}}{K_m}$
Salmine	[FIG. 2.12]	8	16.7	2090
TH(1-12)	PRRRRRSSRPVR	20	41.8	2090
TH(12-23)	RRRRYRRSTVA	28	14.3	511
GA(52-65)	RRRRYGSRRRRRRY	25	11.5	460
Val^{36}I-1(32-37)	RRPIVA	470	6.8	14.3
TH(18-23)	RRSTVA	900	14.3	14.9
Thr^{11}PK(8-13)	RRATVA	1100	7.4	6.6
PK(8-13)	RRASVA	1340	41.8	31.2

Table 2.5 Phosphorylation of Seryl and Threonyl Residues of Thynnine Peptides by PRV PK and Cyclic AMP-dependent Protein Kinase

The peptides were used at a concentration of 1mM and the reaction time was 60 min. 0.05 units of enzyme were used in each case and the reaction mixture was supplemented by a further 0.05 units after 30 min.

The sequences of the peptides TH(12-23) and TH(18-23) are RRRRYRRSTVA and RRSTVA, respectively.

After the phosphorylation of each peptide by the appropriate enzyme, the mixture was hydrolysed, phosphoserine and phosphothreonine were separated by high-voltage paper electrophoresis on Whatman 3MM filter paper. The areas corresponding to [^{32}P]Ser and [^{32}P]Thr were cut out and their radioactivity determined by scintillation spectrometry, all as described in Methods, Sections 7 and 8.

Peptide	Protein kinase	32p (counts/min) incorporated into	
		Ser^{20}	Thr^{21}
TH(12-23)	PRV PK	54,300	22,780
TH(18-23)	PRV PK	23,500	27,450
TH(18-23)	cAMPdPK	26,840	3,300

Table 2.6 Rates of Phosphorylation of Synthetic Peptides with Herpes Simplex Virus Type-1 Protein Kinase (HSV-1 PK)

Peptides were at a concentration of 1mM and the initial rates of their phosphorylation were determined by incubation of the peptide with 0.05 units of enzyme for 30 min at 30°C (Methods, Section 5.1B). Ser and Thr residues are underlined and basic residues are indicated in bold type. The standard one-letter code for amino acids is used.

Peptide	Structure	Rate of phosphorylation (% of that with TH(1-12))
TH(1-12)	PRRRRRSSRPVR	100
TH(12-23)	RRRRYRRSTVA	65
TH(18-33)	RRSTVARRRRRVRRRR	<1
GA(52-65)	RRRRYGSRRRRRRY	37
TH(18-23)	RRSTVA	28
Glu^{22}TH(18-23)	RRSTEA	<1
PK(8-13)	RRASVA	56
Thr^{11}PK(8-13)	RRATVA	28
Val^{36}I-1(32-37)	RRPTVA	43

Table 2.7 Comparison of the Kinetic Constants of PRV PK and of Cyclic AMP-Dependent Protein Kinase for Phosphorylation of Synthetic Peptide Substrates

The concentration of peptides was either in the range of 2-80μM or 0.1-4mM. Assays were performed as described in Methods, Section 5.1B, the duration of each incubation at 30°C was 10 min. Apparent Km and Vmax values were calculated from double reciprocal plots fitted to the Michaelis-Menton and are tabulated in the figure. 0.05 units of the appropriate enzyme were used in each case.

The structure of the peptides are indicated by the standard one-letter code for amino acids.

Peptide	Structure	PRV PK		cAMPdPK	
		Apparent Km (μM)	V_{max} (pmol.min^{-1})	Apparent Km (μM)	V_{max} (pmol.min^{-1})
PK(8-13)	RRASVA	1,340	41.8	17	13.2
TH(18-23)	RRSTVA	900	14.3	800	15.3
TH(12-23)	RRRRYRRSTVA	28	14.3	240	23.5
TH(1-12)	PRRRRRSSRPVR	20	41.8	385	19.7

Fig. 2.17 The Phosphorylation in vivo of Proteins of Vero cells infected with HSV-1(F) and Recombinant virus R7041

$25cm^2$ plaque dishes of Vero cells were either mock-infected or infected with HSV-1(F) or R7041 at a multiplicity of 10-15 p.f.u./cell. 13h after the infection the cells were washed with 199V medium with the phosphate content reduced to 1/10th its usual concentration (Materials, Section 3.3B); Methods Section 1.3). 1h later the cells were labelled with 250µCi $^{32}P_i$, and harvested 3h later (as described in Methods Section 2). Approximately 25µg of protein from the cytoplasm was subjected to electrophoresis under denaturing conditions on an 11.5% polyacrylamide gel cross-linked with DATD.

Replicate dishes were prepared and cells were labelled with [^{35}S]Met 3h after infection for 1-2h with 100-200µCi [^{35}S]Met, and then harvested. Cytoplasmic and nuclear extracts were prepared and loaded on the same gel as markers.

lane 1 [^{35}S]Met-labelled cytoplasmic proteins from cells infected with HSV-1(F).

lane 2 [^{35}S]Met-labelled cytoplasmic proteins from cells infected with R7041.

lane 3 [^{35}S]Met-labelled nuclear proteins from cells infected with HSV-1(F).

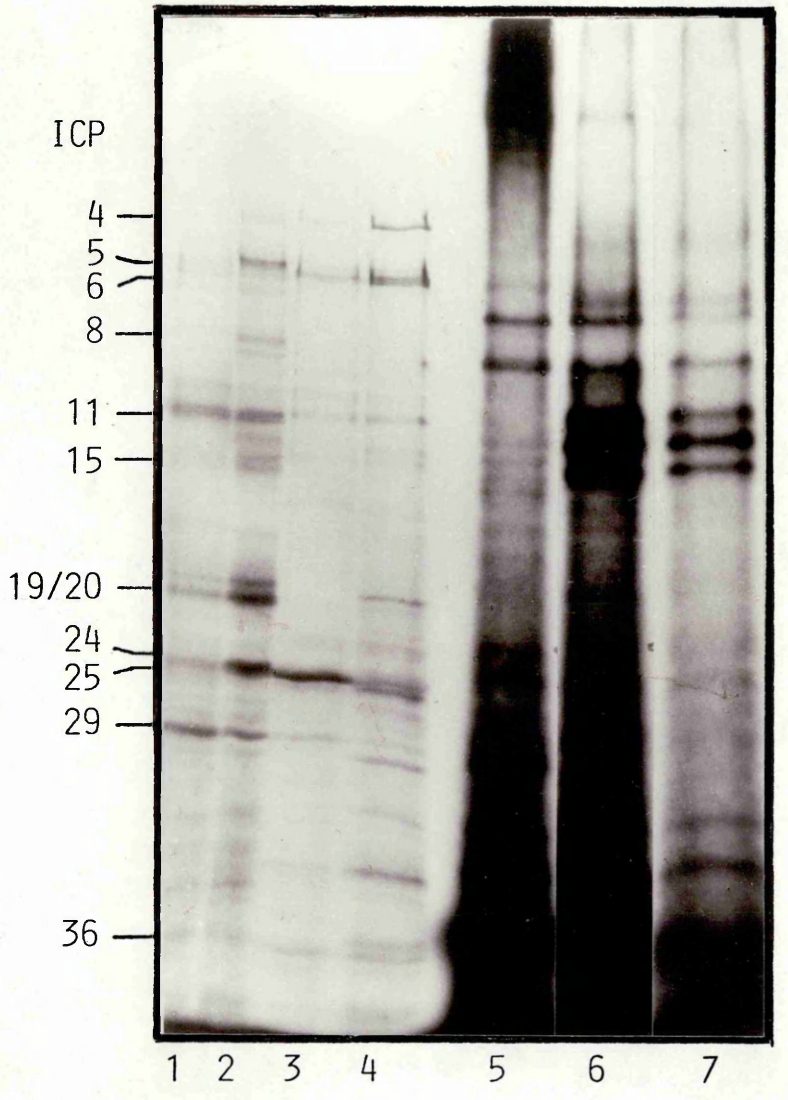
lane 4 [^{35}S]Met-labelled nuclear proteins from cells infected with R7041.

lane 5 [^{32}P]labelled cytoplasmic proteins from mock-infected cells.

lane 6 {^{32}P]labelled cytoplasmic proteins from cells infected with HSV-1(F).

lane 7 [^{32}P]labelled cytoplasmic proteins from cells infected with R7041.

Known herpesvirus proteins are indicated on the left hand side of the gel (ICP).


ICP
4
5
6
8
11
15
19/20
24
25
29
36
1
2
3
4
5
6
7

DISCUSSION

1 THE GENETIC ORIGIN OF THE PROTEIN KINASE INDUCED IN CELLS INFECTED WITH HERPESVIRUSES

The results presented in Section I clearly establish that the new protein kinase present in cells infected with HSV-1 is specified by the virus, and encoded by DNA sequences within the domain of the open reading frame, US3. This conclusion is drawn from consideration of several lines of evidence, both indirect and direct.

At the outset, it was shown that the appearance of the protein kinase in cells infected with either HSV-1 or PRV both required and correlated with the expression of the viral genome (Figs. 1.2, 1.3, 1.8 and 1.9). However, the appearance in infected cells of new proteins dependent on viral gene expression does not necessarily indicate that the proteins are viral. Cellular proteins are also found among those newly detectable after viral infection, and some of these proteins are dependent for their induction on the expression of the viral genome (Latchman and Preston, 1987). These include stress proteins (Notarianni and Preston, 1982), the promoters of which appear to be activated in a similar manner to those of α-genes; and the proteins induced by interferon (Friedman et al., 1984), the production of the latter probably requiring the generation of overlapping viral RNA transcripts.

Comparison of the SDS-polyacrylamide gel mobilities of similar proteins from cells infected with different strains of HSV has frequently been used to provide an indication whether these are of viral origin. To employ this approach for PRV PK and HSV-1 PK, homogeneous preparations of both enzymes would have been needed.

Initially, therefore, a modification of this strategy was used, involving comparison of the chromatographic behaviour of the enzyme activities induced in cells infected with these different herpesviruses. The results of these experiments showed that the protein kinase activity induced in cells infected with HSV-1 differed in chromatographic behaviour from that induced in cells infected with PRV. Such differences were found for chromatography on DEAE-cellulose (DE-52), DEAE-Sephacel, Mono Q and Sephacryl S-200 (Figs. 1.1, 1.6 and 1.7), and were consistent with the enzymes being encoded by the viral genome.

Nevertheless, because of their indirect nature, the experiments just mentioned were still open to possible objections. For example, the different chromatographic behaviour might have been due to interaction of a single host protein kinase with different viral proteins. Alternatively different patterns of specific intracellular partial proteolysis might have generated the two apparently different enzyme activities from a single inactive cellular enzyme. A strong interaction with a viral protein, resisting dissociation at 0.5M KCl, does not seem very likely, but there has been one report suggesting the existence of a possible herpesvirus protease (Dierich et al., 1979).

The publication of the sequence of gene US3 of HSV-1 (McGeoch et al., 1985), and the identification in it of motifs conserved in eukaryotic protein kinases (Fig. I7) (McGeoch and Davison, 1986), allowed for more direct approaches to the question of the genomic origin of this enzyme. In one of these approaches cells were infected with a mutant of HSV-1(F) (R7041) containing a deletion in the US3 gene, and no protein kinase activity was detectable (Fig. 1.12 and Fig. 1.13).

Moreover the protein kinase activity was regained when cells were infected with strains (R7050 and R7051) in which the US3 gene was restored (Fig. 1.12B and Fig. 1.13.).

Although this provided very strong evidence in favour of the protein kinase being the product of the HSV-1 US3 gene, there remained the possibility that the protein kinase was not the product of the US3 gene, but a cellular enzyme phosphorylated and activated by the latter. Such a cascade type of activation is exemplified in many instances in intermediary metabolism, the best characterized being that initiated by activation of the cyclic AMP-dependent protein kinase (e.g. Krebs, 1985). One genetic means of rejecting this possibility of a cascade system is by using a temperature-sensitive mutant of the gene in question whereby one can determine whether the putative gene product is also temperature-sensitive. This approach was used successfully in the case of the tyrosine kinase encoded by the src gene of Rous sarcoma virus (Collett and Erikson, 1978). In the present work, a different approach was used. The fact that the antiserum to a synthetic oligopeptide corresponding to the carboxy-terminal eight amino acids of the US3 gene-product cross-reacted with a component in purified preparations of HSV-1 PK (Fig. 1.16) provided the necessary direct correlation between gene and protein, and effectively eliminated the cascade possibility.

If the results with the antiserum were considered in isolation one could perhaps argue for the possibility of a chance cross-reaction, or criticise the lack of homogeneity of the most purified preparation of HSV-1 (even though the autophosphorylation results are very persuasive). However, when the results of both the genetic deletion and immunochemical approaches are considered together, the objections to either alone lose their force. Thus, although a correlation of

the amino acid sequence of the purified protein with that predicted from the nucleotide sequences is required for ultimate proof, the evidence that the protein kinases are virally coded would seem to be as strong as that for almost any of the other enzymes thought to be encoded by HSV-1. If this conclusion is accepted, a corollary of these results is that the US3 gene of HSV-1 does indeed encode a protein kinase. This point may appear trivial in view of the highly conserved motifs that the predicted protein from the US3 gene sequence shares with other protein kinases. However, the predicted product of the US3 gene of HSV-1 and of the corresponding gene of VZV deviate from the protein kinase consensus sequences in two major respects. These both involve the important nucleotide binding site of the enzyme, which in all protein kinases previously identified contains the hexapeptide sequence GlyXaaGlyXaaXaaYaa (where Yaa is Gly or Ala or Ser) followed 11-28 amino acids later by AlaXaaLys (Hunter and Cooper, 1985). In the predicted US3 gene product however, the corresponding sequences are ThrXaaGlyXaaXaaGly and IleXaaGly (McGeoch et al., 1985).

It is intriguing that such otherwise highly conserved features of protein kinases are not found in the viral enzymes, and one wonders whether this has any functional significance. It would be interesting to use site-directed mutagenesis to convert the sequence of the HSV-1 PK to that of the consensus and see what effect this had on its catalytic activity.

2 IMPLICATIONS OF THE PURIFICATION OF THE VIRAL PROTEIN KINASES

The complete characterisation of any enzyme requires its purification to homogeneity. In the case of PRV PK this has been achieved (Results, Section II, 1). The evidence that PRV PK has been completely purified is as follows:

A single 38kDa protein is the only band visible by staining, with Coomassie Blue or silver, a polyacrylamide gel after electrophoresis under denaturing conditions. That this 38kDa band is the protein kinase is argued by two independent results. The specific activity is in the same range (1000-5000 units/mg) as other homogeneous protein kinases, for example protein kinase C (Parker et al., 1984) and cyclic AMP-dependent protein kinase (Reimann and Beham, 1983). One imagines that this similarity in specific activity reflects a similar catalytic mechanism with an upper limit on its efficiency. The single 38kDa protein observed under denaturing conditions was also the sole species phosphorylated when the purified enzyme preparation was incubated with [γ^{32}P]ATP, a result indicative of autophosphorylation of a pure protein kinase.

The purification of PRV PK has already shown its value. It has allowed unequivocal studies on catalytic and structural properties (see below) as well as important comparisons with other homogeneous protein kinases, including the partially pure HSV-1 PK. For the future the purification has opened other avenues. The fact that PRV PK can be obtained in quantities of 40-50µg per preparation should allow microsequencing to be performed. This will ultimately allow comparison of partial amino acid sequences of tryptic peptides of PRV PK with the amino acid sequence predicted by the nucleotide sequence of the PRV PK gene, when this is known. Microsequencing will also be essential to identify the phosphopeptides, and thus the

amino acids which are phosphorylated during the autophosphorylation reaction. This is of potential importance as it has been shown that some tyrosine protein kinases can regulate their own activities by autophosphorylation (Hunter, 1987).

The purification of HSV-1 PK was not complete despite the adoption of a similar strategy to that employed successfully with PRV PK. The purification was made more difficult because lower initial yields of HSV-1 PK were routinely obtained, as compared with PRV PK. This may be partly due to the lower initial input multiplicity of infection (10 p.f.u./cell) for HSV-1, in contrast to the 20 p.f.u./cell used in the PRV infection (Fig. 1.3). This seems unlikely to be the complete explanation. The HSV-1 PK may simply be more labile during the preparation procedure than PRV PK; or the low yield of HSV-1 PK may reflect a lower expression of the HSV-1 US3 gene. It is not inconceivable that this viral protein kinase need only be present in small quantities in the infected cell, and that the PRV PK is produced in excess of requirement. Such a situation would not be unprecedented as, by definition, the non-rate-limiting cellular enzymes of a metabolic pathway are present in excess. Thus a lower extent of expression from a weaker promoter in HSV-1 might still supply an adequate amount of enzyme. Eventually it should be possible to test the strengths of the PRV and HSV-1 US3 promoters by the construction of chimeric genes.

The best preparation of partially purified HSV-1 PK had a specific activity of 200 units/mg, and two major components of apparent molecular weights 68,000 and 61,000 were visible on an SDS polyacrylamide gel stained with Coomassie Brilliant Blue. As argued in the Results, Section II, 1, one of these components probably corresponds to HSV-1 PK, and the enzyme is thus probably almost pure.

It was essential to purify HSV-1 PK substantially from the cytoplasmic extract in order to correlate the intensity of the signal when the protein was probed with anti-US3 serum, with the purification of the protein kinase activity. The degree of purity achieved proved adequate for this purpose, and, furthermore, allowed certain conclusions regarding the structural and functional properties of the enzyme to be drawn with a fair degree of confidence.

The clearest conclusion regarding the structure of the viral protein kinases can be drawn for PRV PK. The discrepancy between that Mr obtained for PRV PK under denaturing conditions and the native Mr suggests a multimeric structure. The Mr value for the homogeneous enzyme obtained from SDS polyacrylamide gels was 38,000, this representing the Mr under denaturing conditions. Several techniques were used to determine the Mr of the enzyme under native conditions. Sucrose density gradient centrifugation and chromatography on three types of gel filtration chromedia, TSK 3000, G150 (Katan et al, 1985), and Superose 12 (Fig. 2.5), all gave values of approximately 68,000. Sephacryl S-200 gave a somewhat larger value of 90,000 (Fig. 1.7); but this anomalous value was rejected. As the native Mr of 68,000 is quite close to twice the denatured molecular weight (i.e. 76,000) it is most likely that the native form of PRV PK is a homodimer.

The Mr obtained for HSV-1 PK from immunological identification after SDS polyacrylamide gel electrophoresis was 68,000. The native Mr was also investigated using two types of gel exclusion chromatography. The first was Sephacryl S-200, which yielded a value of 200,000 (Fig. 1.7), and the second was Superose 6, which gave an estimation of Mr

in the range between that of aldolase (158,000) and catalase (240,000) (Fig. 2.11). The poor resolution of this column for proteins with an Mr above 100,000 meant that it was not possible to determine an accurate value for HSV-1 PK. The Mr obtained from chromatography on Sephacryl S-200, by corollary with that for PRV PK, is probably too high. A dimer of HSV-1 PK would be expected to have a native Mr of 136,000, whereas the anticipated value for a trimer would be 204,000. In view of the apparent dimeric nature of PRV PK, and the fact that homotrimeric structures are rare because this symmetry is unfavourable, it is tentatively concluded that HSV-1 PK is probably also a homodimer.

The Mr of 68,000 for the US3 protein is considerably higher than the 53,000 predicted from the DNA sequence. However discrepancies of this order of magnitude have frequently been found between estimates of molecular weights of HSV proteins from polyacrylamide gels and the molecular weights indicated by the nucleotide sequence (Perry et al, 1986; McGeoch et al, 1985).

PRV PK would appear to be considerably smaller than HSV-1 PK, as judged by a comparison of the Mr values (38,000 v 68,000) obtained by SDS polyacrylamide gel electrophoresis. This may reflect difficulty in relating apparent Mr on SDS gel electrophoresis to actual size, as indicated above. The most likely cause of this discrepancy in the present instance is charged residues on the proteins; and the magnitude of this effect could differ for PRV PK and HSV-1 PK. Nevertheless it is likely that PRV PK is considerably smaller than HSV-1 PK. There is no reason to regard this as unlikely if the VZV PK is considered. This is predicted to be 44kDa in size, some 17%

smaller than the 53kDa HSV-1 PK. When both the VZV US2 and HSV-1 US3 gene sequences are analysed, the C terminal (catalytic) region in each is approximately the same size: in HSV-1 it contains 287 amino acids, corresponding to approximately 32kDa of protein, and in VZV it contains 297 amino acids corresponding to approximately 33kDa of protein. On the other hand the N-terminal regions differ substantially: in HSV-1 US3 it contains 194 amino acids (approximately 22kDa of protein), whereas in VZV it is considerably smaller, containing only 96 amino acids (approximately 10kDa of protein) (Fig. I6). Hence PRV PK might have a catalytic domain of a similar size to HSV-1 PK, but only a very short N-terminal sequence, perhaps even shorter than that in VZV PK. Such a short N-terminal domain has a precedent in the catalytic subunit of the cyclic AMP-dependent protein kinase. This protein kinase is also quite small (approximately 40.5kDa) and has only 45 amino acids in its N-terminal domain.

3 THE SUBSTRATE-SPECIFICITY OF THE VIRAL PROTEIN KINASES

The site-specificities of several protein kinases have been shown by analysis of residues phosphorylated *in vivo* to depend largely on the amino acid sequence around the target residue. Utilization of synthetic peptides mimicking phosphorylation sites made it possible to define the substrate specificity of several protein kinases *in vitro*. Examples of kinases, the substrate specificity of which has been investigated using this approach, are shown in Table I2.

This strategy was adopted for PRV PK and HSV-1 PK. At the outset there were three main objectives in performing these studies. The first was to allow further comparison of these enzymes with other known protein kinases; the second was to provide a basis from which the amino acid sequences of herpesvirus proteins might be examined to identify potential substrates for these enzymes; and the third was to provide a basis from which to design a specific inhibitor of these protein kinases.

The results presented in Results, Section II 2, show that the herpesvirus protein kinases can catalyse the phosphorylation of both seryl and threonyl residues in peptides that contain several arginyl residues on the amino-terminal side of the target residue. At least two arginine residues are required, and the best substrates examined contained four to six such residues.

Although the results described in Section II 2, do not completely define the determinants of site-specificity for the viral protein kinases they are useful in several different ways. Thus they demonstrated clearly that HSV-1 PK and PRV PK are catalytically similar to one another, but distinct from other known protein

kinases. For example these enzymes differ from protein kinase C in being unable to phosphorylate peptides in which multiple arginine residues are on the carboxy-terminal side of the target residue, or to phosphorylate peptides in which the arginyl residues are replaced by ornithyl residues. Although the viral kinases and the cyclic AMP-dependent protein kinases have several peptide substrates in common, their relative preferences for these (as indicated by Km values) were found to be very different (Results, Section II 2, Table 2.7). The information now available (Table I2) regarding the site specificity of protein kinases clearly distinguishes the viral kinases from other protein kinases besides the cyclic AMP-dependent protein kinases and protein kinase C. These include the cyclic GMP-dependent protein kinase (Glass et al., 1982), myosin light chain kinase and histone H4 kinase (Eckols et al., 1983). This in turn suggests that the function of the viral protein kinases is different from these other protein kinases.

A second way in which the information regarding substrate-specificity is useful is in relation to the prediction of possible substrates for these enzymes. Before discussing this it should be stressed, however, that the total three-dimensional structure of the true physiological substrate could alter the minimum requirement for phosphorylation of the target serine or threonine. Thus proteins capable of being phosphorylated by the viral protein kinases in vitro may not display all the necessary determinants outlined in Results, Section II 2. This point is illustrated by a consideration of protein kinase C (Woodgett et al., 1986), which can phosphorylate substrates which do not have long runs of basic residues on the N and C terminal sides of the target residues, even though the best peptide substrates

for this enzyme in vitro have these characteristics. In the case of the viral protein kinases, although peptides with long runs of arginine residues on the N-terminal side of the target are the best substrates for the enzymes in vitro it is possible that the physiological substrate of the enzymes contains fewer arginine residues on the N-terminal side of the target, the three-dimensional configuration providing additional determinants for good interaction with the enzyme.

Bearing these reservations in mind an attempt will be made to use the results of the studies on site-specificity to assess possible substrates. The fact that HSV-1 PK is encoded by open reading frame US3 (Results, Section I) raises the possibility that its substrate might also be encoded in the Us region of the herpesvirus genome, as functionally related proteins have been found to be clustered at several locations of the genome of HSV-1 (eg the proteins for DNA synthesis (Quinn and McGeoch, 1985; Gibson et al., 1984)).

As the whole sequence of the HSV-1 Us region is known (McGeoch et al., 1985) it was possible to scan the amino acid sequences of the predicted gene products of the Us region to determine possible phosphorylation sites for HSV-1 PK. Two potentially favourable sites for HSV-1 PK were identified in genes US7 and US9 of HSV-1 (Table D1). If these sites are functionally important one would expect them to be conserved in VZV, which also has a protein kinase gene at a corresponding region of its genome. The genes in VZV corresponding to US7 and US9 of HSV-1 are US3 and US1, respectively; but when these were examined it was found that the potentially favourable phosphorylation sites had not been conserved (Table D1).

It is possible that the multiple arginine residues on the N-terminal side of possible target residues in the predicted products of the US7 and US9 genes of HSV-1 arose fortuitously because of the very high (68%) GC content of the DNA of this virus; and the fact that arginyl residues are encoded by the CGN codon family. This feature is not shared by VZV (46% GC), the genome of which should therefore be more amenable to analysis for potential viral substrates for the protein kinases.

As analysis of the Us region of HSV-1 and VZV did not reveal a conserved putative phosphorylation site for the viral protein kinases, the possibility of another viral substrate was examined, particular attention being paid to known phosphoproteins. As the whole of the HSV-1 genome sequence was not yet available the published VZV sequence (Davison and Scott, 1986) was analysed, this having the additional advantage of a lower GC content as already mentioned above. Table D2 shows the best potential sites in the genome of VZV for phosphorylation by the α-herpesvirus protein kinase. The equivalent HSV-1 gene, if any, was identified by reference to Table 1 of Davison and Scott (1986). It can be seen that only for gene 4 is there an equivalent in HSV-1 α27. Inspection of the sequence of α27 (Frame and McGeoch, personal communication) revealed partial conservation of this potential phosphorylation site, and, as α27 is phosphorylated in vivo (Pereira et al., 1977; Hay and Hay, 1980) this protein might be regarded as a possible substrate. However as PRV has only a single immediate-early gene, the equivalent of α4, it is doubtful whether a functional homologue of α27 exists in PRV, excluding it from being the

substrate of PRV PK. Other VZV genes predicted to encode the proteins, the HSV equivalent of which are known phosphoproteins, did not contain such sites. These included the large subunit of the ribonucleotide reductase (gene 19), DNA polymerase (gene 28), the major DNA binding protein (gene 29), immediate-early protein α4 (gene 67, 71) and the exonuclease (gene 48). It is likely that cellular protein kinases, such as the ubiquitous casein kinase II, are responsible for the phosphorylation of these viral proteins (Stevely et al., 1985; Jakubowicz and Leader, 1987b). Indeed typical casein kinase II sites have been found with specific acidic determinants around possible phosphorylation sites of many of these viral proteins. Examples are: HSV-1 US9, α0, α4, α22 and ICP 39.3. Putative sites for casein kinase I, another cellular protein kinase, have been tentatively identified in ICP31, the major 65kDa tegument protein, DNA polymerase and ICP8.

It could be argued that analysis of possible phosphorylation sites by comparison between the proteins of HSV-1 and VZV is invalid as the protein kinases of the two viruses might have different substrate specificites. Although it is not yet possible to exclude this experimentally, the unique nature of the protein kinase gene at a similar position in the genomes of these viruses makes this as unlikely as the putative thymidine kinase and ribonucleotide reductase genes in VZV having different substrates from the corresponding genes in HSV-1.

Even if HSV-1 PK is responsible for catalysing the phosphorylation of sites on some of the known indispensible viral phosphoproteins, this phosphorylation is obviously not necessary for their function, as the mutant of HSV-1(F) with the protein kinase gene deleted is viable in

tissue culture cells. Thus such proteins could not be physiological important targets of the enzyme. In fact initial experiments (Fig. 2.17) have failed to detect any major differences between the ^{32}P-labelling in vivo of viral phosphoproteins of cells infected with wild type HSV-1(F) and those infected with R7041. Therefore the possibility of a cellular substrate for this enzyme now deserves strong consideration.

TABLE D1

ANALYSIS OF THE Us REGION OF HSV-1 FOR POTENTIAL PHOSPHORYLATION SITES CONSERVED IN VZV

HSV-1 gene	Potential phosphorylation sequence	Equivalent VZV gene	Potential phosphorylation sequence (if present)
US1	KRKRPSRPLSS RRSVRQAQ RRSSARWT	RS2 (63)	-
US2	-	-	-
US3	RRRSRDEIG RYRSRAA	US2 (66)	RRSGTHP
US4	RRGRRTHPSVR	-	-
US5	-	-	-
US6	-	-	-
US7	RRYRRSRRPIY KSRRRSSR SRRRSSRTP	US3 (67)	-
US8	RRVSVGEDV	US4 (68)	-
US9	RRRRRTRCVGM	US1 (65)	-
US10	-	RS3 (64)	
US11	-	-	-
US12	-	-	-

References: McGeoch et al., 1985
Davison and Scott, 1986
McGeoch and Davison, 1986

TABLE D2

ANALYSIS OF THE VZV GENOME FOR POTENTIAL PHOSPHORYLATION SITES FOR THE VIRAL PROTEIN KINASES

VZV gene	Potential phosphorylation site	HSV-1 gene	Potential phosphorylation site
4	RRSSRSYNTQ RRRRPTTP	α27	RRRAPRT
9	VRRKTTPSY	-	-
33	RRRRVSP	-	-
38	RRRKKS	-	-
52	RRKYKSTF	-	-

References: Davison and Scott, 1986

McGeoch et al., 1986

McGeoch and Frame (personal communication)

4 POSSIBLE FUNCTIONS OF THE PROTEIN KINASE CODED BY α-HERPESVIRUS

The absence of a suitably conserved potential target for the viral protein kinases in the VZV and HSV genomes suggest a host protein as the most likely substrate. This is also suggested by the viability of the deletion mutant of HSV-1(F) (R7041). Clearly the phosphorylation cannot be necessary for essential viral functions, such as the adsorption of the virus to the surface of the cell, the regulation of the temporal expression of different classes of viral genes, the transport of proteins to the nucleus or the assembly of the virion. If this is true, the viral proteins involved in these processes are likewise excluded as physiologically important substrates.

It is also unlikely that HSV-1 PK plays a role in the latency and reactivation of the virus. This conclusion emerges from some preliminary experiments performed in the laboratory of B. Roizman. The recombinant virus R7041, lacking a functional US3 gene, is still able to establish latency in experimental animals (B. Meigner, R. Longnecker and B. Roizman, personal communication).

Although potential host functions of the viral protein kinase can only be guessed at present, it seems worth discussing a number of general possibilities. One possibility is that the viral protein kinase may function in the shut-off of host cell protein synthesis. Phosphorylation is known to inhibit protein synthesis in certain circumstances. For example, eIF-2 can be phosphorylated by a highly specific protein kinase, preventing it recycling after a round of initiation (Pain, 1986). However the viral protein kinase cannot phosphorylate eIF-2 *in vitro* (Katan *et al.*, 1985), and it is difficult to see how phosphorylation of any of the known initiation

factors could cause inhibition of host cell protein synthesis, while maintaining a high level of viral protein synthesis. Indeed studies of the inhibition of host-cell protein synthesis by HSV-1 indicate that the early phase of inhibition does not require viral gene expression (Fenwick and Clark, 1982) and that the viral genes responsible for the late phase of inhibition do not map in the Us region (Daksis and Chan, 1987).

Another possible role for the protein might be in transcription. Activation of viral RNA synthesis would clearly be an indispensible function, and the viability of the deletion mutant R7041 effectively eliminates this from consideration. However the possibility that the protein kinase may inhibit host cell RNA synthesis or cause degradation of host mRNAs remains to be tested. In the context of current knowledge, the most attractive possibility at present is that the enzyme plays a role in viral DNA replication. This could either be by facilitating viral DNA synthesis or by preventing host cell DNA synthesis. It is striking that most of the other enzymes encoded by α-herpesviruses are involved in DNA replication. Examples are: Alkaline exonuclease, DNA polymerase, DNA binding protein, ribonucleotide reductase, thymidine kinase and dUTPase. Most of these enzymes perform functions very similar to host enzymes, and it is thus conceivable that the viral protein kinase has a cellular homologue, as yet undiscovered. It is pertinent in this regard to point out that several yeast genes that have been shown to encode protein kinases control progression through the cell cycle (Patterson et al., 1986; Reed el at., 1985). It is tempting to speculate that the viral protein kinase may function in a similar manner to one of these host enzymes, perhaps preventing the entry of the host cell

into S phase, thus preventing competition of the host cell with the virus for precursors of DNA.

The fact that these considerations are still speculative should not detract from the results presented here. The HSV-1 protein kinase is the first and only eukaryotic protein kinase shown to be an authentic product of a viral gene. The protein kinases of acute transforming retroviruses such as Rous sarcoma virus, are of course, the products of recently transduced cellular genes. It is obvious that the protein kinase gene of α-herpesviruses would not have been conserved had it not conveyed a selective advantage to the virus that contained it. It is reasonable to expect that the use of mutant viruses lacking a functional protein kinase gene will eventually allow us to discover the nature of this selective advantage and to identify the substrate and function of this intriguing viral enzyme.

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