HERPES SIMPLEX VIRUS DNA-BINDING PROTEINS :

STUDIES ON 21K AND THE 'a' SEQUENCE

by

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SUMMARY

This project has involved the investigation of the polypeptide products of gene U_S11, and their involvement in DNA-protein interactions occurring within the 'a' sequence of HSV-1. The work stems from the observation of Dalziel and Marsden (1984), that polypeptides of apparent molecular weight 21,000 and 22,000 (21K, 22K) specifically interacted with the HSV-1 'a' sequence - a sequence involved in a number of functions, including circularisation of the virus genome, inversion between the L and S segments of the genome, and cleavage and packaging of viral DNA.

To characterise the 21K/22K polypeptides, antisera were raised against synthetic oligopeptides corresponding to five different regions of the predicted U_S11 gene product, since earlier evidence suggested that the 21K and 22K polypeptides were the products of this gene. All five oligopeptides were immunogenic, and four induced antisera which appeared to recognise virus-specific polypeptides. The polypeptides recognised included species with apparent molecular weights of 22K, 21K, 17.5K, 15K, 14K and 11K, and these were shown, by tryptic peptide analysis, to share common amino acid sequences. Timecourse experiments, and the use of phosphonoacetic acid, an inhibitor of viral DNA replication, established that the 21K and 22K polypeptides are true late gene products, and immunoblotting of proteins eluted from DNA cellulose demonstrated that they are also DNA-binding proteins. Immune electron microscopy studies, however, demonstrated that the U_S11 gene products localise strongly to nucleoli of infected cells - an unexpected location for an 'a' sequence binding protein, since viral DNA is absent

from nucleoli (Rixon et al., 1983).

Attempts to purify the 21K polypeptide, with a view to studying in more detail its interaction with the 'a' sequence, met with little success. Therefore, DNase I footprinting experiments on the HSV-1 'a' sequence were carried out, using crude whole cell extracts. The experiments revealed DNA-protein interactions occurring within two regions of the 'a' sequence - the DR_2 repeat elements and the U_b region. These interactions were specific for virus-infected cell extracts. Within the DR_2 repeat region a repetitive pattern was seen, consisting of an area of protection (spanning seven nucleotides - GGGGAGG), flanked on either side by two nucleotides (AG and GG) at which cleavage was enhanced, and separated by one unaffected nucleotide (C). Within the U_b region there was no apparent protection, however, there was a very marked increase in cleavage between a number of nucleotides. The crude whole cell extracts possessed both a 5'-3' exonuclease and a DNase I-like activity which limited the usefulness of the footprinting assay : it was therefore not possible to determine whether specific cleavage events were generating the enhanced cleavages observed.

Use of an HSV-2 deletion mutant lacking gene U_S11 demonstrated that the products of this gene are not involved in these interactions. Timecourse and drug inhibition experiments suggested that early polypeptides are involved, while experiments involving a temperature sensitive mutant tsK showed that immediate-early polypeptides, with the possible exception of Vmw175, are not sufficient alone to produce the observed interactions.

The HSV-1 and HSV-2 'a' sequences are heterogeneous

and share only limited stretches of homology (Davison and Wilkie, 1981). The most extensive region of homology lies within U_b, and overlaps the region of enhancement. The use of a cloned synthetic oligonucleotide representing this homology region, and an intact HSV-2 'a' sequence, suggested that the U_b homology region is involved in the interactions observed.

Enhancement was only observed on 3' end-labelled DNA fragments. For technical reasons, it was only possible to 3' end-label one strand of the intact HSV-1 'a' sequence close to the U_b region, and one strand (the opposite strand) of the cloned synthetic oligonucleotide representing the U_b homology region. Comparison of the patterns of enhancement observed on these two substrates led to the conclusion that the enhanced cleavages could reflect the 5' boundaries of protein binding sites, revealed by an endogenous 5'-3' exonuclease activity. Two putative U_b-binding sites are proposed, one contained entirely within, and the other overlapping, the U_b homology region. These have the sequence : 5'-CC$^{C}_{G}$AAAAC$^{ACA}_{GGG}$CCCCCC-3'.

NON-STANDARD ABBREVIATIONS

It is assumed that the reader is familiar with the more standard abbreviations, such as DNA, RNA, h, min *et cetera*, and these are therefore not listed below.

BHI	brain heart infusion
BHK	baby hamster kidney
bp	base pairs
BPB	bromophenol blue
BSA	bovine serum albumin
CMV	cytomegalovirus
cpe	cytopathic effect
CT	calf thymus
DAB	bis-diazotised benzidine
DMSO	dimethylsulphoxide
DNase	deoxyribonuclease
DR	direct repeat
DTT	dithiothreitol
E	eluate
EBV	Epstein-Barr virus
Fc	crystalisable fragment (of immunoglobulin)
FPLC	fast protein liquid chromatography
FT	flow-through
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
HPLC	high pressure liquid chromatography
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HVS	herpes virus saimiri
I	input
ICP	infected cell polypeptide (HSV-1)
ICSP	infected cell-specific polypeptide (HSV-2)
IE	immediate early
IgG	immunoglobulin G
IR	internal repeat

MCMV	murine cytomegalovirus
MDB	major DNA-binding protein
MDV	Marek's disease virus
2-ME	2-mercaptoethanol
MI	mock-infected
mol. wt.	molecular weight
NA	non-absorbed
NRS	normal rabbit serum
dNTP	any deoxyribonucleoside triphosphate
NZW	New Zealand White (rabbit)
OD	optical density
ori	origin of DNA replication
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PC	post column
pfu	plaque forming units
pi	post infection
PI	pre-immune
pp.21K	partially-purified 21K polypeptide
PRV	pseudorabies virus
RIA	radioimmunoassay
RID	radial immunodiffusion
RNase	ribonuclease
rr_1	large subunit of ribonucleotide reductase
rr_2	small subunit of ribonucleotide reductase
RT	room temperature
TK	thymidine kinase
TR	terminal repeat
ts	temperature sensitive
U	unique
dUTPase	deoxyuridine-5'-triphosphate nucleotidohydrolase
uv	ultra violet
Vmw	virus-specific polypeptide of apparent molecular weight
VZV	varicella-zoster virus
W	Watt

The three-letter amino acid code

amino acid	three-letter code
alanine	Ala
arginine	Arg
asparagine	Asn
aspartic acid	Asp
cysteine	Cys
glutamine	Gln
glutamic acid	Glu
glycine	Gly
histidine	His
isoleucine	Ile
leucine	Leu
lysine	Lys
methionine	Met
phenylalanine	Phe
proline	Pro
serine	Ser
threonine	Thr
tryptophan	Trp
tyrosine	Tyr
valine	Val
any amino acid	XXX, X

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INTRODUCTION

INTRODUCTION

SECTION I : INTRODUCTORY COMMENTS

Herpes simplex virus (HSV) is interesting not only as a human pathogen but also as a useful model for eukaryotic genetics and molecular biology. For these reasons herpes simplex virology is a rapidly advancing field. The isolation and characterisation of conditional lethal virus mutants has been invaluable in elucidating a number of viral gene functions. More recently, this approach has been augmented by the development of plasmid-based systems for the study of both HSV transcription and DNA replication. In addition, the entire HSV type 1 genome is now almost completely sequenced (Duncan J McGeoch, personal communication) and this should allow a more detailed analysis of individual viral genes. Despite these advances, however, many questions still remain unresolved. For example, little is known about the precise mechanism of viral DNA replication, or the mechanism by which the viral DNA is cleaved and packaged into virus particles.

This project has involved the further characterisation of an HSV type 1-encoded polypeptide known as Vmw21. This polypeptide has the interesting property of being a strong DNA-binding protein which appears to specifically interact with a sequence of viral DNA termed the 'a' sequence. The 'a' sequence is involved in a number of important functions, including the cleavage and packaging of viral DNA. This project has also involved the investigation of other apparently sequence-specific interactions occurring within

the 'a' sequence of HSV type 1.

The aim of this introduction is to provide a general overview of HSV biology with particular emphasis on virus-induced DNA-binding proteins and the involvement of DNA-protein interactions in various aspects of the virus life cycle (for example, transcription, DNA replication and packaging). A quite extensive account of the possible functional roles of the 'a' sequence is also provided. For this reason it is not possible to do justice to all aspects of herpes virology, and several important areas, such as latency, pathogenesis and carcinogenesis, are dealt with only briefly, if at all.

SECTION II : GENERAL INTRODUCTION

II.1 The human herpesviruses

Man is the natural host of the five human herpesviruses - herpes simplex virus type 1 and type 2 (HSV-1, HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV). In addition, a novel herpesvirus has recently been isolated from immunocompromised patients (Josephs et al., 1986; Salahuddin et al., 1986), but this work remains to be confirmed and the experiments extended to determine whether this is genuinely a sixth human herpesvirus. Herpes simplex virus is ubiquitous and infects a large percentage of the population. Whitley (1985) has recently reviewed the epidemiology of HSV. The primary infection is frequently asymptomatic or mild, associated with mucocutaneous lesions around the mouth or genital mucosae. More serious, however, is the occasional occurrence of keratoconjunctivitis (which can lead to corneal blindness) or, more rarely, acute necrotising encephalitis, while infection of the newborn can result in severe disseminated disease. (more usually by HSV-2)

A characteristic feature of herpesviruses is their potential to establish a latent infection, periodically reactivating to produce recurrent disease. For example, VZV - the causative agent of chicken pox in children - can reactivate later in life to cause shingles, while CMV, which usually produces an asymptomatic primary infection, is a severe problem in immunocompromised hosts both as a primary and a recurrent disease. Similarly, HSV can reactivate to produce severe, usually localised, lesions in immunosuppressed

patients. In contrast, in normal individuals reactivation of HSV is usually asymptomatic or mild, leading in the latter case to the appearance of 'cold sores' or genital lesions.

Additional clinical interest in these viruses stems from their association with tumours. Epstein-Barr virus is strongly associated with both Burkitt's lymphoma and nasopharyngeal carcinoma (Epstein et al., 1964; zur Hausen et al., 1970; de-Thé et al., 1978; reviewed in de-Thé, 1982), while CMV appears to be associated with the European form of Kaposi's sarcoma (Giraldo et al., 1975). Herpes simplex virus type 2 has been implicated in the aetiology of at least a proportion of cases of cervical carcinoma (Naib et al., 1966; Aurelian, 1973; McDougall et al., 1980; Eglin et al., 1981; Park et al., 1983) but its precise role is uncertain (zur Hausen, 1982; Galloway and McDougall, 1983; Rawls, 1985).

II.2 The family Herpesviridae

The human herpesviruses belong to the family Herpesviridae, which includes over eighty distinct herpesviruses, described in a wide range of animals from fish to man (reviewed in Roizman, 1982) and even in a fungus (Kazama and Schornstein, 1972). Members of the family Herpesviridae share a number of characteristics (reviewed in Roizman and Furlong, 1974). They : replicate within the nucleus of infected cells; possess a double-stranded DNA genome of approximate molecular weight 80-150 x 10^6; have an icosahedral capsid, consisting of 162 capsomeres; have a rather ill-defined layer known as the tegument between the

capsid and the envelope; and have a lipid envelope, usually acquired by budding through the inner nuclear membrane. Thus, herpesviruses cannot be differentiated by their morphology. Instead, they have been divided into subfamilies on the basis of their biological properties (Roizman et al., 1981).

Herpes simplex virus type 1 belongs to the Alpha-herpesvirinae subfamily, as do HSV-2, VZV and pseudorabies virus (PRV), among others. This subfamily is characterised by a variable host cell range both in vivo and in vitro, a relatively short reproductive cycle (<24 hours), and the frequent establishment of latency in vivo, usually in ganglia. In contrast, the Betaherpesvirinae subfamily, which includes both human and murine cytomegalovirus (HCMV, MCMV) shows a narrow in vivo and in vitro host range, a relatively long reproductive cycle which often results in enlargement of infected cells (cytomegalia), and can establish latency in secretory glands and lymphoreticular cells, as well as in other tissues. The Gammaherpesvirinae subfamily, which includes EBV and Marek's disease virus (MDV), also has a narrow host range in vivo, and in vitro infects lymphoblastoid cell lines, being either T or B cell specific. Their reproductive cycle is variable, often leading to a persistent, rather than a lytic, infection, and latency is established in lymphoid tissues.

II.3 The herpes simplex virus genome

Herpes simplex virus type 1 has a double-stranded DNA genome of approximately 155,000 base pairs (Becker et al., 1968; Kieff et al., 1971). The genome consists of a Long (L) and a Short (S) region, each comprising a unique

sequence (U_L, U_S) flanked by inverted repeats (TR_L/IR_L, TR_S/IR_S) (Sheldrick and Berthelot, 1974). This is represented schematically in Figure 1. A direct repeat sequence, known as the 'a' sequence, is present at the genomic termini and in inverted orientation at the L-S junction (Sheldrick and Berthelot, 1974; Grafstrom et al., 1974, 1975; Wadsworth et al., 1976; Wilkie and Cortini, 1976). One to several copies of the 'a' sequence may be present at the L terminus and the L-S junction, but only one copy is usually found at the S terminus (Wagner and Summers, 1978; Locker and Frenkel, 1979a). Inversion of the L and S segments can occur between inverted copies of the 'a' sequence to generate four isomeric forms of viral DNA which are found in equimolar amounts (Sheldrick and Berthelot, 1974; Hayward et al., 1975; Clements et al., 1976; Delius and Clements, 1976; Skare and Summers, 1977). These are shown in Figure 1.

SECTION III : INFECTION WITH HERPES SIMPLEX VIRUS

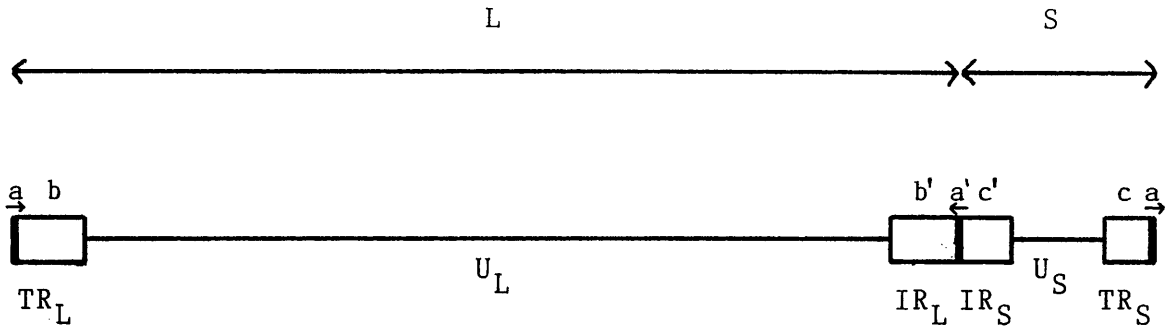
III.1 Adsorption and penetration

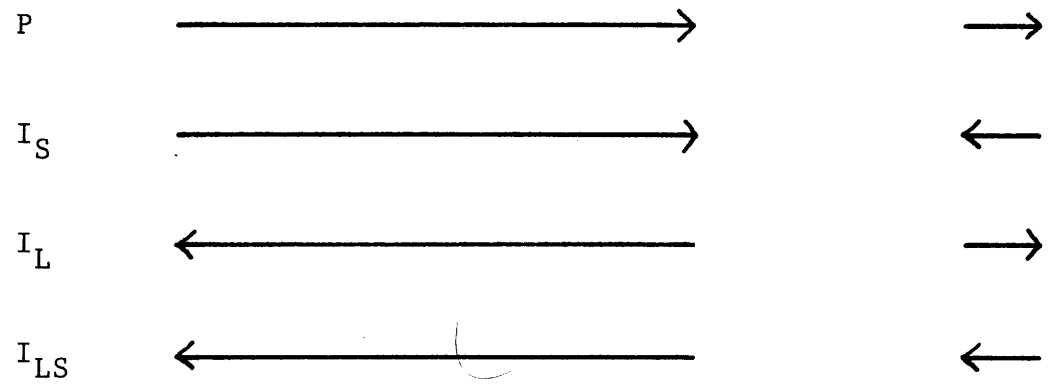
Herpes simplex virus rapidly adsorbs to cells in tissue culture (Hochberg and Becker, 1968; Hummeler et al., 1969; Vahlne et al., 1978) by a process that is not well characterised, but presumably involves attachment to specific cell surface receptors. Interestingly, it appears that HSV-1 and HSV-2 do not use the same cell surface receptor, in that they interfere with adsorption of virus strains of the homologous, but not the heterologous, serotype (Vahlne et al., 1979; Addison et al., 1984).

Following adsorption, virus enters the cell either

FIGURE 1

Schematic representation of the HSV genome. The genome consists of a Long (L) and a Short (S) region, each comprising a unique sequence (U_L,U_S) flanked by inverted repeats (TR_L/IR_L; TR_S/IR_S). The 'a' sequence is present as a direct repeat at the genomic termini, and in inverted orientation at the L-S junction. Inversion of the L and S segments occurs between inverted copies of the 'a' sequence to generate four isomeric forms of viral DNA found in equimolar amounts. These are designated P (prototype), I_S (inversion of the short segment), I_L (inversion of the long segment) and I_{LS} (inversion of the long and short segments).


L
S
a b
b' a' c'
c a
U_L
U_S
TR_L
IR_L IR_S
TR_S


P
I_S
I_L
I_{LS}

by a process of endocytosis (Hummeler et al., 1969) or by fusion of the virus envelope with the cell membrane (Morgan et al., 1968), and the virus DNA is then rapidly transported to the nucleus (Hummeler et al., 1969) where transcription of the viral DNA takes place (Wagner and Roizman, 1969).

Rosenthal et al. (1984) demonstrated changes in the mobility of cell surface proteins during adsorption and penetration. Initially the membrane became less mobile, suggesting a multivalent attachment between the virus and cell receptors. In contrast, there was an increase in mobility above control levels during penetration, which would be compatible with either extensive endocytosis or the fusion of viral and cell membranes.

III.2 The effect of HSV infection on host cell metabolism

Virus infection has a profound effect on host cell metabolism, cell DNA, RNA and protein synthesis all being significantly impaired (Roizman and Roane, 1964; Roizman et al., 1965; Spear et al., 1970). The reduction in host protein synthesis is contributed to by a number of factors. Host polyribosomes disaggregate rapidly following infection (Sydiskis and Roizman, 1966) and later reassemble to form new polyribosomes with an altered sedimentation rate (Sydiskis and Roizman, 1968) which appear to preferentially associate with virus-specified mRNA (Stringer et al., 1977; Silverstein and Engelhardt, 1979). Host RNA synthesis is decreased following infection (Roizman et al., 1965; Pizer and Beard, 1976) and the mRNA released from the polyribosomes is degraded (Nishioka and Silverstein, 1977; Schek and Bachenheimer, 1985). Although some cell mRNA does

associate with polysomes late in infection (Stringer et al., 1977) the translation of these mRNAs appears to be suppressed (Silverstein and Engelhardt, 1979). This may be due to modifications to the mRNA itself, since cell proteins were translated in vitro less well than viral proteins from purified, deproteinised infected cell RNA (Inglis and Newton, 1981; Fenwick and McMenamin, 1984), although Schek and Bachenheimer (1985) suggested that this could simply be explained by the extensive degradation of cellular mRNA.

At least two factors appear to be involved in the inhibition of host polypeptide synthesis - a virion component(s), and a protein(s) synthesised later in infection (Honess and Roizman, 1975; Fenwick and Walker, 1978; Fenwick and Clark, 1982; Read and Frenkel, 1983). The virion-associated component is non-essential in tissue culture (Fenwick and Clark, 1982; Read and Frenkel, 1983), while the delayed component can function in the absence of a functional virion component (Fenwick and Clark, 1982; Read and Frenkel, 1983), and is required for the full inhibitory effect on host protein synthesis.

The disaggregation of host polyribosomes, decrease in RNA synthesis and degradation of cellular mRNA all appear to involve a component of the virion (Fenwick and Walker, 1978; Nishioka and Silverstein, 1978; Schek and Bachenheimer, 1985), although in some cell types protein synthesis is required for the degradation of cellular mRNA (Nishioka and Silverstein, 1978). Whether one or more virion components are involved, and whether it/they are the same as those involved in inhibition of host polypeptide synthesis, is unclear.

Fenwick and Walker (1979) and Kennedy et al. (1981)

reported the phosphorylation of cellular proteins associated with the small ribosomal subunit during infection. This phosphorylation was dependent on virus-specified protein synthesis (Fenwick and Walker, 1979), and therefore may not play a major role in altering ribosomal function, at least early in infection.

It was observed that HSV-2 shut off host protein synthesis more rapidly and efficiently than HSV-1 (Powell and Courtney, 1975; Pereira et al., 1977). Although this generalisation does not hold true for all strains of HSV-1 and HSV-2, and in particular strain HG52 (Marsden et al., 1978), the HSV-2 strain used in these studies, the differential shut-off has been used to map this virion function to between 0.52 and 0.59 map units on the viral genome (Morse et al., 1978). A similar location was found using strain $17syn^+$ x strain HG52 intertypic recombinants (Howard S Marsden, unpublished observations). The inhibition of host cell DNA synthesis also appears to involve a virion component (Fenwick and Walker, 1978) and to be greater following HSV-2 infection than following HSV-1 infection (Fenwick et al., 1979). The latter authors have similarly mapped this function to between 0.52 and 0.59 map units on the viral genome. However, the nature of the product, or products, involved in these functions is, as yet, unknown.

A number of morphological changes occur within cells following viral infection, including reduplication and thickening of nuclear membranes and the appearance of filamentous or tubular structures within nuclei (Nii et al., 1968; Schwartz and Roizman, 1969; Atkinson et al., 1978). In addition, margination of host cell chromatin and

disaggregation of nucleoli occur early in infection (Nii et al., 1968; Schwartz and Roizman, 1969; Cabral and Schaffer, 1976), coinciding with the inhibition of host cell DNA synthesis and ribosomal RNA synthesis and processing (Wagner and Roizman, 1969).

SECTION IV : HERPES SIMPLEX VIRUS TRANSCRIPTION

IV.1 General features of HSV transcription

The transcription and processing of HSV-specific RNAs is very similar to that of cellular RNA. The viral transcripts are synthesised in the nucleus (Wagner and Roizman, 1969), a process which appears to involve the host cell RNA polymerase II at all stages of virus infection (Alwine et al., 1974; Ben-zeev and Becker, 1977; Costanzo et al., 1977). They are capped at their 5' ends and internally methylated in a similar manner to host cell mRNA (Bartkoski and Roizman, 1976; Moss et al., 1977), and most transcripts are polyadenylated at their 3' ends (Bachenheimer and Roizman, 1972; Silverstein et al., 1973, 1976).

Thus, it is hardly surprising that viral genes have been found to possess 5' and 3' regulatory sequences similar to those of cellular genes (McKnight, 1980; Frink et al., 1981; Everett, 1983; Preston et al., 1984; Rixon et al., 1984; Rixon and McGeoch, 1985; Bzik and Preston, 1986), including 5' promoter elements such as the 'TATA' box and 'CAAT' box sequences (Gannon et al., 1979; Wasylyk et al., 1980; Benoist et al., 1980; Efstratiadis et al., 1980), and the 3' sequence AATAAA important for cleavage and polyadenylation of pre-mRNAs (Proudfoot and Brownlee, 1974, 1976; Fitzgerald and Shenk, 1981; Zarkower et al.

1986). In addition, a consensus sequence 'YGTGTTYY' has been found which occurs about 10 base pairs (bp) downstream from the polyadenylation site in a number of eukaryotic genes (Taya et al., 1982; McLauchlan and Clements, 1983; McLauchlan et al., 1985), and this sequence has been shown to be required for the efficient formation of mRNA 3' termini (McLauchlan et al., 1985).

However, in contrast to the situation for cellular genes, splicing is a relatively rare event in HSV transcription (Wagner, 1985), and only a few spliced transcripts known to code for viral polypeptides have been described (Watson et al., 1981; Rixon and Clements, 1982; Frink et al., 1983; Costa et al., 1985; Perry et al., 1986).

IV.2 Temporal regulation of HSV gene expression

The synthesis of viral proteins is temporally regulated and can be broadly divided into three main phases : immediate-early (IE), early and late. This closely parallels the temporal regulation of viral RNA transcription (reviewed in Wagner, 1985). Immediate-early (or α) polypeptides, as the name suggests, are synthesised immediately following viral infection and their synthesis is not dependent on the de novo synthesis of other viral polypeptides (Honess and Roizman, 1974). Thus, infection under conditions which inhibit protein synthesis allows the specific accumulation of IE mRNAs. These mRNAs are transcribed from only a limited portion of the virus genome (Clements et al., 1977; Jones et al., 1977). The locations and orientations of the major IE transcripts are shown later (see section VIII). There are five IE polypeptides : Vmw175 (or ICP4), Vmw110

(ICP0), Vmw68 (ICP22), Vmw63 (ICP27) and Vmw12 (ICP47); these correspond to IE genes 3, 1, 4, 2 and 5, respectively (Preston et al., 1978; Clements et al., 1979; Watson et al., 1979; Marsden et al., 1982). With the exception of Vmw12, these are all phosphoproteins, found predominantly within the nucleus of infected cells, and are capable of binding to calf thymus DNA, at least within crude whole cell extracts (Pereira et al., 1977; Preston, 1979a; Hay and Hay, 1980; Marsden et al., 1982).

The precise function of the IE gene products is not clear. At least one of these (Vmw175) plays an essential role in HSV transcription (Preston, 1979a; Dixon and Schaffer, 1980; Watson and Clements, 1980), while another (Vmw110) plays a very significant, if perhaps not absolutely essential, role in virus replication (Stow and Stow, 1986; Sacks and Schaffer, 1987). A virus mutant with a deletion in the gene for Vmw68 is impaired for growth in some cell types, but not others (Sears et al., 1985), suggesting that a cellular polypeptide may complement this function. Sacks et al. (1985) described a conditional lethal virus mutant containing a temperature-sensitive (ts) lesion within Vmw63, suggesting that this polypeptide is essential for virus replication. A virus mutant which fails to synthesise detectable levels of Vmw63 under IE conditions was recently described (MacLean and Brown, 1987), but the expression of this polypeptide under early or late conditions remains to be investigated. Finally, virus mutants which lack the gene for Vmw12 appear to grow normally in a number of cell types (Mavromara-Nazos et al., 1986a; Umene, 1986; Brown and Harland, 1987), suggesting that this polypeptide is not essential for virus growth, at least

in tissue culture.

In contrast to the IE gene products, the synthesis of early (or β) polypeptides is absolutely dependent upon the prior synthesis of IE polypeptides (Honess and Roizman, 1974). Early polypeptides are synthesised prior to, and often play a role in, viral DNA replication. These include enzymes such as thymidine kinase and ribonucleotide reductase, and proteins more directly involved in replication, such as DNA polymerase and the major DNA-binding protein (see section V).

The late (or γ) polypeptides require not only both IE and early polypeptide synthesis but also viral DNA replication for their efficient expression (Honess and Watson, 1977; Jones and Roizman, 1979; Holland et al., 1980). This class include a number of structural proteins, such as the major capsid protein (Vmw155, VP5), glycoprotein C (gC) and the U_S11 gene product, Vmw21 (Honess and Roizman, 1973; Marsden et al., 1976; Costa et al., 1981; Frink et al., 1983).

This classification, however, is not absolute, and several polypeptides display kinetics of synthesis which are intermediate between these phases. For example, the large subunit of ribonucleotide reductase (rr_1), although classified as an early gene, often appears under IE conditions; another early protein, glycoprotein D, although synthesised in significant levels in the absence of DNA replication, requires DNA replication for optimal expression (Gibson and Spear, 1983; Johnson et al., 1986), and hence gD is sometimes termed an early-late, or βγ gene; in addition, late genes appear to be divisible into two groups, leaky-late (γ_1) and true-late (γ_2), which differ in their

degree of dependence on DNA replication for expression (Powell et al., 1975; Holland et al., 1980; Wagner, 1985).

IV.3 Control of HSV transcription

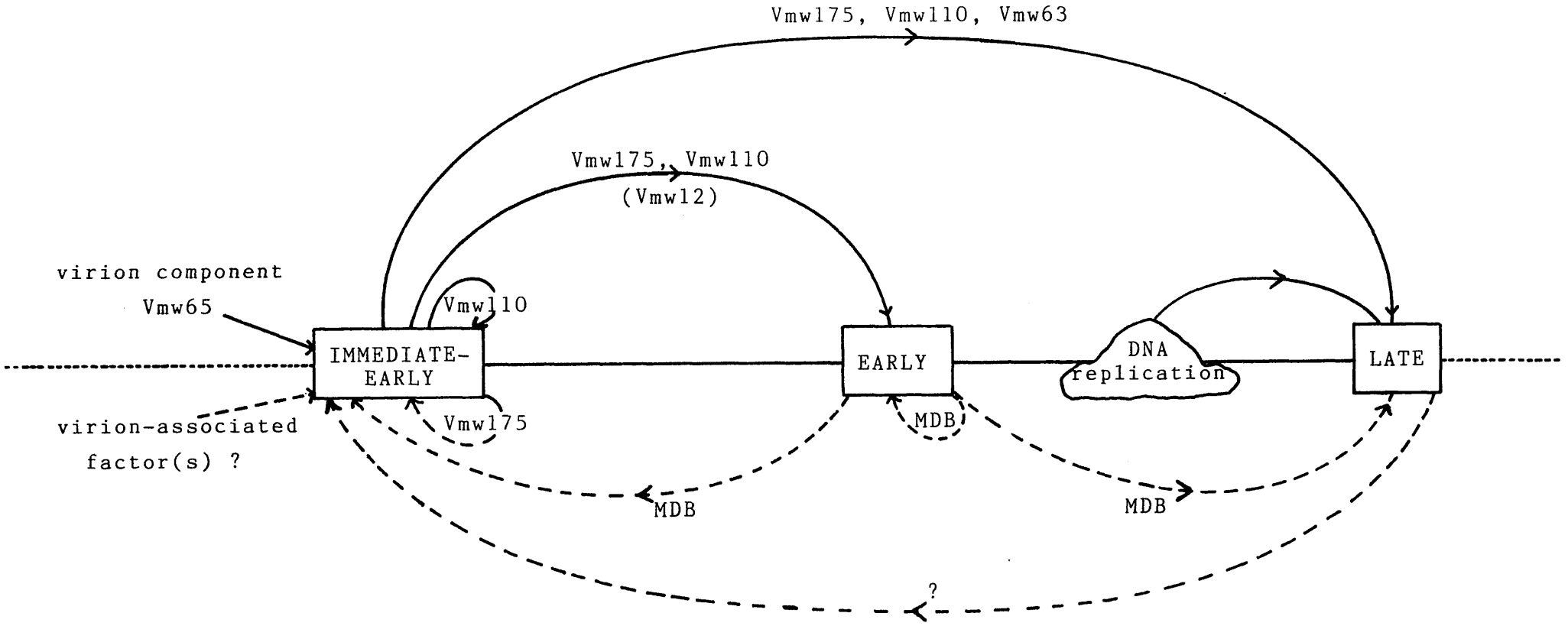
The regulation of HSV transcription is a complex process and, at best, is poorly understood. However, some of the factors involved have been elucidated, mainly through studies involving ts mutants and, more recently, through the use of short term transfection assays, or biochemically-transformed cell lines, to examine the ability of cloned viral gene products to transactivate plasmid-borne promoters. In general, transfection experiments involving wild-type and ts viral gene products as transactivators have produced results very compatible with their phenotypes in situ (DeLuca and Schaffer, 1985), although some apparent discrepancies have arisen (see below). These various studies have suggested that there are a number of both positive and negative regulatory factors influencing the different temporal phases of HSV transcription. A summary is shown in Figure 2.

Although IE transcription appears to involve the cellular RNA polymerase II (Costanzo et al., 1977) and does not require prior synthesis of viral polypeptides, it is significantly enhanced by a component of the virus particle (Post et al., 1981; Batterson and Roizman, 1983). The trans-activating virion component has been identified as the major tegument protein, Vmw65 (Campbell et al., 1984). In addition, Read and Frenkel (1983), on the basis of studies involving ts mutants, suggested that factor(s) present in their virus stocks had a negative influence on IE gene expression, reducing the stability of IE mRNA. However,

FIGURE 2

Regulation of HSV transcription. A summary of the viral polypeptides believed to influence viral gene expression. These are discussed fully in the text. Factors shown above the horizon, and indicated by a solid line (——), have a positive influence in the direction shown, while those shown below the horizon, and indicated by the dashed line (- - -), have a negative influence. The role of Vmw12 in early gene expression remains to be established.

MDB : major DNA-binding protein


Vmw175, Vmw110, Vmw63
Vmw175, Vmw110
(Vmw12)
virion component
Vmw65
Vmw110
IMMEDIATE-
EARLY
EARLY
DNA
replication
LATE
virion-associated
factor(s) ?
Vmw175
MDB
MDB
MDB
?

these authors did not attempt to establish whether this factor was also a virion component.

The IE gene products themselves regulate transcription. Both transfection assays and studies involving ts mutants suggest that Vmw175 has a strong suppressive effect on IE gene transcription (Dixon and Schaffer, 1980; Watson and Clements, 1980; DeLuca et al., 1985; O'Hare and Hayward, 1985a; Gelman and Silverstein, 1986), although at very low levels it may serve to activate IE promoters (DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985a; Gelman and Silverstein, 1986). In contrast, Vmw110 appears to stimulate IE transcription, at least in transfection assays (O'Hare and Hayward, 1985a; Gelman and Silverstein, 1986). Despite this difference in behaviour with respect to IE transcription, Vmw175 and Vmw110 can both independently stimulate early gene transcription, while in combination they appear to act synergistically on early promoters (Everett, 1984a, 1986; Gelman and Silverstein, 1985, 1986; O'Hare and Hayward, 1985b; Quinlan and Knipe, 1985). One report suggests that the presence of Vmw12 may augment this synergistic effect (O'Hare and Hayward, 1985b). However, this remains to be confirmed.

The ability of Vmw110 to transactivate early gene promoters in transient expression assays contrasts with the observation that most ts mutants in Vmw175 fail to induce early gene expression (Watson and Clements, 1978, 1980; Dixon and Schaffer, 1980). Although it is possible that early gene expression is inhibited by the altered polypeptide (DeLuca and Schaffer, 1985; Gelman and Silverstein, 1986), two deletion mutants in Vmw175 also fail to induce early polypeptides, and their deleted gene products have neither positive nor negative regulatory

activity in transfection assays, either alone or in combination with other IE polypeptides (DeLuca et al., 1985). Thus, it would appear that during normal infection Vmw110 cannot functionally substitute for Vmw175.

Recently, virus mutants with deletions in both copies of the gene for Vmw110 have been isolated (Stow and Stow, 1986; Sacks and Schaffer, 1987). Although the deleted genes were inactive in transfection assays (Perry et al., 1986; Sacks and Schaffer, 1987) the viruses were capable of replication in tissue culture, in a number of different cell lines, producing yields 10-100 fold reduced compared to wild-type virus (Stow and Stow, 1986; Sacks and Schaffer, 1987). The effects of deletion were most apparent at low multiplicities of infection (Stow and Stow, 1986), and the plating efficiency of the mutants was increased 15-50-fold when titrated on cell lines biochemically transformed with the functional gene for Vmw110 (Sacks and Schaffer, 1987) - i.e. a major defect of these viruses would appear to be at the level of initiation of plaque formation. Thus, although there would appear to be no absolute requirement for Vmw110, at least in tissue culture, this polypeptide would appear to be essential for normal virus growth, particularly at low multiplicities of infection.

Although Vmw175 is clearly essential for early gene expression, it may not necessarily be sufficient. Additional factors appear to be required for efficient expression from at least some early promoters. Persson et al. (1985) found that of seven early genes examined only two were almost fully expressed in response to Vmw175 alone, whereas the others varied in expression from intermediate to very low levels. Therefore, normal viral replication in vivo probably

requires a balanced interaction between a number of IE, and possibly other, viral gene products.

Studies involving ts and deletion mutants have suggested that both Vmw175 (Preston, 1979a; Watson and Clements, 1980; DeLuca et al., 1984, 1985) and Vmw63 (Sacks et al., 1985) are involved in the activation of late gene expression. This is supported by the recent finding, in transfection assays, that a combination of Vmw175, Vmw110 and Vmw63 was required for significant activation of a late promoter (Everett, 1986).

Factors other than IE gene products may also be involved in the regulation of HSV gene expression : DNA replication is required for the efficient expression of late genes; Godowski and Knipe (1985, 1986) have shown, using ts mutants, that the major DNA-binding protein, an early protein, may have a negative regulatory role in IE, early and late gene expression - an effect independent of its role in DNA replication; and an unknown late gene function also appears to be involved in the negative regulation of IE gene transcription (DeLuca et al., 1984).

IV.4 cis-acting sequences required for the regulation of HSV transcription

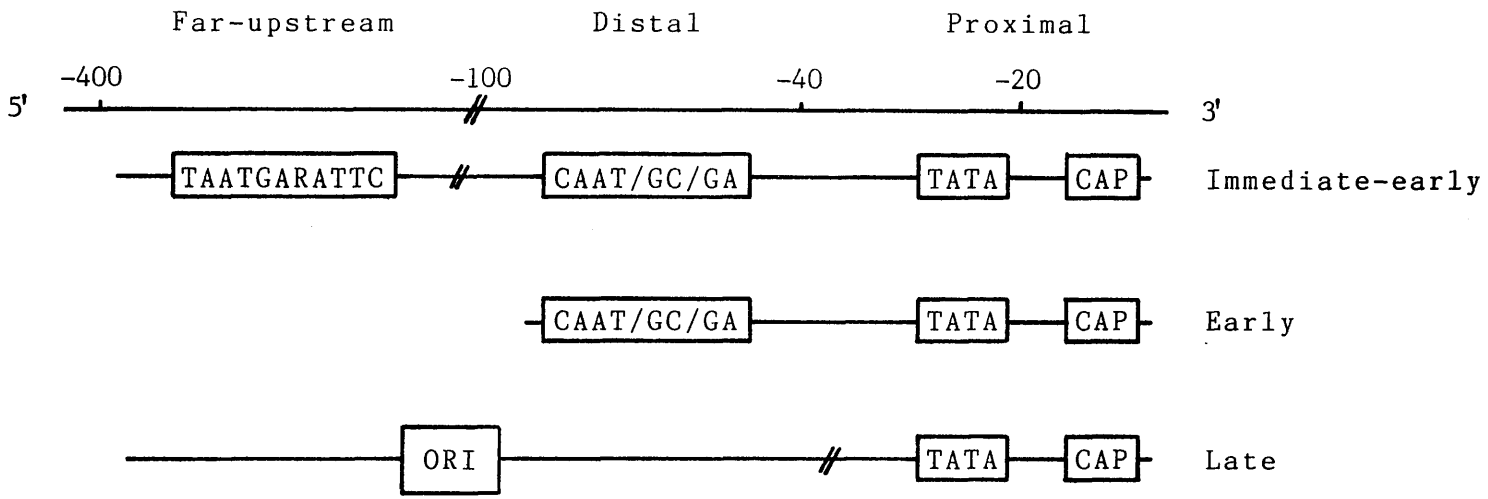
The mechanism by which these various factors regulate HSV gene expression is unknown. It is well established that control is primarily, although not exclusively, at the level of transcription (Preston, 1979b; Everett, 1984a; Johnson and Spear, 1984; O'Hare and Hayward, 1985a, 1985b; Coen et al., 1986; Gelman and Silverstein, 1986). Therefore, one possible mechanism for control would be specific

interactions between the transcription factors and specific DNA sequences present in the promoter or 5' upstream regulatory regions which are characteristic of and possibly unique to each temporal class of gene. The features believed to be important for IE, early and late promoter activity, respectively, can be seen in Figure 3.

A consensus sequence 'TAATGARATTC' is found in the far upstream region of all IE genes, but no early or late genes (Mackem and Roizman, 1982; Whitton et al., 1983; Whitton and Clements, 1984a) - i.e. this sequence is characteristic of, and apparently unique to, IE genes. The 'TAATGARATTC' element has been shown to mediate responsiveness to the virion transactivating factor, Vmw65 (Preston et al., 1984; Gaffney et al., 1985) although full responsiveness required the presence of a GA-rich element itself poorly active alone (Bzik and Preston, 1986). Could Vmw65 therefore interact directly with the 'TAATGARATTC' sequence? Probably not, since Vmw65 neither binds to calf thymus DNA nor to DNA containing a 'TAATGARATTC' element (Muller, 1987; Marsden et al., 1987; C M Preston, M C Frame and M E M Campbell, submitted for publication). Kristie and Roizman (1987) have shown that cellular polypeptides interact with DNA containing the 'TAATGARATTC' element, while, more recently, Preston and coworkers, using both a gel retardation assay in conjunction with specific antibodies or with immuno-affinity purified Vmw65, and DNase I protection/ methylation inhibition experiments, have demonstrated an interaction between Vmw65 and the 'TAATGARATTC' element via a cellular polypeptide(s) (Preston et al., submitted for publication). Since the complexes containing Vmw65 bound the 'TAATGARATTC' elements with a much higher affinity

FIGURE 3

cis-acting signals required for the regulation of HSV transcription. Taken from Johnson and Everett (1986a). Promoter element requirements appear to decrease, the later the temporal expression of a gene. Thus, late promoters consist of only a 'TATA-box'-capsite region (CAP), although they require an active origin of DNA replication (ORI) in cis for efficient expression; early genes also require a distal promoter element, containing at least one of either a 'CAAT-box', GC-rich motifs, or GA-rich motifs; while immediate-early genes require, in addition, a far upstream element, including the consensus sequence, TAATGARATTC, as well as flanking modulatory sequences, for response to the virion transactivator, Vmw65. This diagram is not drawn to scale.


Far-upstream
Distal
Proximal
-400
-100
-40
-20
5'
3'
TAATGARATTC
CAAT/GC/GA
TATA
CAP
Immediate-early
CAAT/GC/GA
TATA
CAP
Early
ORI
TATA
CAP
Late

than cellular polypeptides alone, these authors concluded that Vmw65 must interact with DNA by modifying a cellular factor(s).

Cellular transcription factors, not surprisingly, do appear to play a role in HSV transcription. For example, the transcription factor Sp1 appears to bind to GC-rich sequences within the distal promoter region of IE genes and stimulates transcription from these genes *in vitro* (Jones and Tjian, 1985). In addition, the sequences required both for *cis*- and *trans*-activation of an early HSV gene (the gene for thymidine kinase) encompass binding sites for Sp1, CAAT-binding protein and TATA-box-binding protein (McKnight and Kingsbury, 1982; Coen *et al.*, 1986). Since no additional sequences appear to be specifically required for transactivation of early genes (Everett, 1983, 1984b; Smiley *et al.*, 1983; Coen *et al.*, 1986) it has been suggested that IE genes may also act indirectly, by altering the specificities of cellular transcription factors. As mentioned earlier, IE polypeptides, with the exception of Vmw12, can bind to calf thymus DNA (Hay and Hay, 1980). However, whether they interact directly or indirectly with DNA remains to be established. Indeed, one report suggests that purified Vmw175 fails to bind to DNA in the absence of cellular polypeptides (Freeman and Powell, 1982). Using a gel retardation assay, Kristie and Roizman (1986a) recently demonstrated the presence of Vmw175 in complexes formed between infected cell polypeptides and the promoter-regulatory domains of three IE genes, a true late gene and, to a lesser extent, the early thymidine kinase gene. A number of binding sites appeared to be present in some of these promoter elements. Muller (1987) demonstrated an

interaction between Vmw175 and its own transcription start site, and suggested that this could be involved in the repression of IE gene transcription. In addition, Beard *et al.* (1986) showed that Vmw175 interacted with DNA sequences adjacent to the gD gene promoter, appearing to interact with at least three separate domains within this region. More interestingly, purified Vmw175 appeared to stimulate *in vitro* transcription from this promoter, acting at the level of initiation of transcription. Subsequently, these authors also demonstrated that partially-purified Vmw175 could stimulate *in vitro* transcription from late genes (gC and VP5), while it inhibited transcription from an IE gene, that for Vmw68 (Pizer *et al.*, 1986). Again, whether these are direct or indirect effects remains to be determined.

Therefore, at present the general consensus of opinion appears to favour an indirect role for IE polypeptides, in altering the specificity of cellular transcription factors. However, the IE polypeptide Vmw12 differs from the others in that it is non-phosphorylated, does not appear to bind to DNA, and is located predominantly in the cytoplasm of infected cells (Preston, 1979a; Marsden *et al.*, 1982; Palfreyman *et al.*, 1984). Thus, if Vmw12 does play a role in augmenting the effects of Vmw175 and Vmw110 on early gene transcription, then it is more likely to do so at a post-transcriptional level, for example, by influencing mRNA stability or translational efficiency.

Efficient transcription from a true late promoter requires even fewer regulatory sequences. Only the 'TATA'-box /capsite region appears to be required (Homa *et al.*, 1986; Johnson and Everett, 1986a). Studying the U_S11 gene

promoter, Johnson and Everett (1986a) have shown that an active origin of replication is required in *cis* for efficient expression from this true late promoter, and that the DNA sequences required for the fully efficient, regulated expression of U_S11 lie within 31 bp of the RNA capsites. In addition, by deleting the distal promoter region from the gD promoter this classical early gene was converted to a late gene phenotype. In agreement with these findings Homa *et al.* (1986) have shown a similar sequence requirement for the gC gene promoter, another true late gene.

The requirement for DNA replication for the efficient expression of late genes may reflect more than an increase in copy number compensating for a weak promoter. Johnson and Everett (1986b) have shown that in the early stages following replication the increase in expression from the U_S11 gene promoter was greater than the increase in plasmid copy number, whereas in later stages these increased more or less in parallel. Thus, replication *per se* may account for an increase in late promoter activity. An alteration in the genome structure or in the factors binding to viral DNA during replication may influence late gene expression (Silver and Roizman, 1985; Tack and Beard, 1985; Johnson and Everett, 1986b).

The mechanisms discussed above for the activation of viral genes could equally well apply to the suppression of viral gene expression. Thus, for example, factors binding to the distal promoter region of early genes could serve to inhibit early gene expression late in infection, while factors binding to the 'TAATGARATTC' element of IE genes could specifically inhibit expression from this class of genes.

In summary, a combination of viral proteins, cellular factors and regulatory DNA sequences control the sequential activation of HSV genes, but the precise interactions between these factors remains to be determined.

SECTION V : HSV-INDUCED ENZYME ACTIVITIES

V.1 Nomenclature for HSV-induced polypeptides

Following infection of cells with HSV a large number of polypeptides are induced. These include the IE polypeptides which appear to function primarily in HSV transcription, virus-induced enzymes and DNA-binding proteins involved in various aspects of viral replication, structural polypeptides which compose the virus particles, and glycoproteins which play important roles in the adsorption and penetration of virus particles into cells, and in the induction of immune responses following infection *in vivo*. Nomenclature used in the literature for these polypeptides is confusing, since a number of different systems co-exist. In this thesis I intend mainly to adopt the system of Marsden *et al.* (1976), whereby polypeptides were originally named according to their apparent molecular weight in SDS-polyacrylamide gels. Thus, for example, a polypeptide with an apparent molecular weight of 21,000 was designated Vmw21. However, reference will be made to other systems, particularly that of Honess and Roizman (1973) in which polypeptides have been designated infected cell polypeptide (ICP) numbers, according to their relative position on SDS-polyacrylamide gels. Where possible, when using the ICP system, the most probable corresponding polypeptide identified in the system of Marsden *et al.* is provided. For glycoproteins, I have used the system proposed

at the eighth International Herpesvirus Workshop, held in Oxford 1983, which has now been universally accepted. In this system, glycoproteins arising from different genes are given a unique letter : presently seven glycoproteins have been identified, and are designated gB, gC, gD, gE, gG, gH and gI, respectively (see section VII.2).

As already mentioned, virus-specific protein synthesis can be divided into IE, early and late temporal stages. Among the early polypeptides are a number of virus-induced enzyme activities, many of which play a direct or indirect role in viral DNA replication.

V.2 HSV-induced enzymes involved in nucleic acid metabolism

(a) Thymidine kinase

Virus-induced enzymes indirectly influence viral DNA replication by modifying nucleic acid metabolism, most notably pyrimidine metabolism (see Figure 4). Herpes simplex virus encodes an enzyme, thymidine kinase (TK), (Dubbs and Kit, 1964; Munyon *et al.*, 1971) which phosphorylates, in addition to thymidine, deoxycytidine (Jamieson and Subak-Sharpe, 1974; Jamieson *et al.*, 1974) and thymidylate (Chen and Prusoff, 1978), using nucleoside triphosphate, usually ATP, as a phosphate donor. Thus, it possesses both deoxypyrimidine kinase and thymidylate kinase activities. However, exogenously added deoxycytidine, unlike thymidine, fails to be incorporated into DNA, suggesting that reduction of CDP, rather than phosphorylation of dCMP, is the major route of dCDP, and hence dCTP, synthesis following infection (Jamieson and Subak-Sharpe, 1976).

The HSV TK has also been associated with a nucleoside

PYRIMIDINE METABOLISM

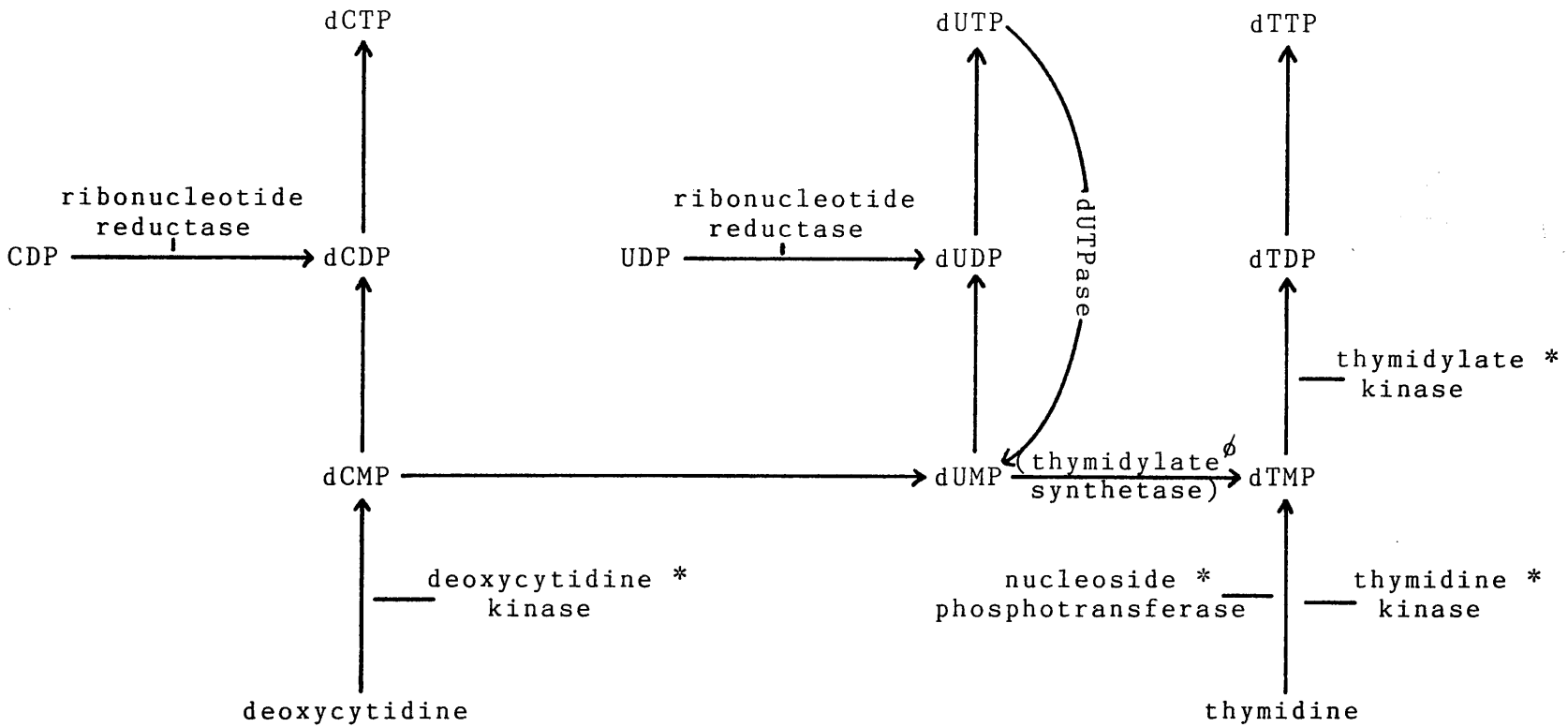


FIGURE 4

Virus-induced enzymes involved in pyrimidine metabolism. A simplified diagram of pyrimidine metabolism is provided, with the possible sites of action of a number of virus-induced enzyme activities. A thymidylate synthetase activity (ø) does not appear to be encoded by HSV, but is encoded by other herpesviruses, including VZV and HVS (see text). The virus-encoded enzyme thymidine kinase is associated with a number of different enzyme activities - these are indicated (*).

phosphotransferase activity (Jamieson et al., 1976; Falke et al., 1981) which is capable of utilising AMP, and possibly ADP, as a phosphate donor to convert thymidine to thymidylate (dTMP). This activity, however, also appears to require a component of the cellular thymidine kinase system (Jamieson et al., 1976).

(b) Deoxyuridine-5'-triphosphate nucleotidohydrolase

Deoxyuridine-5'-triphosphate nucleotidohydrolase, or dUTPase, is an enzyme which catalyses the conversion of dUTP to dUMP and pyrophosphate. The host cell dUTPase, by reducing intracellular levels of dUTP, acts to minimise incorporation of this nucleotide into DNA. Deoxy-UMP can be methylated by the enzyme thymidylate synthetase to dTMP, providing additional substrate for thymidylate synthesis. The HSV-induced dUTPase has been shown to be virus-encoded (Preston and Fisher, 1984). Although HSV, HCMV, PRV and EBV appear not to encode a thymidylate synthetase enzyme, two other herpesviruses do - herpesvirus saimiri (HVS) and VZV (Davison and Scott, 1986; Honess et al., 1986; Thompson et al., 1987). In contrast to HSV, both HVS and VZV possess an A+T rich genome, possibly suggesting a greater requirement by these viruses for thymidylate synthesis.

(c) Uracil-DNA glycosylase

Virus infection also appears to induce a virus-specific uracil-DNA glycosylase (Caradonna and Cheng, 1981). Host cell uracil-DNA glycosylase functions to remove deaminated cytosine residues from DNA, thus reducing the probability of G:C to A:T transitions occurring during replication (deaminated dCMP forms stable G:U base pairs). However,

although the virus-induced enzyme differs biochemically from the host cell enzyme (Caradonna and Cheng, 1981) it has yet to be shown to be virus encoded.

The function of these viral enzymes is thought to be analogous to their cellular counterparts. Virus mutants lacking TK (Dubbs and Kit, 1964; Jamieson et al., 1974) and/or dUTPase (Fisher and Preston, 1986) are unimpaired for growth in growing tissue culture cells. However, TK, but not dUTPase, is essential for growth in serum-starved resting cells (Jamieson et al., 1974; Fisher and Preston, 1986), suggesting the importance of the former enzyme in cells with a low level of de novo pyrimidine synthesis. Thus, it is possibly not surprising that TK-negative virus mutants are less pathogenic and less able to undergo latent infections in vivo than wild-type virus (Field and Wildy, 1978).

(d) Ribonucleotide reductase

Recently, interest has focussed on another viral enzyme involved in nucleotide metabolism. Ribonucleotide reductase is an enzyme which catalyses the reduction of all four ribonucleoside diphosphates to their corresponding deoxyribonucleoside diphosphates, and is essential for DNA replication in both prokaryotic and eukaryotic cells (Thelander and Reichard, 1979). A ribonucleotide reductase activity which differs biochemically and immunologically from the cellular enzyme is induced following HSV infection (Cohen, 1972; Ponce de Leon et al., 1977; Averett et al., 1983; Huszar et al., 1983). Using a ts mutant, Dutia (1983) demonstrated that the virus-induced enzyme is at least in

part virus encoded. This mutation resulted in a 100-fold reduction in virus growth over 24 hours at the non-permissive temperature, suggesting that this enzyme is essential for viral replication (Preston et al., 1984). Like its cellular counterpart, it appears to be composed of two subunits (Cohen et al., 1985; Frame et al., 1985; Bacchetti et al., 1986), a large subunit (rr_1) and a small subunit (rr_2), whose interaction is believed to be essential for enzyme activity (Frame et al., 1985). Synthetic oligopeptides corresponding to the carboxy-terminus of Vmw38 (rr_2) specifically inhibit the viral enzyme, and are thought to do so by inhibiting this interaction (Cohen et al., 1986; Dutia et al., 1986). This finding has initiated an exciting new approach to anti-viral therapy - the use of synthetic oligopeptides, or their analogues, to inhibit specific protein-protein interactions essential for viral replication. Furthermore, since antisera raised against the carboxy-terminal peptide from the HSV-1 Vmw38 recognised analogous polypeptides from HSV-2, VZV, PRV and equine herpesvirus type 1 (Dutia et al., 1986), it may be possible to derive an anti-viral agent effective against a number of members of the herpesvirus family.

V.3 HSV-induced enzymes involved or potentially involved in virus DNA replication

(a) DNA polymerase

The enzymes described above all indirectly influence viral DNA replication. Other virus-induced enzymes may have a more direct effect. The HSV-1-induced DNA polymerase activity is associated with a polypeptide of apparent

molecular weight (mol. wt.) 150K (Powell and Purifoy, 1977). This enzyme differs both biochemically and immunologically from the host cell DNA polymerase (Keir et al., 1966a, 1966b), contains both a DNA polymerase and a 3'-5' exonuclease activity (Weissbach et al., 1973; Knopf, 1979) and has been shown to be virus-encoded and essential for viral replication (Purifoy et al., 1977). Although apparently active as a monomer in vitro (Knopf, 1979; Ostrander and Cheng, 1980) the viral polymerase may form a functional complex in vivo with other viral proteins, including the major DNA-binding protein and the alkaline exonuclease (Purifoy and Powell, 1981; Littler et al., 1983; Chiou et al., 1985) and a protein known as ICSP34/35 (Vaughan et al., 1985) or $65K_{DBP}$ (H S Marsden, M Murphy, R G Hope, D J M Purifoy, P J Vaughan and K L Powell, manuscript in preparation). This putative complex will be discussed in more detail later (section VI.4)

(b) Alkaline exonuclease

An alkaline exonuclease activity, with properties distinct from those of cellular nucleases, is induced following HSV infection (Keir and Gold, 1963; Morrison and Keir, 1968; Hay et al., 1971; Hoffmann and Cheng, 1978). This enzyme has been shown to possess an endonuclease activity in addition to its 5' and 3' exonuclease activities (Hoffmann and Cheng, 1979; Hoffmann, 1981). Marker rescue of ts lesions (Moss et al., 1979) and in vitro translation experiments (Preston and Cordingley, 1982) suggest that it is virus-encoded, corresponding to the virus-specific polypeptide Vmw85 (Marsden et al., 1978). It appears to be essential for viral replication (Moss, 1986), but its

function *in vivo* is poorly understood. Its possible association with the DNA polymerase (Francke and Garrett, 1982) may suggest a direct role in viral DNA replication. Alternatively, it may be involved in the degradation of host cell DNA thought to occur during viral infection (Wildy *et al.*, 1961; Hoffmann and Cheng, 1978).

(c) Topoisomerase

An HSV-induced topoisomerase activity has also been reported (Biswal *et al.*, 1983; Leary and Francke, 1984; Muller *et al.*, 1985). Topoisomerases are enzymes which interconvert topological isomers of DNA by transient breakage and rejoining of DNA strands. They produce either transient single-strand breaks (type I topoisomerases) or double-strand breaks (type II topoisomerases). Thus, for closed circular DNA, a type I topoisomerase will change the linkage number of a given topoisomer in steps of one, whereas a type II topoisomerase will do so in steps of two. Topoisomerases, or proteins with topoisomerase-like activity, have been implicated in DNA replication, transcription and recombination. (Reviewed in Gellert, 1981).

Biswal and coworkers (1983) described an HSV-1 induced topoisomerase activity which copurified with the viral DNA polymerase through several chromatographic steps, while Muller *et al.* (1985) suggested that a type I topoisomerase activity was a component of the virion envelope or tegument structure. Whether these activities belong to the same protein, and whether they are virus-encoded, remains to be determined, as does their role in virus replication.

V.4 Other HSV-induced enzyme activities

Protein kinase

In addition to these enzymes, virus-induced protein kinase activities have also been described. It has been predicted, from DNA sequence analysis, that HSV-1 gene U_S3 encodes a protein kinase (McGeoch and Davison, 1986). Following infection at least one, and possibly several, protein kinase activities are induced (Blue and Stobbs, 1981) and one of these has been shown to differ biochemically from known cellular enzymes (Purves et al., 1986). This latter enzyme has been extensively purified and shown to react with an anti-peptide antiserum directed against the carboxy-terminus of the predicted product of gene U_S3, suggesting that gene U_S3 indeed encodes a functional protein kinase activity (M C Frame, F C Purves, H S Marsden, D J McGeoch and D P Leader, submitted for publication). A virion-associated protein kinase activity has also been described (Rubenstein et al., 1972; Lemaster and Roizman, 1980) although it now appears possible that this represents the cellular enzyme, casein kinase II (Stevely et al., 1985).

SECTION VI : HSV-INDUCED DNA-BINDING PROTEINS

VI.1 DNA-binding proteins in HSV-infected cell extracts

Infection with HSV induces at least seventeen polypeptides with the ability to bind to DNA (Bayliss et al., 1975; Purifoy and Powell, 1976). Among these are the IE polypeptides and the DNA polymerase and alkaline exonuclease activities described earlier (Bayliss et al., 1975; Purifoy and Powell, 1976; Hay and Hay, 1980), some polypeptides

which comigrate with virion components, and a predominant polypeptide of apparent mol. wt. 21,000 (Bayliss et al., 1975; Powell and Purifoy, 1976). These early studies identified HSV-induced DNA-binding proteins by DNA cellulose chromatography, employing calf thymus or salmon sperm DNA. This approach identifies non-specific DNA-binding proteins, and does not distinguish between the ability of a protein to interact directly with the DNA itself, or to bind DNA indirectly via protein-protein interactions.

Figure 5 shows an example of HSV-induced polypeptides eluted by high salt from a native DNA cellulose column. Some polypeptides are obviously present in high abundance, for example, those labelled MDB, 87, 65 and 21. The protein designated MDB, also known as the major DNA-binding protein (see below, section VI.2), has been purified to homogeneity and shown to bind directly to DNA (Powell et al., 1981), as has the polypeptide designated 65 (Vaughan et al., 1985; see below, section VI.3). The 87K polypeptide probably represents the alkaline exonuclease. Purification procedures used to obtain apparently pure preparations of both the alkaline exonuclease and the DNA polymerase involved as the final step DNA cellulose chromatography (Powell and Purifoy, 1977; Banks et al., 1983), suggesting that these two enzymes probably interact directly with the DNA. Although the polypeptide designated 21 (Vmw21) is obviously one of the more abundant DNA-binding proteins in this example, it is often seen at even higher levels, similar in intensity to the MDB and 65K polypeptide (compare, for example, Bayliss et al. (1975), Figure 6). Vmw21 has yet to be shown to bind directly to DNA.

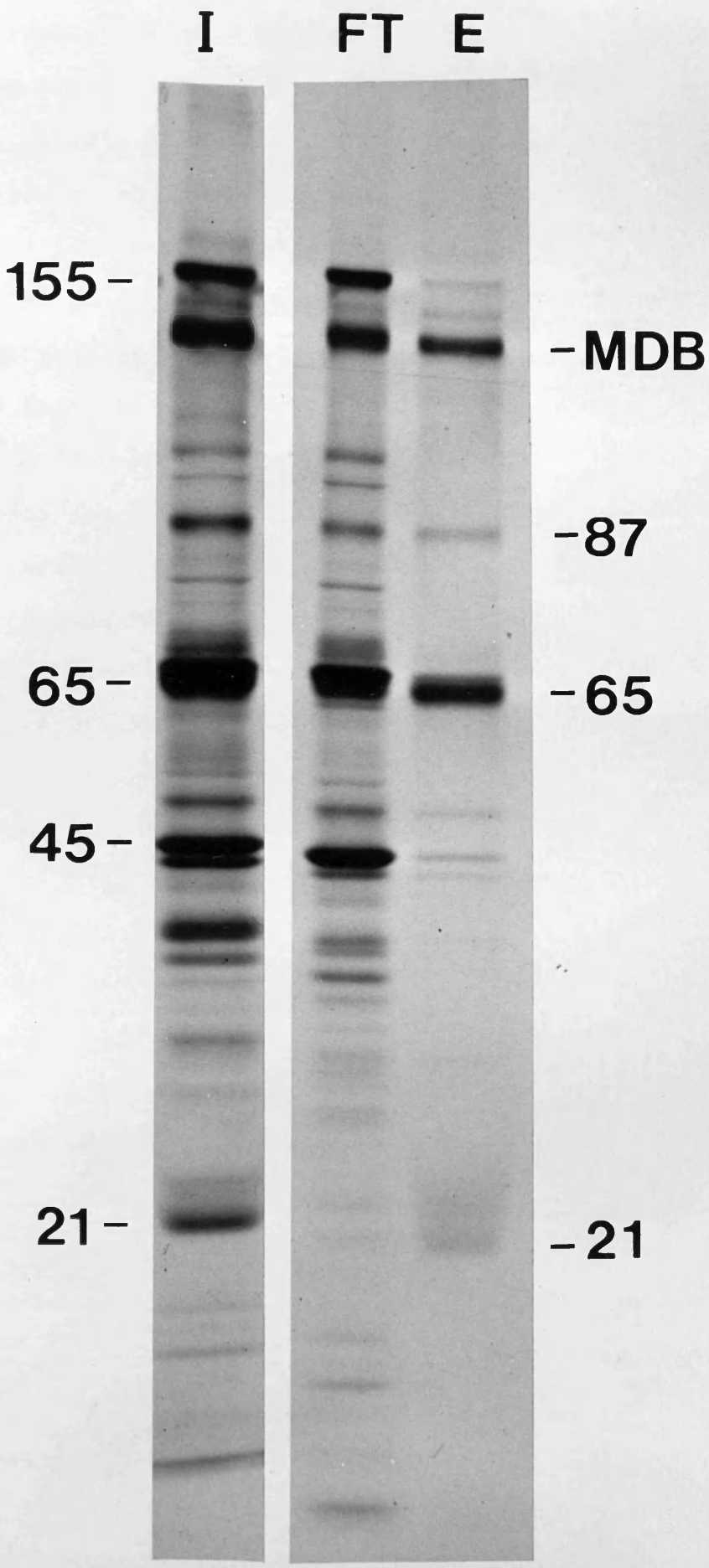
FIGURE 5

Herpes simplex virus-induced DNA-binding proteins. DNA cellulose chromatography was carried out as described in method section V.3. HSV-1-infected cell extracts, harvested by the high salt extraction procedure (method section I.7b) were applied to a double-stranded calf thymus DNA cellulose column in B2 buffer, and the flow-through material collected. Following extensive washing of the column, bound proteins were eluted using 2M NaCl.

I input

FT flow-through

E eluate


I
FT
E
155
65
45
21
MDB
87
65
21

VI.2 The major DNA-binding protein

Until recently the only HSV-induced DNA-binding protein studied in any detail was the so-called major DNA-binding protein (MDB, Vmw130, ICP8). The major DNA-binding protein has been implicated not only in the regulation of viral gene expression (see section IV.3) but also in viral DNA replication. The purified protein has been shown to bind preferentially to single-stranded DNA (Ruyechan and Weir, 1984; Lee and Knipe, 1985), to bind co-operatively to single-stranded DNA, holding it in an extended configuration (Ruyechan, 1983; Lee and Knipe, 1985), to denature poly-deoxyadenylic acid-polydeoxythymidylic acid duplexes at 40°C (Powell *et al.*, 1981), and to stimulate the activity of the HSV DNA polymerase *in vitro* (Ruyechan and Weir, 1984). These are properties shared by helix destabilising proteins, some of which, such as the bacteriophage T4 gene 32 product, and the bacteriophage T7 helix destabilising protein, have also been implicated in DNA replication (Champoux, 1978). In addition, antibody specific for the major DNA-binding protein will inhibit DNA replication in isolated infected cell chromatin (Powell *et al.*, 1981), and *ts* mutants whose lesions map within or near this gene fail to synthesise HSV DNA at the non-permissive temperature (Conley *et al.*, 1981; Littler *et al.*, 1983; Weller *et al.*, 1983).

Although the major DNA-binding protein exhibits no preference for HSV DNA *in vitro* (Powell *et al.*, 1981; Lee and Knipe, 1985), it is thought to do so *in vivo* (Knipe and Spang, 1982; Lee and Knipe, 1983; Leinbach and Casto, 1983; Quinlan *et al.*, 1984). Localisation studies have demonstrated that the protein rapidly migrates to the

nucleus, where its precise location depends on the presence or absence of viral DNA replication. In the presence of viral DNA synthesis it localised to large globular areas within the nucleus (Quinlan et al., 1984) a pattern resembling that seen for pulse-labelled viral DNA (Rixon et al., 1983). A DNase-sensitive nuclear association was described under these conditions for this protein (Knipe and Spang, 1982). In contrast, in the absence of viral DNA synthesis the protein localised to small discrete foci throughout the nucleus, corresponding to a high salt labile association with the nuclear matrix (Quinlan et al., 1984). These different nuclear locations were termed 'replicative' and 'pre-replicative' sites, respectively, by these authors. Temperature shift experiments involving mutants containing ts lesions within the major DNA-binding protein, suggest that this protein can move freely between these different structures. Thus, inhibition of viral DNA replication by shifting to the non-permissive temperature resulted in redistribution of the major DNA-binding protein to the pre-replicative sites, whereas re-initiation of DNA synthesis by shifting down to the permissive temperature resulted in localisation of the protein to the replicative compartment (Quinlan et al., 1984). This occurred both in the presence and absence of continued protein synthesis. The major DNA-binding protein is also present in deoxyribonucleoprotein complexes isolated from infected cell nuclei, and immuno-precipitation using a monoclonal antibody against this protein demonstrated that both intact viral DNA and cell DNA were present in these complexes (Leinbach and Casto, 1983).

In conclusion, therefore, the major DNA-binding protein

would appear to be both involved in, and essential for, viral DNA replication. This may, however, not be its only function. The PRV major DNA-binding protein has been shown to be important in stabilising progeny viral DNA molecules in infected cells, possibly by providing protection against nuclease attack (Ben-Porat et al., 1983), while Ruyechan and Weir (1984) demonstrated that the HSV-1 major DNA-binding protein could bind polyriboadenylic acid with an affinity intermediate between that for single-stranded and double-stranded DNA, suggesting that it may also bind to polyA$^+$ mRNA. This latter observation raises the possibility that the major DNA-binding protein could act to regulate gene expression at a post-transcriptional level. Although Godowski and Knipe (1986) demonstrated that the major DNA-binding protein could influence gene expression at the level of transcription, they did not exclude the possibility of an additional effect at a post-transcriptional level.

VI.3 The 65K DNA-binding protein

The polypeptide ICSP34,35 has been shown to be strongly associated with the DNA polymerase (Vaughan et al., 1984,1985). It is the HSV-2 counterpart of the HSV-1 polypeptide 65K DNA-binding protein ($65K_{DBP}$)(Marsden et al., manuscript in preparation) and is distinct from the 65K transactivating factor (Marsden et al., 1987). It is a strong DNA-binding protein (Bayliss et al., 1975; Powell and Purifoy, 1976; Figure 5), and can bind to DNA in the absence of other proteins, with the possible exception of the HSV DNA polymerase (Vaughan et al., 1985). The function of this polypeptide is at present unknown, but it has recently been shown to be one of seven genes essential for the replication in vivo of plasmid molecules

containing an HSV-1 origin of DNA replication (Challberg, 1986; McGeoch, 1987)

VI.4 Protein complexes involving HSV-induced DNA-binding proteins

Multiprotein complexes are believed to be involved in a number of DNA replication systems (Alberts et al., 1983) and there is now a growing body of evidence to suggest that similar complexes may function in HSV DNA replication. There is both genetic and biochemical evidence for a functional complex involving at least four virus-specific polypeptides - the major DNA-binding protein, the alkaline exonuclease, the DNA polymerase and ICSP34,35 ($65K_{DBP}$). Purification of the DNA polymerase through a series of chromatographic stages was often found to result in the purification of a major 150K polypeptide apparently contaminated with a lower mol. wt. protein later identified as ICSP34,35 (Powell and Purifoy, 1977; Knopf, 1979; Ostrander and Cheng, 1980; Vaughan et al., 1985). That this polypeptide was strongly associated with the DNA polymerase was confirmed by Vaughan et al. (1984) who used monoclonal antibodies against the major DNA-binding protein, alkaline exonuclease and ICSP34,35 for immuno-affinity chromatography of infected cell extracts. These authors demonstrated (i) that a monoclonal antibody against the alkaline exonuclease would purify not only the alkaline exonuclease but also the major DNA-binding protein, and to a lesser extent the DNA polymerase and ICSP34,35; (ii) that a monoclonal antibody against the major DNA-binding protein purified, in addition to the major DNA-binding protein, the alkaline exonuclease and ICSP34,35; and (iii) that a monoclonal antibody against ICSP34,35

resulted in the purification of the DNA polymerase and major DNA-binding protein, as well as ICSP34,35, with a low level also of the alkaline exonuclease. Thus, these authors suggested that there was a very strong association between the major DNA-binding protein and the alkaline exonuclease, and between the DNA polymerase and ICSP34,35 while there was an association of intermediate strength between the major DNA-binding protein and ICSP34,35.

That this putative complex is functional *in vivo* is suggested by studies involving *ts* mutants. A mutant of HSV-2 containing a *ts* lesion within the alkaline exonuclease gene was found to induce a DNA polymerase activity which was also temperature-sensitive *in vivo* (Francke and Garrett, 1982). In addition, *ts* lesions within the major DNA-binding protein have been shown to destabilise both the alkaline exonuclease and DNA polymerase activities (Purifoy and Powell, 1981; Littler *et al.*, 1983), and to induce secondary mutations within the DNA polymerase gene, resulting in altered sensitivity to the DNA polymerase inhibitors, phosphonoacetic acid and aphidicolin (Chiou *et al.*, 1985). These secondary mutations may be compensatory, perhaps restoring a functional interaction between the DNA polymerase and the altered major DNA-binding protein.

VI.5 Interactions between HSV DNA and virus-induced polypeptides

The association of virus-induced polypeptides with HSV DNA *in vivo* has not been extensively studied. Herpes simplex virus DNA does not show a predominantly nucleosomal structure and is therefore sensitive to nuclease attack on isolated nuclei (Leinbach and Summers, 1980). Pignatti *et al.* (1979) have purified what they term

'replication complexes' from HSV infected cells, and shown that these contain viral, but not cellular, DNA polymerase activity, and intact viral DNA. Isolated, non-deproteinised HSV DNA was found to be relatively resistant to 3', but not 5', exonuclease attack, leading to the suggestion that protein(s) might be bound at the 3' hydroxyl terminus of HSV DNA (Kudler and Hyman, 1979). This suggestion was supported by the electron microscopy observations of Wu *et al.* (1979) who found evidence for protein association with the direct terminal repeat regions of HSV DNA (the 'a' sequence), and the corresponding internal repeat regions. The sensitivity of this association to alkaline denaturation of the DNA led these authors to suggest that this was not a covalent attachment. Four polypeptides attached non-covalently to HSV DNA were described by Hyman (1980) following *in vitro* iodination of purified viral DNA. These polypeptides had apparent mol. wt. of 71K, 50K, 20K and 16K but their identity remains unknown. It is to be expected that proteins involved in, for example, the replication or packaging of HSV DNA, would be found to be associated with viral DNA *in vivo*, but little is known about the functions of the above described polypeptides.

VI.6 Sequence-specific DNA-protein interactions in HSV

Sequence-specific interactions might be expected between viral DNA sequences and polypeptides directly involved in processes such as transcription, recombination, replication and packaging of viral DNA. Only recently has evidence been provided for such interactions. Thus, IE polypeptide Vmw175 has been shown to specifically interact

with promoter-regulatory regions of a number of HSV genes (Beard et al., 1986; Faber and Wilcox, 1986; Kristie and Roizman, 1986); Vmw21 and Vmw22 have been shown to specifically interact with the HSV-1 'a' sequence, a sequence involved in recombination and packaging of the virus genome (Dalziel and Marsden, 1984); and a virus-induced protein(s) has been shown to specifically interact with an HSV-1 origin of viral DNA replication, ori_S, (Elias et al., 1986).

(a) Specific interactions between promoter-regulatory regions and Vmw175

As already mentioned (section IV) Vmw175 is phosphorylated, located predominantly within the nucleus of infected cells, and binds relatively strongly to DNA (Powell and Purifoy, 1976; Pereira et al., 1977; Marsden et al., 1978, 1982; Hay and Hay, 1980; Wilcox et al., 1980). The use of monoclonal antibodies and a gel retardation assay demonstrated the presence of Vmw175 in specific complexes formed between promoter-regulatory domains of selected IE, early and late genes and infected cell polypeptides (Kristie and Roizman, 1986a). Multiple binding sites were thought to be present in at least some of these promoter-regulatory regions. Using a similar assay, and by DNase I footprinting, Muller (1987) demonstrated an association between Vmw175 and its own transcription start site. Beard et al. (1986) demonstrated that Vmw175 in a partially-purified form (claimed to represent approximately 5% of the total protein) would form complexes with sequences spanning the 5' end of the gD gene, as assessed by immunoprecipitation of radiolabelled fragments of DNA. Again several binding sites were indicated, the strongest association being with a

fragment spanning the region -263 to +11 with respect to the mRNA start site, with adjacent fragments being more weakly bound. Footprinting experiments demonstrated that partially purified preparations of Vmw175 protected a region from -81 to -111 with respect to the gD RNA capsite from DNase I digestion (Faber and Wilcox, 1986). Two additional binding sites located at the 5' end of the tetracycline resistance gene of pBR322 were also protected in footprinting experiments and showed sequence homology with the protected gD region. Thus, a consensus sequence 5'-ATCGTCNNNNYCGRC-3' was suggested to be an essential component of the putative Vmw175 binding site. Interestingly, Kristie and Roizman (1986b) identified a binding site within the promoter region of IE gene 1, protected from digestion by exonuclease III, which showed considerable sequence homology with this consensus.

The relationship between this gD binding site and transcriptional activation by Vmw175 is unclear. Beard *et al.* (1986) demonstrated that partially purified Vmw175 stimulated transcription from this gene *in vitro*, acting at the level of transcription initiation. Interestingly, they also found that purified Vmw175 could stimulate *in vitro* transcription from the HSV gC and VP5 genes (two late genes) but inhibited *in vitro* transcription from IE gene 4 (Pizer *et al.*, 1986), consistent with the known activity of Vmw175 *in vivo* (see section IV). However, Everett (1983) previously demonstrated that sequence elements required for fully regulated transcription from the gD gene reside within 83 bp of the RNA capsite, outwith the binding site identified by Faber and Wilcox. In addition, by scanning HSV sequence data the latter authors concluded

that although sequences homologous with their consensus site were found upstream from IE genes 1 and 3, they could not find similar sequences upstream from the other IE genes nor from the TK gene. Kristie and Roizman (1986a, 1986b), using gel retardation assays, demonstrated the presence of Vmw175 in complexes formed between infected cell polypeptides and the promoter-regulatory domains of IE gene 2, and with a fragment derived from the promoter region of IE gene 3 which showed no apparent homology with the consensus sequence. Thus binding sites other than the consensus sequence must be involved in the interaction between Vmw175 and HSV DNA.

Although there is no evidence for a strong association between Vmw175 and other polypeptides - partially-purified Vmw175 appearing to exist as a homodimer (Metzler and Wilcox, 1985) - it is unclear at present whether Vmw175 can bind directly to DNA. Freeman and Powell (1982) demonstrated that purified Vmw175 failed to bind to DNA in the absence of added cellular proteins, but did so in their presence, suggesting that Vmw175 may only bind to DNA via a cellular polypeptide(s).

(b) Specific interaction between the HSV-1 'a' sequence and Vmw21

The HSV-1 'a' sequence plays an important role in recombination and packaging of HSV DNA (discussed more fully in section X). Using a DNA competition binding assay, Dalziel and Marsden (1984) demonstrated that two virus-induced polypeptides of apparent mol. wt. 21,000 and 22,000 could be preferentially eluted from salmon testes DNA cellulose columns by DNA fragments containing the HSV-1

'a' sequence, but not by control fragments lacking this sequence. The 21K polypeptide was by far the more predominant. Whether this represented a direct or indirect interaction was unknown, and occasionally, but not consistently, three other polypeptides of 140K, 75K and 33K were also eluted. The 21K/22K polypeptides were synthesised predominantly late in infection, and were tentatively identified as Vmw21 and Vmw22 (Marsden et al., 1976). Vmw21 has previously been shown to be a major HSV DNA-binding protein (Bayliss et al., 1975). The relationship between these apparently 'a' sequence-specific DNA-binding proteins and the protein complexes described by Wu et al. (1979) at the ends and joint region of HSV DNA, and also with the 20K polypeptide found by Hyman (1980) to be strongly associated with purified HSV DNA, remains unknown.

(c) Specific interaction between a viral origin of DNA replication and a virus-induced polypeptide(s)

The HSV genome contains three potential origins of DNA replication -ori$_S$, present in two copies, and ori$_L$ (see later, section IX). Elias et al. (1986), using a nitrocellulose filter-binding assay, demonstrated that protein(s) present in nuclear extracts from infected cells could specifically bind fragments of DNA containing the HSV-1 ori$_S$ sequence. This protein(s) was partially purified by phosphocellulose chromatography and shown to elute separately from the DNA polymerase and major DNA-binding protein, while time course experiments indicated that it has similar induction kinetics to these two polypeptides. In DNase I footprinting experiments (Elias et al., 1986) the partially-purified protein protected a region of 18 bp which over-

lapped one of the two 45 bp inverted repeats which form the 90 bp minimal functional origin (Stow and McMonagle, 1983). However, it has yet to be established whether this ori_S-binding protein plays a direct role in the initiation of viral DNA replication.

SECTION VII : VIRION POLYPEPTIDES

VII.1 Structural polypeptides and the HSV virion

Herpes simplex virus virions are composed of four main structural elements, the core, capsid, tegument and envelope (reviewed recently by Dargan, 1986; see Figure 6A). Within the nuclei of infected cells empty nucleocapsids devoid of any core structure can be seen, in addition to the full nucleocapsids containing DNA - the A and B capsids, respectively, of Gibson and Roizman (1972). Mature enveloped virions are seen only in the cytoplasm of infected cells. The protein composition of the different capsid forms is summarised in Table 1.

The core of the HSV virion consists of DNA spooled around a central cylindrical mass with regular 4-5nm spacing (Furlong et al., 1972). Gibson and Roizman (1972) suggested that the core protein might be the virion polypeptide VP21, since this was the only protein absent from empty nucleocapsids but present in full nucleocapsids and virions. VP21 is probably equivalent to the virion polypeptide Vmw43. Interestingly, Vmw43 comigrates in SDS-polyacrylamide gels with a major HSV-1 DNA-binding protein (Bayliss et al., 1975). The cylindrical core structure appears to be connected to the inner poles of the capsid, and so may provide a mechanical support for the orderly winding of

FIGURE 6

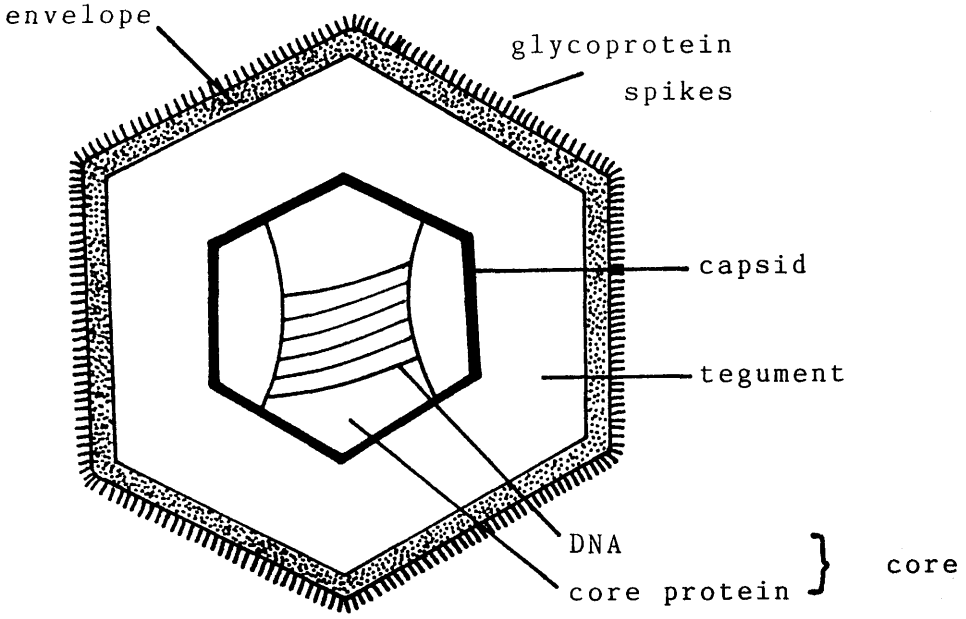
Herpes simplex virus virions.

A. Schematic diagram of HSV virions. The virions are composed of four main structural elements - the core, capsid, tegument and envelope. Within the core, viral DNA is spooled around a cylindrical protein mass. The virion envelope contains a number of glycoproteins, visible on electron microscopy as spikes protruding from the envelope.

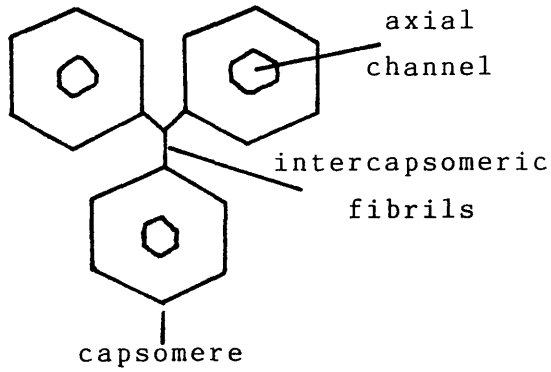
B. Intercapsomeric fibrils. The capsid is composed of 150 hexameric and 12 pentameric capsomeres. Adjacent capsomeres appear to be connected at their vertices by a matrix of intercapsomeric fibrils.

C. Six-fold symmetry of the hexameric capsomeres. Hexameric capsomeres are believed to have six-fold symmetry, probably consisting of six molecules of the major capsid protein, Vmw155.

A.


envelope
glycoprotein
spikes
capsid
tegument
DNA
core protein
core

B.


axial
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intercapsomeric
fibrils
capsomere

C.

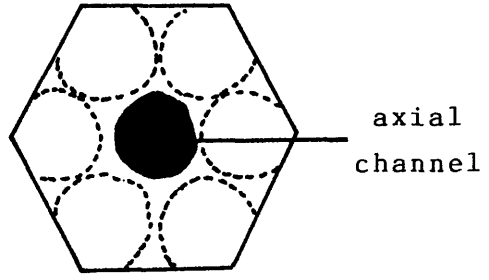

axial
channel

Table 1 : Protein composition of nucleocapsids

Protein nomenclature is given as in references 1, 2 and 3. The presence (+) or absence (-) of a given protein from empty or full nucleocapsids (Gibson and Roizman, 1972) is indicated.

References

1. Gibson and Roizman, 1972
2. Cohen et al., 1980
3. Heilman et al., 1979
4. Spear and Roizman, 1972
5. Marsden et al., 1976
6. Vernon et al., 1981
7. Steven et al., 1986
8. Braun et al., 1984b
9. Heine et al., 1974
10. Braun et al., 1984a
11. Gibson and Roizman, 1974
12. Zweig et al., 1979a
13. Zweig et al., 1980
14. Preston et al., 1983
15. Knopf and Kaerner, 1980
16. Frazer J Rixon, personal communication

Table 1 : Protein composition of nucleocapsids

PROTEIN (1)	(2)	(3)	PRESENCE IN NUCLEOCAPSIDS : EMPTY	FULL	NOTES
VP5	NC-1	p155	+	+	Major capsid protein (4,5). Probably major hexameric constituent (6,7).
VP19C	NC-2	p50	+	+	DNA-binding protein and possibly internal capsid protein - thus suggested may function in packaging and anchoring virus DNA in capsid (8). Present at similar molarity to VP5 (9). Also been suggested as possible pentameric constituent (6).
VP21	-	-	-	+	Minor component and possible core protein (1). Possibly unprocessed form of ICP35 family (VP22a/VP22) (8,10).
VP22a	NC-3 (NC-4)	p40	-	+	(1,10). Absent from virions (1), possibly processed to VP22 (11). Believed to coat surface of full capsids (6,10), and thus may interact with tegument proteins and facilitate envelopment (1).
			+	-	(16). Location, however, controversial. Multiple processed forms (10,11,12,13); processing is essential for virus DNA encapsidation (14).
VP23	NC-5	p32	+	+	Present at surface of empty capsids (10).
VP24	NC-6	p25	+	+	Minor component (1), possibly a phosphoprotein (15).

the viral DNA (Nazerian, 1974; Ŝmíd et al., 1977).

The core is enclosed within an icosahedral capsid, consisting of 150 hexameric and 12 pentameric capsomeres (Wildy et al., 1960). These are described as hollow, elongated polygonal prisms whose axial channel tapers in width from the outer to the inner capsid surface (Wildy et al., 1960; Steven et al., 1986). Adjacent capsomeres appear to be connected at their vertices by a matrix of intercapsomeric fibrils (Vernon et al., 1974; Palmer et al., 1976; see Figure 6B). The hexameric capsomeres are generally believed to have six-fold symmetry (Furlong, 1978; Steven et al., 1986; see Figure 6C) and it is probable that each capsomere is composed of six molecules of the major capsid protein, Vmw155 or p155 (Spear and Roizman, 1972; Marsden et al., 1976; Vernon et al., 1981; Steven et al., 1986). In addition, the virion protein p50 (VP19, NC2) may form part of the pentameric capsomeres (Vernon et al., 1981). Indeed, Zweig et al. (1979b) have shown that p50 and the major capsid protein, p155, may be present in a disulphide-linked complex in HSV-2 nucleocapsids. Similarly, p40 (VP22a) appeared to form disulphide-linked complexes, but the components of these could not be resolved. Surface iodination experiments (Braun et al., 1984a) suggested that p40 coats the surface of full but not empty nucleocapsids, strengthening the previous suggestion that this protein might be involved in the binding of tegument proteins and the subsequent envelopment of the nucleocapsids (Gibson and Roizman, 1972). However, immune electron microscopy studies, using two different monoclonal antibodies, have localised p40 on partially-cored rather than full nucleo-capsids, and shown this polypeptide to be present on

nucleocapsids formed at the non-permissive temperature following infection with DNA-positive, packaging-negative ts mutants (Frazer J Rixon, personal communication). The apparent absence of p40 in these studies from full nucleocapsids would appear to be in direct contrast to the findings of Gibson and Roizman (1972) and Braun et al. (1984a). One possibility is that the procedure used by these latter authors to purify nucleocapsids may have resulted in preparations containing both full and partially-cored nucleocapsid structures. The true location of the p40 polypeptide is therefore at present unresolved. Studies involving a ts mutant containing a lesion within the gene for p40 suggest that processing of this polypeptide may be essential for the encapsidation of viral DNA (Preston et al., 1983).

The tegument is a rather ill-defined layer which lies between the capsid and the envelope of the virion. Proteins believed to reside within the tegument include the very high molecular weight polypeptides VP1-3 (>200K) which are thought to intercalate between the capsid and the envelope (Gibson and Roizman, 1972) and the 65K virion transactivating factor (Batterson and Roizman, 1983; Campbell et al., 1984). A protein kinase activity has also been associated with capsid-tegument structures (Lemaster and Roizman, 1980).

Only those nucleocapsids containing full genome-length fragments of DNA become enveloped. The envelope, acquired by budding through the inner nuclear membrane (Darlington and Moss, 1968) contains several viral glycoproteins, and these have a number of important roles in the virus replicative cycle.

VII.2 Glycoproteins specified by herpes simplex virus

Herpes simplex virus type 1 encodes at least seven glycoproteins, gB, gC, gD, gE, gG, gH and gI (Spear, 1976; Marsden et al., 1978, 1984; Baucke and Spear, 1979; Buckmaster et al., 1984; Roizman et al., 1984; Longnecker et al, 1987; McGeoch et al., 1987), all of which, except gI, have been shown to be present in virions (Spear, 1976; Para et al., 1980; Buckmaster et al., 1984; Frame et al., 1986; Richman et al., 1986). An HSV-2 counterpart has been identified so far for gB, gC, gD, gE and gG (reviewed in Spear, 1985; Marsden, 1987). Four of the glycoproteins, gC, gE, gG and gI, are not essential for growth in tissue culture (Hoggan and Roizman, 1959; Heine et al., 1974; Cassai et al., 1975/1976; Holland et al., 1984; Zezulak and Spear, 1984; Longnecker and Roizman, 1986, 1987; Longnecker et al., 1987; Weber et al., 1987). The properties of the glycoproteins are summarised in Table 2.

No single glycoprotein has been identified as a virus receptor. Adsorption to cells appears to be mediated by at least two glycoproteins -gB and gD. Antibodies to gB, and to a lesser extent to gD and gC, inhibited the attachment to cells of virosomes (liposomes containing purified virion polypeptides), while virosomes depleted in either gD or gB bound to cells less efficiently than non-depleted virosomes (Johnson et al., 1984). Although both antibodies against gC and high levels of Fc fragments of IgG can inhibit HSV adsorption (Fuller and Spear, 1985) it seems unlikely that either gC, gE or gI (the latter two glycoproteins form an Fc-binding complex, see below) are essential components of the virus receptor, since all three are dispensable for growth in tissue culture.

Glycoprotein B may also be involved in virus penetration, since ts mutants containing lesions within the gB gene

Table 2 : Properties of the HSV-encoded glycoproteins

GLYCOPROTEIN	POSSIBLE FUNCTIONS
gB	Essential for replication in tissue culture. Implicated in adsorption, penetration and cell fusion.
gC	Not essential for replication in tissue culture. Implicated in adsorption, penetration and cell fusion. gC-1 can act as a C3b-receptor.
gD	No ts mutants yet described. Implicated in adsorption, and in cell fusion.
gE	Not essential for replication in tissue culture. Implicated in adsorption. Interacts with gI to form an Fcɣ-receptor.
gG	Not essential for replication in tissue culture.
gH	Essential for replication in tissue culture. Implicated in cell fusion and in cell-to-cell spread of infectious virus.
gI	Not essential for replication in tissue culture. Interacts with gE to form an Fcɣ-receptor.

adsorb to cells but fail to penetrate (Sarmiento et al., 1979; Little et al., 1981). However, ts lesions lying outwith the gB gene also affect penetration (Addison et al., 1984), suggesting that other polypeptides may also be involved.

Syncytium formation, fusion between individual cells to form large multinucleated cells (syncytia), appears to involve a large number of viral functions, since at least seven, and possibly eight, loci have been mapped to date (reviewed in Spear, 1985; Marsden, 1987). These include gB, gD and gH. Glycoprotein H also appears to be involved in egress of virus from infected cells. A monoclonal antibody against gH inhibits plaque formation, suggesting a possible role for gH in the cell-to-cell spread of infectious virus (Buckmaster et al., 1984).

The HSV-induced glycoproteins are the major virus-specific polypeptides recognised by the host immune response following infection, generating both humoral and cell-mediated immunity. Antibodies capable of neutralising infectious virus in vitro have been described against all the identified HSV glycoproteins, except gI, and passive protection of animals against a potentially lethal dose of HSV is conferred by monoclonal antibodies against gB, gC, gD, gE and gH (reviewed by Marsden, 1987).

It is interesting that three glycoproteins which are apparently non-essential in tissue culture - gC, gE and gI - can interact with key elements of the humoral immune system. Thus, gC-1, but not gC-2, has the capacity to bind to the C3b component of the complement system (Cines et al., 1982; Friedman et al., 1984; Cohen et al., Abstract, 10th International Herpesvirus Workshop, p158), while gE and gI form a functional complex which can bind to the constant (Fc) region of immunoglobulin G (IgG) (Johnson D.C., Stow N.D., Feenstra V., Cross A.C. and Frame M.C., manuscript in preparation). This Fcγ-

receptor binds to IgG from a number of animal species, including man (Baucke and Spear, 1979; Para et al., 1982; Johansson et al., 1984). Although it is not known whether these represent the physiological ligands for gC and the gE/gI complex in vivo, such a function would not seem unreasonable, since these receptors could function in vivo to protect infected cells against immune cytolysis. However, there is no evidence as yet that the presence of these glycoproteins confers a selective advantage in vivo. Indeed, evidence is accumulating which suggests that gC-negative mutants do not show reduced pathogenicity in vivo compared to wild-type virus (Dix et al., 1983; Johnson et al., 1986). In vivo studies involving gE deletion mutants have not yet been reported.

SECTION VIII : STRUCTURE AND ORGANISATION OF THE HSV GENOME

The genome structure of HSV has already been described (section II.3). The organisation of mRNA transcripts for some of the HSV polypeptides can be seen in Figure 7. There is no gross clustering of genes specifying similar functions. Thus, for example, glycoproteins can be found in both L and S, enzyme activities are found in both L and S, and there is no apparent organisation into 'early' and 'late' regions. However, some clustering is evident. The IE genes are all located in or close to the repeat elements, while a group of five consecutive genes within U_S (U_S4-U_S8, see Figure 8) encode known or potential membrane proteins. The significance of this clustering is uncertain.

Analysis of the DNA sequence of U_S and mapping of the mRNA transcripts therein (Rixon and McGeoch, 1984, 1985; McGeoch et al., 1985) reveals a compact organisation of the

FIGURE 7

Organisation of the HSV genome. This diagram shows the HSV-1 genome divided into 0-1 map units. The three origins of viral DNA replication, ori_L and ori_S (see text), are indicated (▼). Below, the locations and orientation of IE genes 1-5, and the genes for a number of other viral polypeptides - including the seven known glycoproteins, some virus-induced enzyme activities and DNA-binding proteins, and a few structural polypeptides - are shown.

exo alkaline exonuclease; pol DNA polymerase; $65K_{DBP}$ 65K DNA-binding protein (ICSP34,35); PK protein kinase; $65K_{TIF}$ virion polypeptide, Vmw65 (65K trans-inducing factor).

Taken from Wagner (1985) and Schaffer et al. (1987).

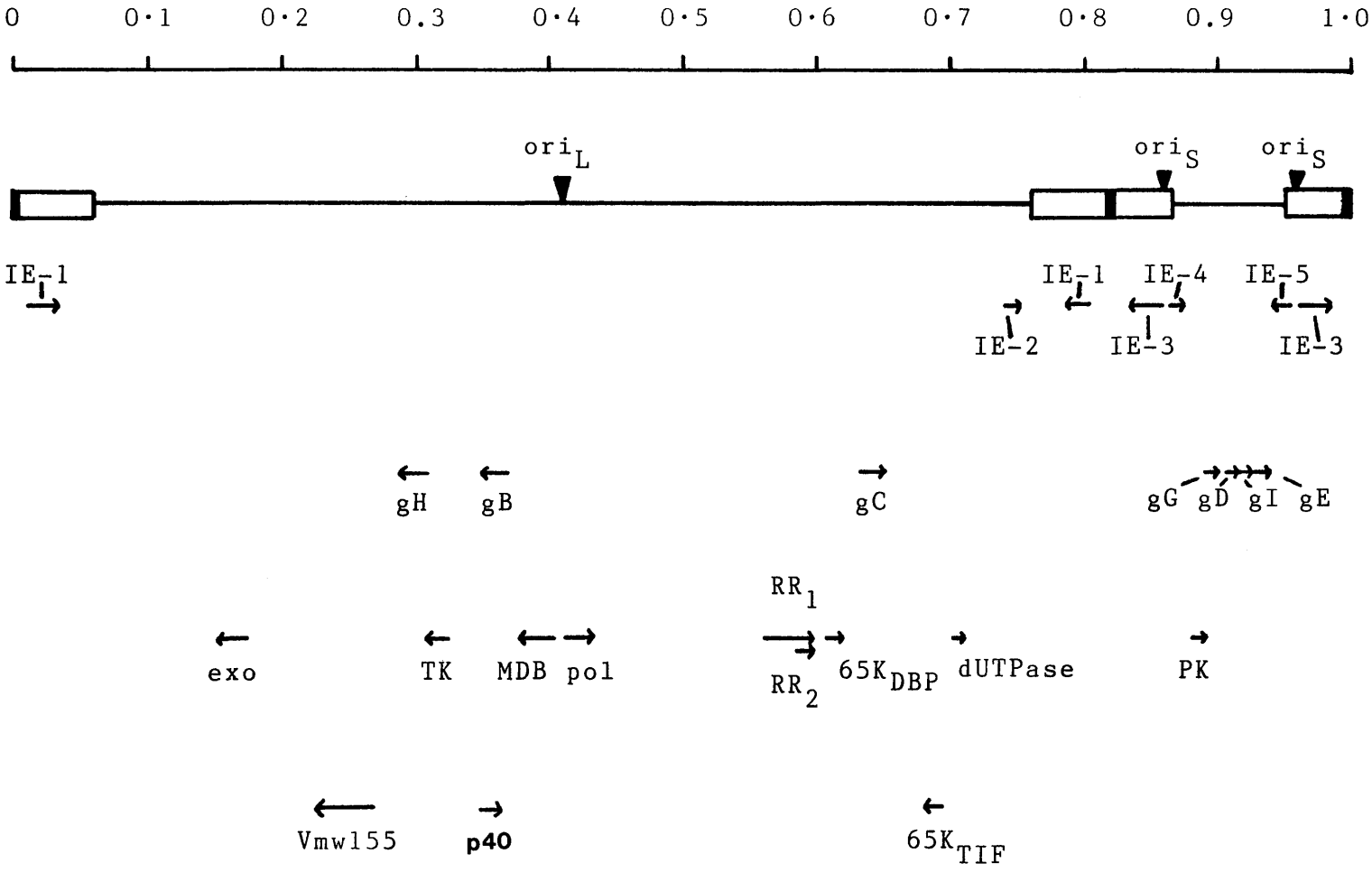
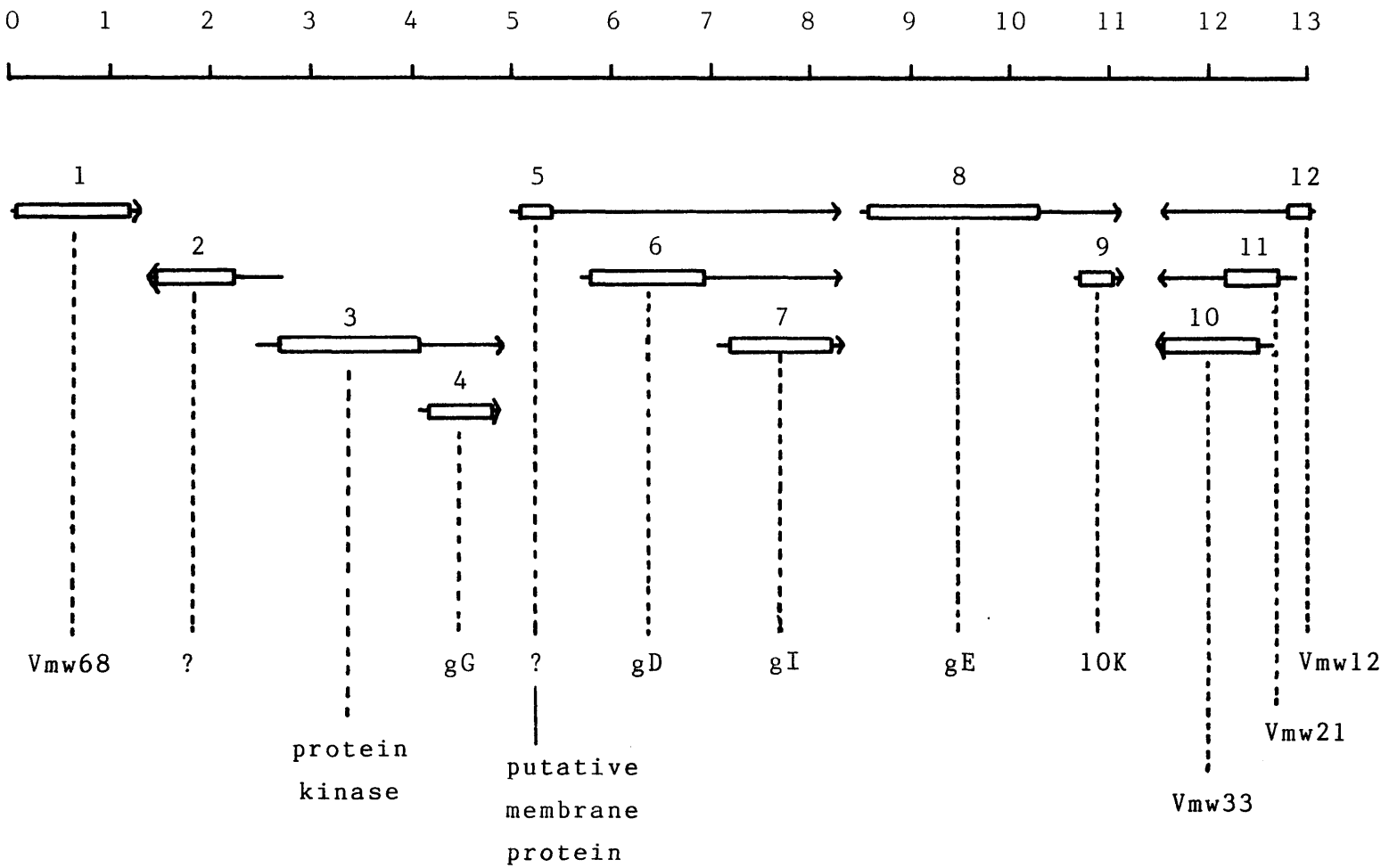

0
0·1
0·2
0.3
0·4
0·5
0·6
0·7
0·8
0.9
1·0
ori L
ori S
ori S
IE-1
IE-1
IE-4
IE-5
IE-2
IE-3
IE-3
gH
gB
gC
gG
gD
gI
gE
RR 1
exo
TK
MDB
pol
RR 2
65K DBP
dUTPase
PK
Vmw155
p40
65K TIF

FIGURE 8

Genome organisation within the short unique region. Adapted from McGeoch *et al.* (1985). The short unique region, approximately 13,000 bp in length, is shown. The transcripts mapped to this region and their predicted coding regions (boxes) are indicated. Where known, the polypeptides encoded by these genes are given.


0 1 2 3 4 5 6 7 8 9 10 11 12 13
1
2
3
4
5
6
7
8
9
10
11
12
Vmw68
?
protein kinase
gG
?
putative membrane protein
gD
gI
gE
10K
Vmw33
Vmw21
Vmw12

HSV genome (Figure 8), and illustrates the point that 3' co-terminal families of mRNAs occur relatively frequently in HSV (Wagner, 1985). One interesting observation concerns the location of the putative origins of DNA replication - ori_S, which is present in two copies, and ori_L. Both origins are located between divergently transcribed genes, but the significance of this location is unclear (Figure 7). These are discussed in more detail below.

SECTION IX : HERPES SIMPLEX VIRUS DNA REPLICATION

The precise mechanism by which HSV DNA replicates is poorly understood. Following infection viral DNA rapidly accumulates in the nucleus, where it appears to circularise (Davison and Wilkie, 1983; Poffenberger and Roizman, 1985). Large tangled masses of replicating DNA have been described on electron microscopy (Hirsch et al., 1977; Jacob and Roizman, 1977), and late in infection HSV DNA is found in a very rapidly sedimenting form, relatively lacking in termini (Jacob et al., 1979; Jongeneel and Bachenheimer, 1981). Thus, viral DNA may replicate via a rolling circle mechanism, generating long head-to-tail concatemers of viral DNA. However, whether the circular HSV DNA is initially amplified via bidirectional, or theta type, replication - as may be the case for PRV (Ben-Porat and Tokazewski, 1977; Jean et al., 1977) - is unknown.

Electron microscopic studies (Friedmann et al., 1977; Hirsch et al., 1977) and studies on defective virus DNA (Frenkel et al., 1975; Kaerner et al., 1979) suggested that there were at least two separate cis-acting signals for HSV DNA replication, one near the middle of U_L (ori_L)

and one in the short repeat region (ori_S) and hence present in two copies in the virus genome. Direct evidence that these regions contained a functional origin of DNA replication was provided by Frenkel and coworkers, who showed that purified monomeric units of defective virus DNA could be amplified by cotransfection with helper wild-type virus DNA, producing long concatemers of head-to-tail repeating units (Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982; Spaete and Frenkel, 1985). Extension of this approach to a plasmid system, using cloned fragments of HSV DNA, has since more finely mapped these origins (Mocarski and Roizman, 1982a; Stow, 1982; Weller et al., 1985), and both have now been sequenced (Gray and Kaerner, 1984; Whitton and Clements, 1984; Quinn and McGeoch, 1985; Weller et al., 1985). Interestingly, both ori_L and ori_S are located between two divergently transcribed genes, ori_S lying between the promoter-regulatory regions of the IE genes 3 and 4/5, while ori_L lies between the 5' ends of two early genes, those encoding the DNA polymerase and major DNA-binding protein (Whitton and Clements, 1984; Quinn and McGeoch, 1985). The significance of this location for either replication or transcription is unclear.

Deletion analysis of ori_S has defined a 90 bp fragment which contains all the cis-acting signals required for a functional HSV-specific origin of replication (Stow and McMonagle, 1983). The sequence of this fragment is shown in Figure 9. The minimal origin region contains a 45 bp imperfect palindrome, at the centre of which is an 18 bp A+T rich sequence which appears to be an essential component (Stow, 1985). Sequences lying outwith the palindrome (towards the left hand side in Figure 9) are also essential for

FIGURE 9

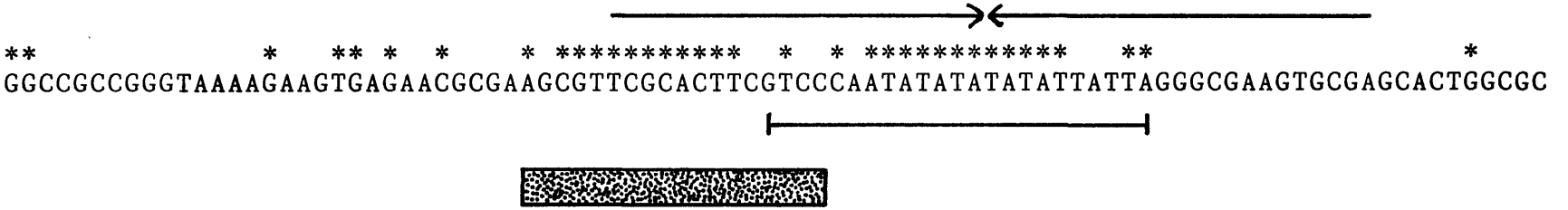
The 90 bp minimal ori_S sequence. The sequence of the 90 bp fragment containing the *cis*-acting sequences required for ori_S function (Stow and McMonagle, 1983) is shown. This represents the upper strand, in the 5'-3' direction, of the ori_S sequence within the short internal repeat.

→← represents the 45 bp imperfect palindrome within this region.

* represents bases conserved between the VZV and HSV ori_S fragments.

⟝⟞ represents the sequence deleted by Stow (1985), resulting in a plasmid which fails to replicate.

 represents the bases on this strand protected by the ori_S-binding protein (Elias *et al.*, 1986).

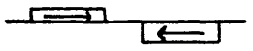

** * ** * * * ********** * * ********** ** *
GGCCGCCGGGTAAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAATATATATATATTATTAGGGCGAAGTGCGAGCACTGGCGC

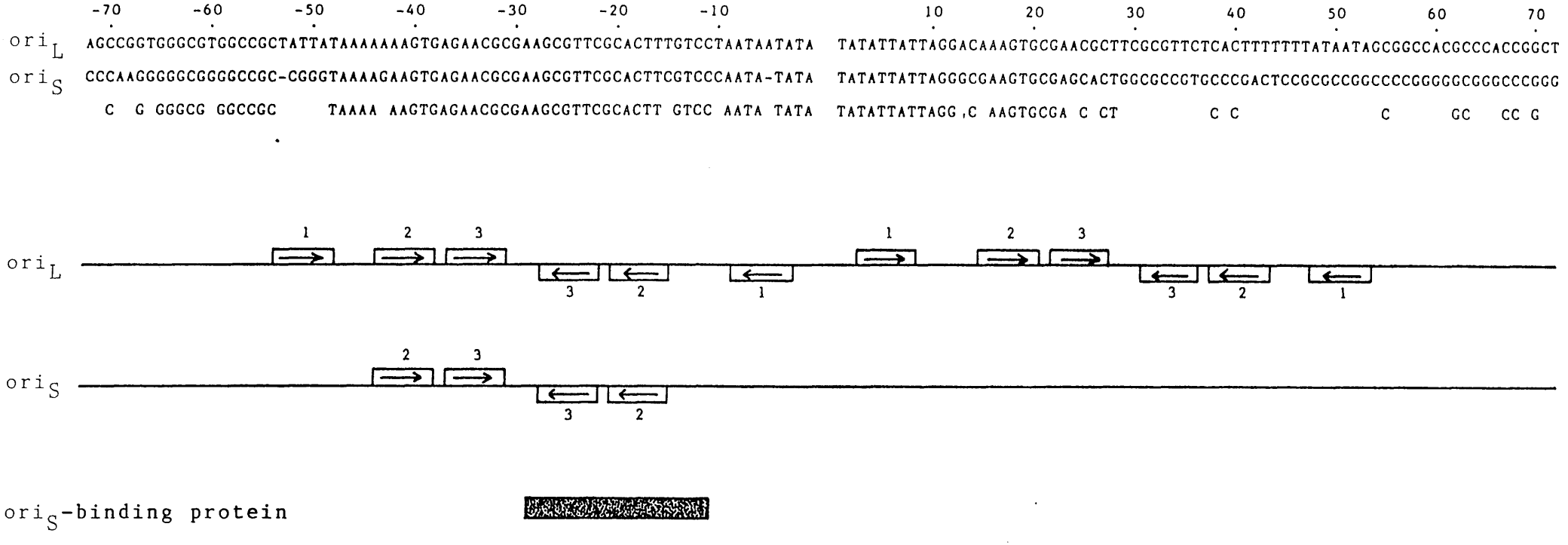
function (Stow and McMonagle, 1983).

Varicella-zoster virus also possesses a functional origin of DNA replication in an analogous position to the HSV-1 ori_S (Stow and Davison, 1986). Comparison of these sequences shows two regions of extensive homology (see Figure 9), the A+T rich region and an 11 bp region to the left of this, overlapping the left hand end of the palindrome. This latter region falls within the region of ori_S protected in the DNase I footprinting studies of Elias et al. (1986). Interestingly, Stow and Davison (1986) found that HSV-1 could stimulate replication from the VZV ori_S.

Considerable homology also exists between the HSV-1 ori_S and a 144 bp perfect palindrome found in the region of ori_L. Weller et al. (1985) demonstrated that a functional ori_L mapped to 0.398-0.413 map units on the virus genome, and within this 425 bp fragment was a perfect 144 bp palindrome. A plasmid containing a 55 bp deletion within this palindrome failed to replicate, suggesting that sequences from within this region are essential for origin function. Out of the 144 bp comprising the palindrome, 90 were shared with ori_S, the greatest homology being near and to the left of the centre of symmetry (see Figure 10). Weller et al. (1985) also described short inverted repeat sequences within each arm of the palindrome (Figure 10), and suggested that these might reflect recognition sites for a DNA-binding protein. Interestingly, ori_S shares two of the three inverted repeat sequences located on the left side of the palindrome, but not those on the right, leading to the suggestion that these differences might reflect directionality of function - i.e. that replication from ori_L could be bidirectional, but that from ori_S unidirectional

FIGURE 10

Comparison between ori_L and ori_S of HSV-1. The 144 bp perfect palindrome from ori_L is compared with the similar region in ori_S. Conserved bases are shown below the two sequences. This comparison is taken from Weller *et al.* (1985). The position of the 3 inverted repeat sequences present in the arms of the ori_L palindrome are indicated  . Two similar inverted repeat sequences found in ori_S are shown below. These are present only in the left hand side of the sequence shown. The 18 bp region protected from DNase I digestion by the ori_S-binding protein is indicated below .


-70 -60 -50 -40 -30 -20 -10 10 20 30 40 50 60 70
ori L AGCCGGTGGGCGTGGCCGCTATTATAAAAAAAGTGAGAACGCGAAGCGTTCGCACTTTGTCCTAATAATATA TATATTATTAGGACAAAGTGCGAACGCTTCGCGTTCTCACTTTTTTTATAATAGCGGCCACGCCCACCGGCT
ori S CCCAAGGGGGCGGGGCCGC-CGGGTAAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAATA-TATA TATATTATTAGGGCGAAGTGCGAGCACTGGCGCCGTGCCCGACTCCGCGCCGGCCCCGGGGGCGGGCCCGGG
C G GGGCG GGCCGC TAAAA AAGTGAGAACGCGAAGCGTTCGCACTT GTCC AATA TATA TATATTATTAGG,C AAGTGCGA C CT C C C GC CC G
ori L
ori S
ori S-binding protein

* (now U_L42 and U_L52, respectively)

φ (U_L30 and U_L29, respectively)

(Quinn and McGeoch, 1985; Weller et al., 1985). Again, it is interesting to note that the region protected from DNase I digestion by the putative ori_S-binding protein includes the leftward reading inverted repeats, as shown in Figure 10. Although Elias et al. (1986) stated that they found no evidence for other sites of protein binding within their ori_S fragment, it is not clear whether these authors also carried out DNase I footprinting on the lower strand. Thus, it is possible that protein binding on the rightward reading inverted repeats may have remained undetected.

The known proteins involved in HSV DNA replication have already been discussed. However, recently Challberg (1986) utilised the plasmid replication system to identify the viral genes required for DNA replication. By cotransfecting with various cloned fragments of HSV DNA an ori_S-containing plasmid could be replicated. This approach identified the DNA polymerase and major DNA-binding protein as essential components of the system. Additional unpublished work by this author, in conjunction with DNA sequence information from Duncan J McGeoch, has identified seven viral genes essential for DNA replication - genes U_L5, U_L8, U_L9 and two genes temporarily designated GC.2L and 63.2L, as well as the DNA polymerase and major DNA-binding protein (McGeoch, 1987). The ribonucleotide reductase and IE genes 1, 2 and 3 were required for optimal effect. The gene designated GC.2L (U_L42) has recently been shown to encode the 65K DNA-binding protein (D S Parris, A Cross, L Haarr, D McGeoch, A Orr, M C Frame, M Murphy and H S Marsden, manuscript in preparation), but the products of the other four genes are at present unknown.

SECTION X : THE STRUCTURE AND FUNCTION OF THE HSV 'a' SEQUENCE

X.1 The 'a' sequence

The 'a' sequence is the direct terminal repeat sequence of the HSV genome and ranges in size from about 250 to 500 bp, varying both between and within strains (Grafstrom et al., 1974, 1975; Sheldrick and Berthelot, 1974; Wadsworth et al., 1975, 1976; Wilkie and Cortini, 1976). It is also present in an inverted orientation at the L-S junction. The structure of the 'a' sequence is summarised in Figure 11. The 'a' sequence itself is terminally redundant, possessing a short (17-21 bp) direct repeat sequence termed direct repeat 1 (DR_1), adjacent to which are two unique regions known as U_b and U_c by virtue of their proximity to the flanking b' and c' sequences, respectively. Between the unique sequences are two further repeat elements, DR_2, a 12 bp repeat unit present in 1 to at least 22 copies and DR_4, a 37 bp sequence present in 1-3 copies (Davison and Wilkie, 1981; Mocarski and Roizman, 1981). Although the precise nucleotide sequence of the 'a' sequence varies from strain to strain, some regions are quite highly conserved. These are depicted in Figure 11. With respect to the virus strains used in this study, the 'a' sequence of HSV-1 strain 17syn$^+$ is approximately 400 bp in length and contains one copy of a DR_4 homology, whereas that of HSV-2 strain HG52 is approximately 250 bp in length and contains one DR_2 and one DR_4 homology (Davison and Wilkie, 1981). These will be described in more detail in later sections.

A single copy of the 'a' sequence is found at the S terminus, but more than one copy may be present at the L

FIGURE 11

Structure of the HSV-1 'a' sequence. A prototype virus genome with, below, an expansion showing the structure of the 'a' sequence in the orientation found at the L-S junction, is presented.

U_b a unique sequence located toward the b' sequence.

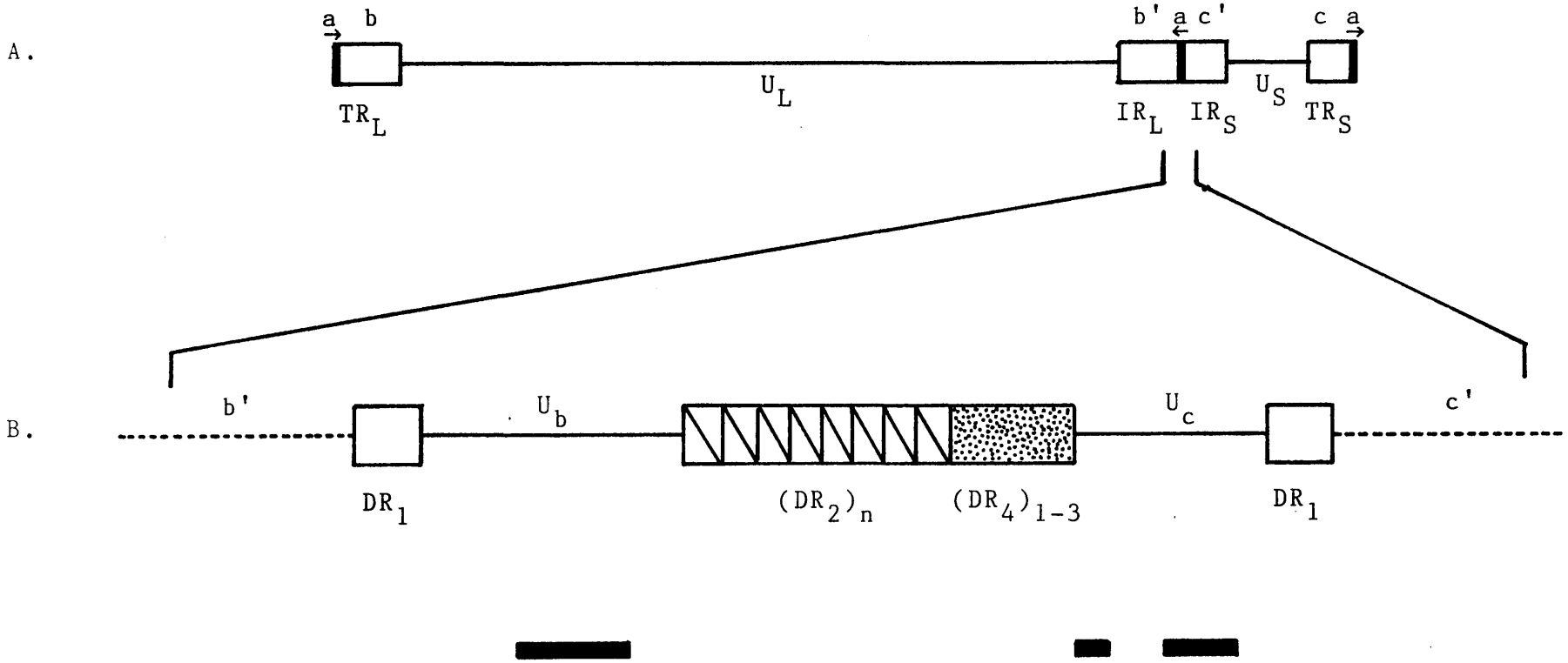
U_c a unique sequence located toward the c' sequence.

DR_1 a 17-21 bp element, present as a direct repeat at the ends of the 'a' sequence.

DR_2 a 12 bp repeat element, present in 1 to at least 22 copies.

DR_4 a 37 bp repeat element, present in 1-3 copies.

▬ indicates regions of unique DNA sequence highly conserved between different strains of HSV.


A.
a b
b' a c'
c a
TR_L
U_L
IR_L
IR_S
U_S
TR_S
B.
b'
U_b
U_c
c'
DR_1
(DR_2)_n
(DR_4)_1-3
DR_1

terminus and L-S junction (Summers and Skare, 1977; Wagner and Summers, 1978; Locker and Frenkel, 1979a). Tandem copies of the 'a' sequence share a single intervening DR_1 (Mocarski and Roizman, 1981), while at the L and S termini the DR_1 element is incomplete (see Figure 12). Mocarski and coworkers (Mocarski and Roizman, 1982b; Mocarski et al., 1985) found that the L termini of both HSV-1 strains F and Justin possess 18.5 bp of a 20 bp DR_1 element (i.e. 18 bp plus a single nucleotide 3' extension) while the S termini possess the remaining 1.5 bp of the DR_1 element. A similar situation was found for HSV-1 strain $17syn^+$ (Davison and Rixon, 1985). In this case the S termini contain only 0.5 bp of the 21 bp DR_1 sequence while the L termini contain the remaining 20.5 bp. In each case the L and S termini together form a complete DR_1.

The 'a' sequence plays an important role in a number of events during the virus life cycle, including circularisation of the genome following infection, site-specific inversion to generate the four genome isomers and cleavage and packaging of unit length monomers from the long linear concatemers generated during viral replication. In addition, the 'a' sequence contains the promoter-regulatory domain of a gene located within the adjacent 'b' sequence (Chou and Roizman, 1986).

X.2 Circularisation

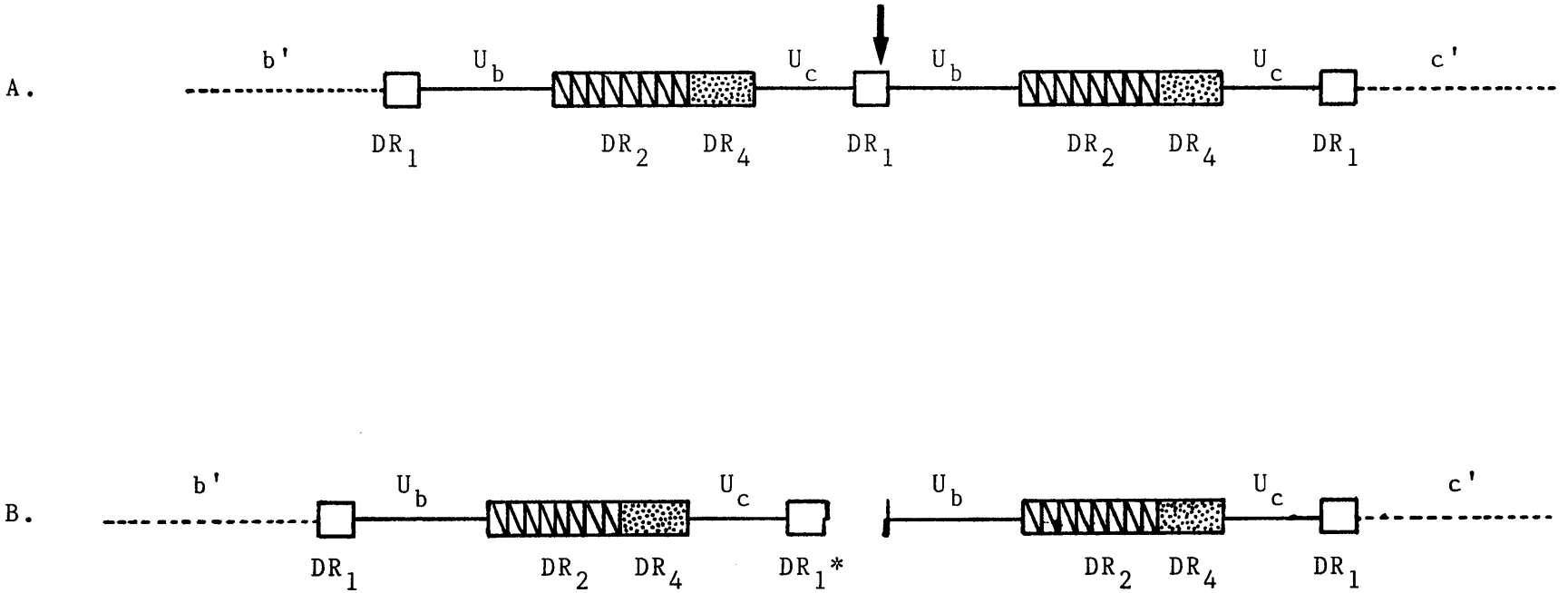
The HSV genome circularises rapidly following infection and a small proportion of packaged viral DNA may already be in this form (Poffenberger and Roizman, 1985). Although it was originally proposed that circularisation could occur via exonuclease digestion of terminal 'a' sequences

FIGURE 12

Structure of L and S termini.

A. Tandem copies of the 'a' sequence share an intervening DR_1. The 'a' sequences are shown in the orientation seen in Figure 11. Cleavage through the intervening DR_1 element (↓) would generate the termini shown in (B).

B. Structure of the L and S termini. Termini are shown in the same orientation as (A). L and S termini possess only partial terminal DR_1 elements (DR_1*), with single nucleotide 3' extensions, such that annealing of an L and an S terminus would form a complete DR_1, as in (A).


A.
b'
U_b
U_c
U_b
U_c
c'
DR_1
DR_2
DR_4
DR_1
DR_2
DR_4
DR_1
B.
b'
U_b
U_c
U_b
U_c
c'
DR_1
DR_2
DR_4
DR_1*
DR_2
DR_4
DR_1
L terminus
S terminus

followed by annealing of the exposed single-stranded ends (Roizman, 1979), it is more likely that circularisation results from direct ligation of termini (Davison and Wilkie, 1983). This latter mechanism is compatible with the structure of the termini shown in Figure 12 and with the observation that intertypic HSV-1/HSV-2 recombinants with heterotypic 'a' sequences at the L and S termini are viable (Davison and Wilkie, 1983). In addition, not all herpesviruses are terminally redundant, for example VZV and PRV (Davison, 1984; Davison and Rixon, 1985) and therefore the genomes of at least these herpesviruses would not be circularised following exonuclease attack. Interestingly, circular PRV DNA molecules have been described following infection (Jean and Ben-Porat, 1976; Jean et al., 1977; Ben-Porat et al., 1980).

Circularisation of the HSV genome is not dependent on viral protein synthesis (Poffenberger and Roizman, 1985) suggesting that ligation of the termini involves either a host or a virion factor. Poffenberger and Roizman (1985) suggested that rapid circularisation of the HSV genome (this was maximal by 30 minutes post-infection, the earliest time point analysed) might be facilitated if the ends of the genome were held closely together following encapsidation, possibly by proteins bound to the terminal 'a' sequences. Proteins which appear to bind to the 'a' sequence have been discussed (section VI).

X.3 Inversion

Inversion of the HSV genome results in the generation of four equimolar populations differing only in the relative orientation of the L and S segments. Whether inversion

occurs simply as a consequence of the structure of the virus genome or whether it plays a biologically important role is unknown. The four genome isomers appear to be functionally equivalent at least in tissue culture, since variants have been isolated which are 'frozen' in P, I_S and I_{LS} configurations, respectively (Preston et al., 1978; Poffenberger et al., 1983; Jenkins and Roizman, 1986). Single plaque isolates of wild-type virus yield equimolar amounts of all four forms, indicating that each isomer must be capable of giving rise to all others. Site-specific recombination need not be postulated for the generation of all four genome isomers. Two of the four isomers could be generated simply through a choice of cleavage sites on concatemeric DNA : i.e. both P and I_{LS} could be generated by cleavage of either a P or I_{LS} concatemer, while both I_S and I_L could be generated by cleavage of either an I_S or I_L concatemer.

Insertion of a copy of the 'a' sequence into the virus TK gene results in additional inversion events between inverted repeats of the 'a' sequence, and deletion events between directly repeated copies (Mocarski et al., 1980; Mocarski and Roizman, 1981, 1982b; Smiley et al., 1981). Smiley et al. (1981) noted that the deleted segments were packaged as appropriately-sized dimers, in approximately equal numbers to complete genomes. Since the deleted segments each contained an origin of DNA replication, it was proposed that the segment was first deleted and then replicated to produce concatemeric molecules from which dimers - of approximately one normal genome length in size - were cleaved and packaged. On the assumption that the recombination events leading to segment deletion were very

similar to those leading to segment inversion, the authors suggested that isomerisation could occur prior to the formation of concatemeric DNA. The site-specific inversion events appear to require the expression of viral genes (Mocarski and Roizman, 1982a).

Deletion studies (Chou and Roizman, 1985) suggest that the unique sequences U_b and U_c are not required for site-specific inversion, nor are two intact copies of DR_1. Inversion was significantly impaired following deletion of the DR_4 sequences, and abolished by further deletion of the DR_2 sequences. Analysis of the products of inversion events suggested that the crossover occurs within DR_2 and possibly DR_4. Thus the signals required for site-specific inversion appear to reside within the direct repeat elements, DR_2 and DR_4. The 'a' sequence of HSV-2 strain HG52, however, contains only a single copy of the DR_2 and DR_4 elements, suggesting that the events leading to inversion are not simply a consequence of the presence of tandem repeats.

Although a number of unique sequences, when inserted in an inverted orientation within the TK gene, do not induce genome rearrangements (Mocarski et al., 1980; Lee et al., 1982; Gibson and Spear, 1983; Pogue-Geile and Spear, 1986), the ability to mediate genome inversion events is not restricted to the 'a' sequence. Sequences from within HSV-1 map co-ordinates 0.706-0.744 induced inversion when inserted in the opposite orientation into the HSV TK gene (Pogue-Geile et al., 1985), as did a fragment derived from the 'c' sequence of the short repeat, albeit at a low frequency (Varmuza and Smiley, 1985). In addition, a virus mutant lacking a junctional 'a' sequence and frozen predominantly in the I_S configuration, generated a minor population of I_{LS} molecules, presumably through recombination

between 'b' sequences within the long repeat (Longnecker and Roizman, 1986). Whether all these inversion events occur by a similar mechanism, or whether there is a specific mechanism operating on the 'a' sequence, remains to be determined.

X.4 Cleavage and packaging

Cleavage of concatemers into unit length genome molecules and packaging of these unit length genomes into viral capsids are tightly linked (Ladin et al., 1980; Deiss and Frenkel, 1986). Thus, DNA-positive ts mutants which fail to encapsidate viral DNA also fail to cleave it, accumulating 'endless' DNA. Temperature shift experiments demonstrated that the appearance of monomeric genome DNA was concomitant with the appearance of full nucleocapsids (Ladin et al., 1980).

Insertion of additional 'a' sequences into the virus genome results in the appearance of novel termini which correspond in position to the inserted 'a' sequence (Smiley et al., 1981; Mocarski and Roizman, 1982b; Varmuza and Smiley, 1985). In addition, although plasmid constructs containing an HSV origin of DNA replication can be replicated they only become packaged and thus propagated in virus stocks as defective particles or 'amplicons' if they also contain an 'a' sequence (Stow et al., 1983; Deiss and Frenkel, 1986). Thus, the HSV 'a' sequence contains signals for the cleavage and packaging of viral DNA.

As mentioned already, the sequence of standard viral L and S termini suggests that cleavage occurs through the shared DR_1 component of two adjacent 'a' sequences (Figure 12). However, nucleotide sequencing of a Justin defective

particle demonstrated that only 4 bp of the DR_1 element were present at the novel U_S-'a' joint, suggesting that a complete DR_1 was not required at each boundary for the cleavage and packaging of the defective DNA (Mocarski et al., 1985). These authors suggested that the recognition signal for cleavage may therefore not reside within the DR_1 repeat itself but rather at an internal site, with cleavage occurring at a measured distance from the internal signal.

By inserting various subfragments of the 'a' sequence into the viral TK gene, Varmuza and Smiley (1985) have shown that the SmaI F fragment (the location of which is indicated later, in Figure 14) contains a functional cleavage and packaging signal. Sequence analysis demonstrated that the novel termini arose by cleavage events outwith the SmaI F insert. Thus, the novel L terminus contained 7 bp of 3' flanking TK DNA, whereas the novel S terminus contained 36 bp of 5' flanking TK DNA. Both termini contained an intact SmaI F fragment. (This is illustrated in Figure 13). Since the precise nucleotide sequences of these termini were unrelated this suggested that the novel L and S termini arose by two distinct cleavage events. Comparison of the relative position of these termini with the standard viral termini (see Figure 13) suggested that their location was dictated by their distance from a signal(s) within the SmaI F fragment (Varmuza and Smiley, 1985). Since the number of DR_2 and DR_4 repeats within different 'a' sequences can vary considerably, and since a subfragment of SmaI F containing the intact DR_2 repeat element was inactive, it was suggested that two separate cleavage/maturation signals reside within the unique U_b and U_c sequences. Furthermore, two subfragments containing an intact U_b and

FIGURE 13

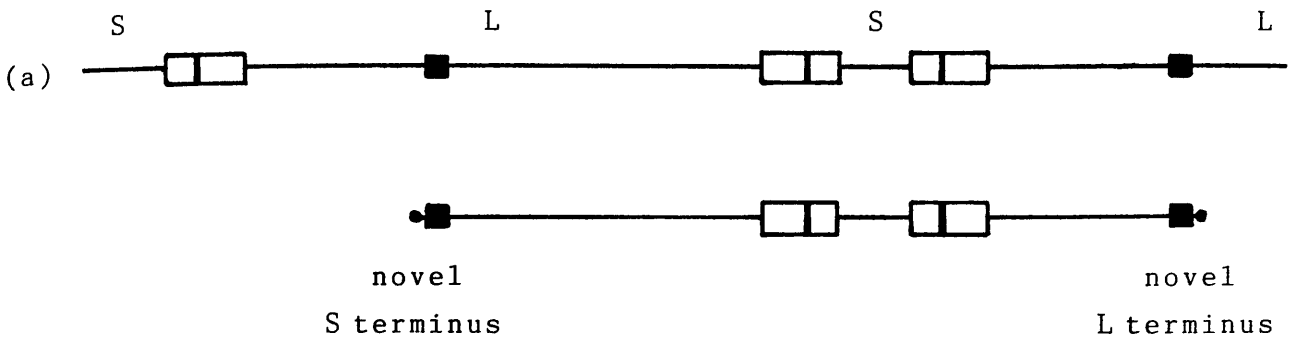
Generation of novel termini by insertion of additional copies of the 'a' sequence into the HSV-1 genome. This figure summarises the results of Varmuza and Smiley (1985). The Sma I F fragment (see Figure 14c) was inserted into the TK gene of HSV-1 strain KOS. Novel L and S termini, generated by cleavage outwith the Sma I F fragment, were detected.

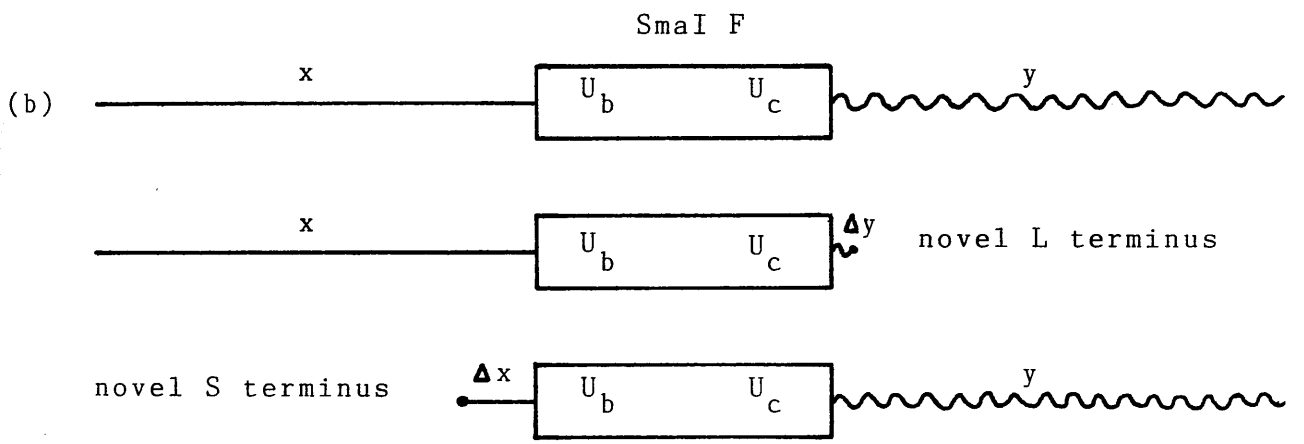
(a) Structure of a virus genome compatible with the novel termini. ■ indicates the Sma I F insert.

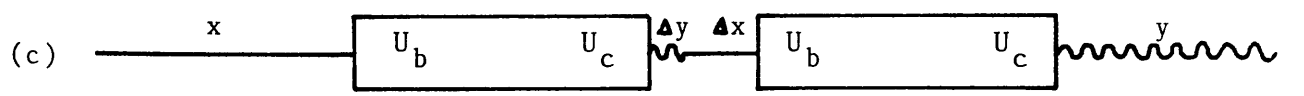
(b) Structure of the novel termini. The Sma I F fragment, inserted into TK, is shown. The flanking sequences are arbitrarily designated x and y. The novel L terminus contains an intact Sma I F fragment, plus 7 bp of flanking y sequence (Δy). The novel S terminus contains an intact Sma I F fragment plus 36 bp of flanking x sequence (Δx).

(c) Structure of a double Sma I F joint. Sequencing and restriction enzyme analysis of double Sma I F joints suggested that these joints had been formed by direct ligation of the novel L and S termini - i.e. the joint contained 2 Sma I F fragments, separated by the Δy and Δx sequences.

(d) Alignment of novel termini with standard viral termini. Although no sequence homology exists between the cleavage sites, their position relative to the Sma I F fragment is well conserved. Standard termini are located 37 bp (S) and 5 bp (L) from the Sma I F fragment, compared with the novel termini which are located 36 bp (S) and 7 bp (L) from the Sma I F fragment.


S
L
S
L
(a)
novel
S terminus
novel
L terminus


SmaI F
(b)
x
U_b
U_c
y
x
U_b
U_c
Δy
novel L terminus
novel S terminus
Δx
U_b
U_c
y


(c)
x
U_b
U_c
Δy
Δx
U_b
U_c
y


(d)
S_{37}
S_{36}
SmaI F
L_5
L_7

an almost intact U_c, respectively, were individually inactive, suggesting that these two signals acted in concert.

These conclusions were supported by the recent deletion analysis of Deiss et al. (1986), who found that HSV amplicons containing 'a' sequences with deletions within U_c failed to be packaged, while those containing 'a' sequences deleted within U_b were only packaged if they first acquired an insertion of a wild-type 'a' sequence derived from the helper virus DNA.

The role of the DR_2 and DR_4 repeat elements is unclear. Since the HSV-2 strain HG52 contains only a single copy of the DR_2 and DR_4 repeat elements, it follows that one copy of these elements must be sufficient for cleavage and encapsidation of viral DNA. In addition, different strains of HSV can efficiently package heterologous 'a' sequence-containing DNA (Vlazny and Frenkel, 1981; Deiss and Frenkel, 1986) suggesting that the cleavage and packaging signals might reside within those regions of the 'a' sequence which are highly conserved. Davison and Wilkie (1981) described an approximately 28 bp region within U_b which was highly conserved between HSV-1 and HSV-2. Homologous sequences were subsequently found to be present in VZV, PRV (Davison and Rixon, 1985), EBV and HVS (Deiss et al., 1986). Deiss et al. (1986) termed this region of homology pac-1, and described a second, less strong, homology within U_c, which they termed pac-2. The pac-1 and pac-2 'consensus' sequences are shown in Figure 14. Interestingly, Spaete and Mocarski (1985) had previously noted sequence homology between CMV and HSV within the region of pac-1, and suggested that this might be responsible for the observed ability of a CMV 'a' sequence to serve as a cleavage/packaging signal for HSV.

FIGURE 14

Conserved regions within the 'a' sequence.

(a) Structure of the HSV-1 'a' sequence.

(b) Unique DNA sequences conserved between different strains of HSV.

(c) The Sma I F fragment of the 'a' sequence.

(d) Pac-1 and pac-2 regions, as defined by Deiss *et al.* (1986). These regions are conserved between a number of herpesviruses (see text).

For HSV, pac-1 has the sequence :

$[C_5][G_6][TGTGTTT^{C}_{T}][G_8]$ HSV-1

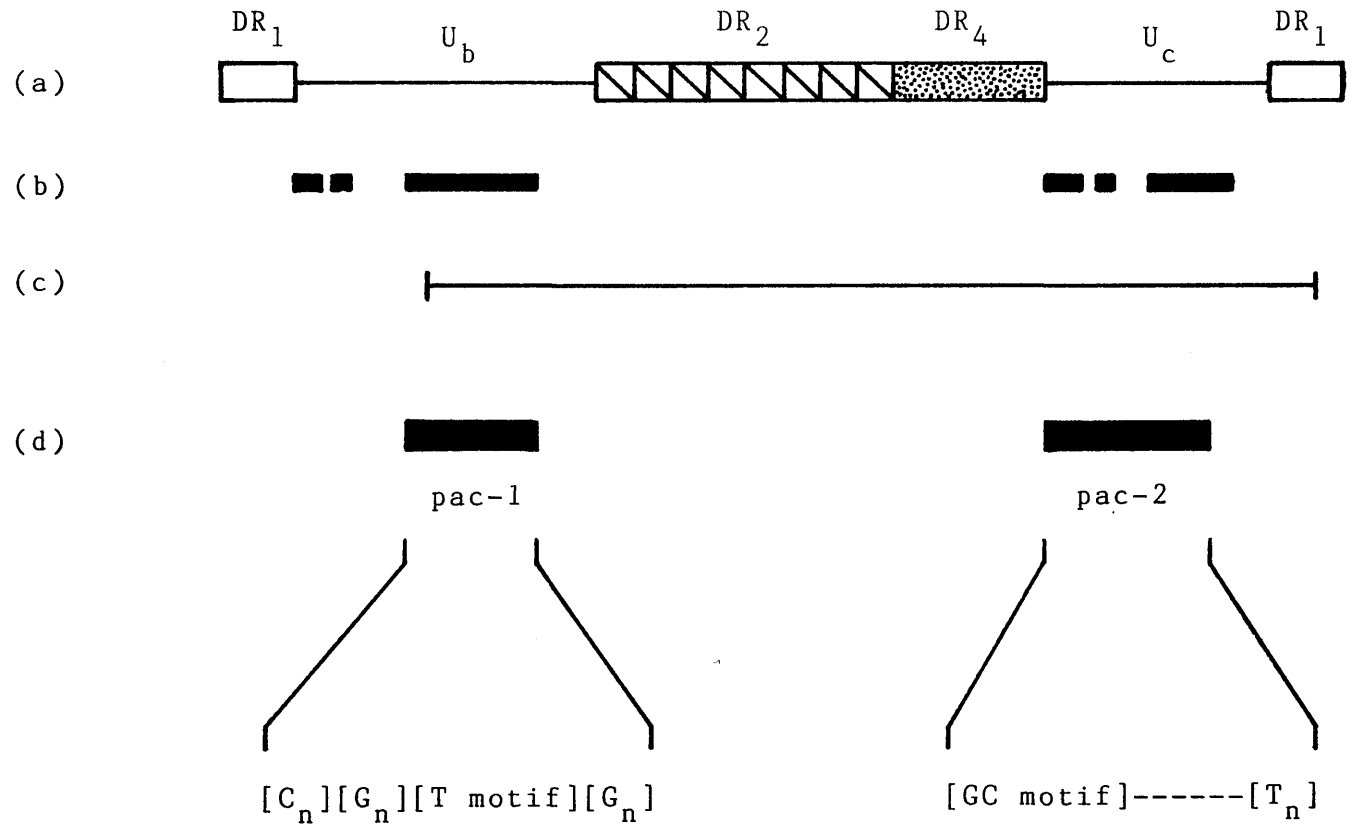
$[C_5][G_8][TGTTTTTT][G_9]$ HSV-2

For HSV, pac-2 has the sequence :

$[CGCCGCG]\text{-}n_{31}\text{-}[T_6]$ HSV-1

$[CGCCGCG]\text{-}n_{29}\text{-}[T_6]$ HSV-2

where n = any nucleotide.


(a)
DR1
Ub
DR2
DR4
Uc
DR1
(b)
(c)
(d)
pac-1
pac-2
[Cn][Gn][T motif][Gn]
[GC motif]------[Tn]

X.5 Models for cleavage/packaging of viral DNA

The mechanism of cleavage/maturation is however unclear. Several models have been proposed and none is proven. These models obviously have to take into account the various products generated by the cleavage and packaging process. Certain features are common to each model. Firstly, the assumption that HSV DNA is packaged directionally from L to S, and that it is the first 'a' sequence encountered that is cleaved, would account for the almost uniform presence of S termini with only a single 'a' sequence (Deiss et al., 1986). As yet, no evidence exists either for or against a directional model for packaging. However, the absolute requirement for the U_c signal (Deiss et al., 1986) might suggest that cleavage and possibly packaging are initiated by this signal, and would support the directional model for packaging. Secondly, following encapsidation cleavage occurs at the next directly repeated 'a' sequence. That a 'headfull' mechanism does not play an overriding role in packaging is suggested by studies with defective genomes (Vlazny et al., 1982), or amplicons (Stow et al., 1986). A significant proportion of defective genomes or amplicons, both of which consist of multiple head-to-tail repeat units, are packaged into nucleocapsids as correctly-terminated fragments containing an integral number of repeat units from one upwards. However, capsids containing these less-than-genome-size molecules do not become enveloped and remain in the nucleus, suggesting a possible 'headfull' mechanism of control at the level of envelopment and transport from the nucleus. Thirdly, L termini appear to be generated by cleavage through the DR_1 element adjacent to U_c, presumably via a U_c-located cleavage signal, while

S termini appear to be generated by cleavage through the DR_1 element adjacent to U_b, presumably via a U_b-located cleavage signal (Mocarski and Roizman, 1982b; Varmuza and Smiley, 1985; Deiss et al., 1986).

A simple model for the cleavage and packaging of HSV DNA is outlined below. Each end of the HSV genome contains an 'a' sequence terminating in an incomplete DR_1 element. These ends become directly ligated following infection, to generate a double 'a' sequence joint, sharing the intervening DR_1. Replication by a rolling circle mechanism then generates concatemeric DNA molecules with interspersed double and single 'a' sequence joints (assuming that the input genome contained a single 'a' sequence at each terminus and at the L-S junction). A putative cleavage/packaging complex encounters a U_c signal and directs a staggered cleavage event through the adjacent DR_1. Packaging ensues, possibly in the L-S direction, and the U_b signal on the next directly repeated 'a' sequence then directs a second staggered cleavage event, again through the adjacent DR_1. Cleavage at the double 'a' sequence joints will generate two single 'a' sequence-containing termini, with no requirement for amplification of 'a' sequences.

Cleavage is probably not restricted to double 'a' sequence joints. Monomeric units from defective molecules or amplicons, containing a single 'a' sequence, are propagated as concatemeric molecules containing an 'a' sequence at each terminus (Deiss and Frenkel, 1986; Stow et al., 1986). A number of models have been suggested to explain this apparent amplification of the 'a' sequence. These are summarised in Figure 15.

FIGURE 15

Models for the cleavage and packaging of viral DNA.

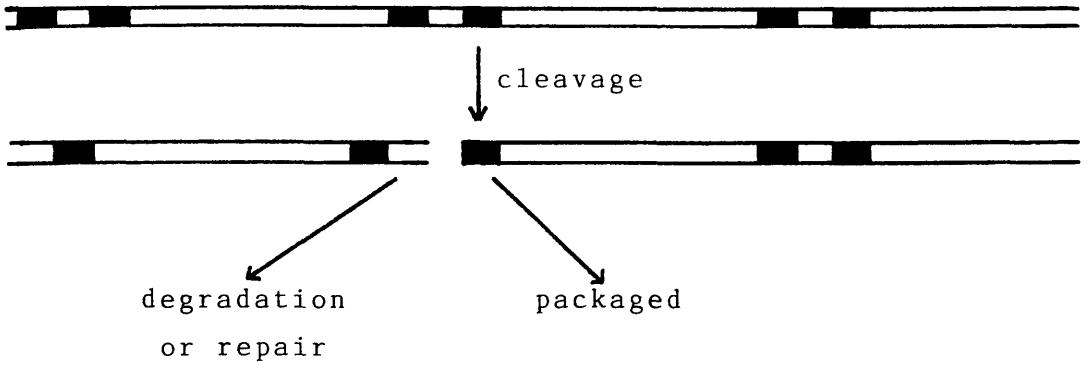
(a) The wastage model (Varmuza and Smiley, 1985; Deiss et al., 1986) proposes that cleavage at a single 'a' sequence generates one functional terminus, containing an 'a' sequence, which is packaged, and one non-functional terminus, lacking an 'a' sequence, which is either rapidly degraded or repaired.

(b) The single-strand nick/repair model (Varmuza and Smiley, 1985) proposes that the cleavage event generates two single-strand nicks at either end of a single 'a' sequence, the strands separate, and are then repaired to generate two 'a' sequence-containing termini which could then be packaged.

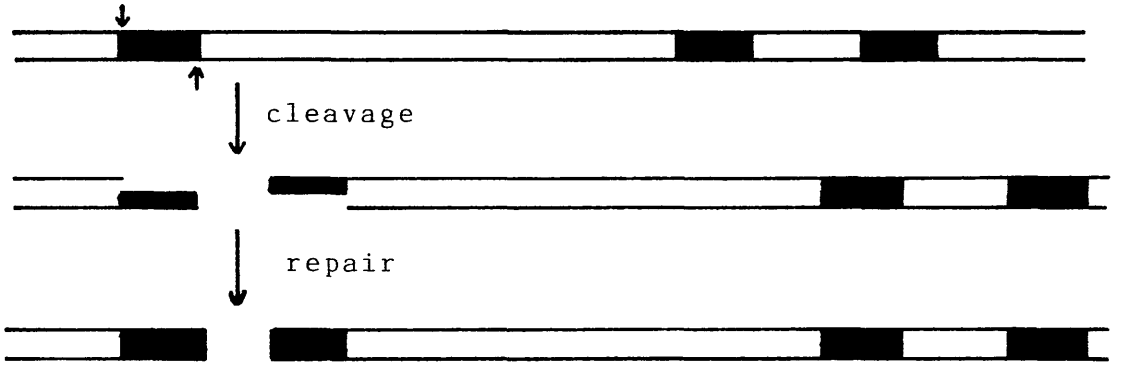
(c) The double-strand gap/repair model (Deiss et al., 1986) is more complicated, and is detailed in Figure 16. Simply, DNA is packaged prior to cleavage, the two directly repeated 'a' sequences then align and are amplified, by a double-strand break/repair mechanism, to generate two double 'a' sequence-containing junctions. Cleavage between the double 'a' sequences then generates 4 'a' sequence-containing termini.

'a' sequences are designated by ▬, or ▭ in (c).

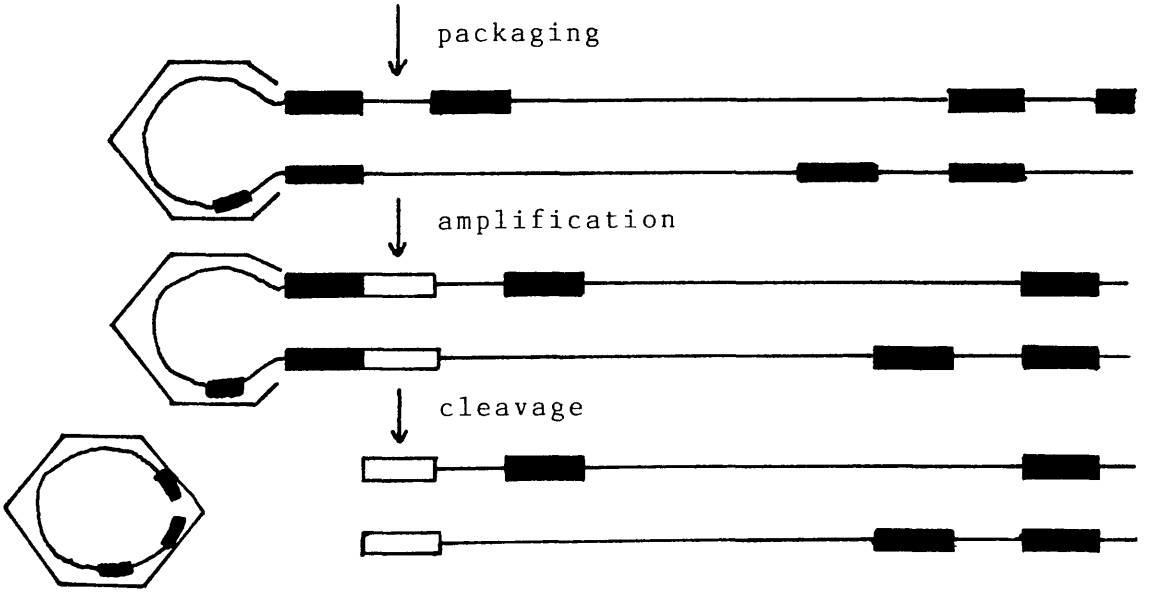
(a) Wastage model



(b) Single-strand nick/repair model



(c) Double-strand gap/repair model



(a) Wastage model

This is the simplest model (Varmuza and Smiley, 1985; Deiss et al., 1986). Cleavage occurs at the single 'a' sequence as before, generating a packaged molecule containing an 'a' sequence at each terminus, but in the process also generating two unpackaged termini lacking an 'a' sequence. Since terminal fragments lacking an 'a' sequence have never been detected, these must be rapidly degraded.

(b) Single-strand nick/repair model

Two other models have been proposed. The single-strand nick/repair model was proposed by Varmuza and Smiley (1985). This model suggests that the putative cleavage/packaging complex recognises the U_b and the U_c signals to generate two single-strand nicks, one at each end of the 'a' sequence. The strands separate and repair synthesis then regenerates an 'a' sequence at both termini. This model would therefore not generate termini lacking an 'a' sequence and would allow amplification of 'a' sequences. However, this model fails to account for the generation of the 3' single nucleotide extensions found at the L and S termini.

(c) Double-strand gap/repair model

The double-strand gap/repair model was proposed by Deiss et al. (1986), and is detailed in Figure 16. In this model, packaging is initiated as before, but without cleavage. When the next directly-repeated 'a' sequence is encountered the two 'a' sequences become aligned and a U_c-directed double-strand cleavage occurs through the DR_1 element of, for example, the L-terminal 'a' sequence (designated junction A in Figure 16). This is followed by

FIGURE 16

Double-strand gap/repair model. Taken from Deiss *et al.* (1986).

1. DNA is packaged prior to cleavage, and the directly-repeated 'a' sequences which will form the L and S termini then align (junctions A and B, respectively).

2. A U_c-directed double-strand cleavage occurs through the DR_1 (▬) of junction A. The cleaved ends align with the DR_1 elements of junction B.

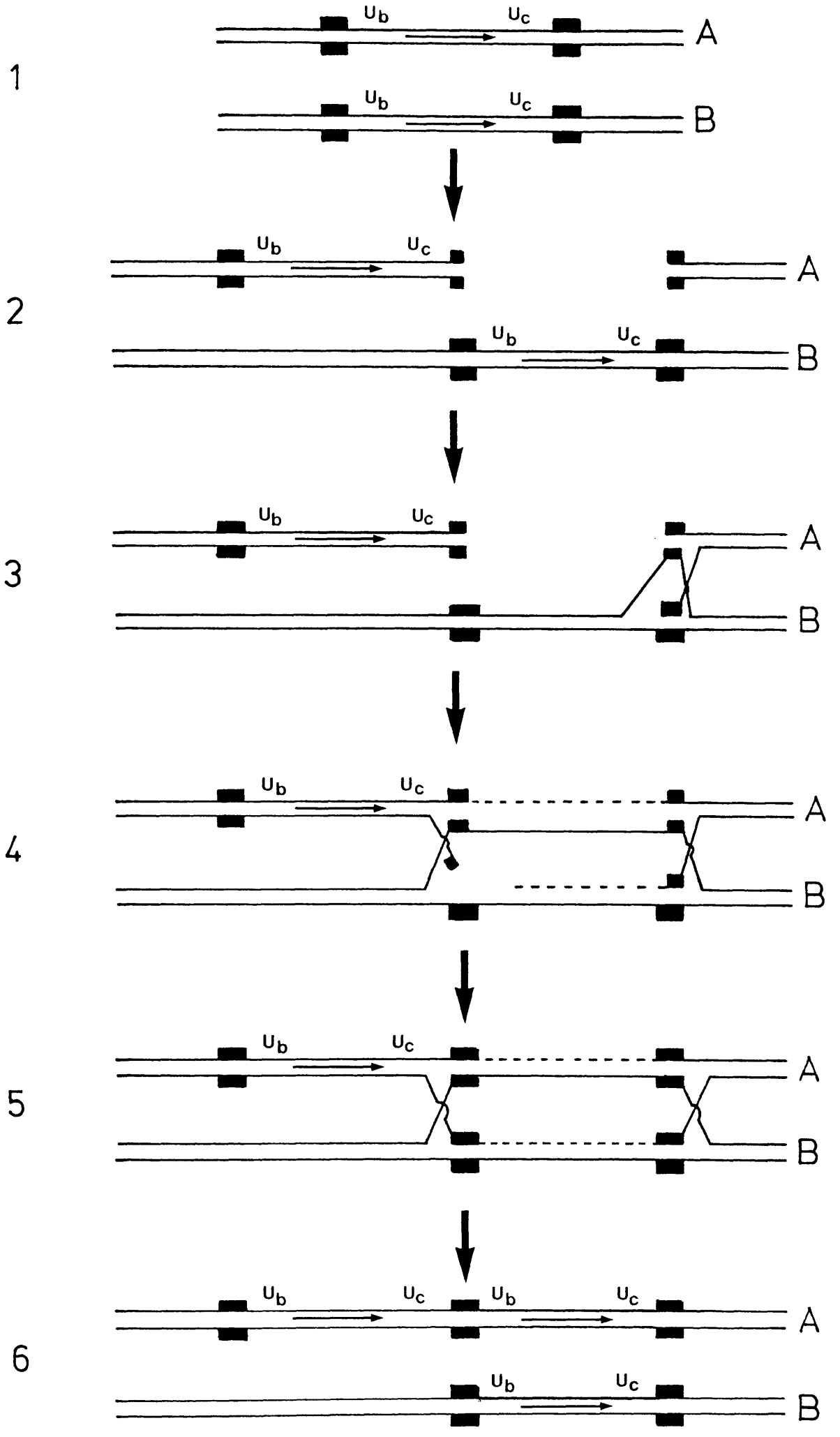
3. One strand from junction A then invades junction B, displacing the equivalent strand. (For simplicity this diagram is drawn with the top and bottom strands of junction B reversed).

4. The displaced strand from junction B aligns with the partial DR_1 elements of junction A, and serves as a template for repair synthesis.

5. Similarly, the non-displaced strand from junction B serves as a template for repair synthesis between the invading partial DR_1 elements.

6. Resolution of the Holliday structure results in the presence of two directly repeated copies of the 'a' sequence at junction A.

7. A similar process is then envisaged to take place for junction B. Cleavage between the adjacent 'a' sequences then generates the L and S termini of the packaged DNA.


1
U_b
U_c
A
U_b
U_c
B
2
U_b
U_c
A
U_b
U_c
B
3
U_b
U_c
A
B
4
U_b
U_c
A
B
5
U_b
U_c
A
B
6
U_b
U_c
U_b
U_c
A
U_b
U_c
B

strand invasion by the free terminus into the intact 'a' sequence, copying of that 'a' sequence with resultant displacement of the upper strand, and the use of this displaced strand as template to repair the first junction. Resolution of the Holliday structure at or near DR_1 then leaves the first junction with a second copy of the 'a' sequence. A similar event is then envisaged to take place at the second junction, so that two adjacent 'a' sequences are present at each terminus. Cleavage then occurs between adjacent 'a' sequences. Therefore, as with the single-strand gap/repair model, this model does not generate termini lacking 'a' sequences and allows amplification of 'a' sequences. However, this model would appear to depend on sequence homology at the DR_1 elements of the 'a' sequence to allow correct insertion of a complete second copy of the 'a' sequence. Therefore, it can not then account for amplification of terminal sequences such as those found by Varmuza and Smiley (1985), where the L and S termini ended in different sequences outwith the SmaI F fragment, and only a single partial copy of DR_1 was present. The sequence of a double SmaI F-containing joint fragment suggested that it had arisen by direct ligation of the termini (see Figure 13) and restriction enzyme analysis of 24 such junctions suggested that only 2 had arisen via recombination events.

SECTION XI : Vmw21 AND AIMS OF THE PROJECT

XI.1 Virus polypeptide Vmw21

Two polypeptides of apparent mol. wt. 21,000 and 22,000 (Vmw21, Vmw22) are made in abundance late in infection (Marsden et al., 1976) and have been mapped to the short region of the genome, map co-ordinates 0.93-0.99 (Marsden et al., 1978). Synthesis of these polypeptides is highly dependent on viral DNA replication (Marsden et al., 1976; Honess and Watson, 1977; Johnson et al., 1986), a feature of 'true' late gene products. Hybrid-arrested translation experiments (Rixon and McGeoch, 1984) suggested that gene U_S11, which lies in the short unique region (McGeoch et al., 1985; see Figure 8) might encode the 21K polypeptide (and possibly the 22K polypeptide), and this was confirmed using an antiserum raised against a synthetic oligopeptide representing the carboxy-terminal seven amino acids of the predicted polypeptide product of this gene (Dalziel, 1984; Johnson et al., 1986). The predicted product of gene U_S11 has a highly unusual amino acid composition. The carboxy-terminal portion of the protein contains 24 tandem repeats of the sequence 'X-Pro-Arg', where X is any one of nine amino acids - Ala, Asp, Gln, Glu, Ile, Pro, Ser, Thr, Val (Rixon and McGeoch, 1984). The repeating unit represents approximately 45% of the protein.

Vmw21 is phosphorylated and may be a minor virion component (Marsden et al., 1976). Vmw21, Vmw22 are equivalent to polypeptides designated ICP47,48 (Honess et al., 1980) and both have been shown to exhibit interstrain variability in apparent mol. wt. (Lonsdale et al., 1979; Honess et al., 1980). Since Vmw21 and Vmw22 consistently show similar alterations in mol. wt. (Lonsdale et al., 1979), map to a similar

region of the genome (Marsden et al., 1978) and both interact with the anti-peptide sera to the U_S11 gene products (Johnson et al., 1986), it would seem likely that these polypeptides are products of the same gene - gene U_S11. The reason for the interstrain variability in apparent mol. wt. is unclear, as there is no extensive reiteration within the DNA coding sequences of gene U_S11, since all the potential codons for proline and arginine are used (Rixon and McGeoch, 1984). However, a short 18 bp sequence is present as three tandem repeats in HSV-1 strain 17syn$^+$ (Rixon and McGeoch, 1984), but only as two tandem repeats in HSV-1 strain Patton (Watson and Vande Woude, 1982), and it has been suggested (Rixon and McGeoch, 1984) that variation in the number of repeats of this 18 bp sequence may account for this interstrain variability in apparent mol. wt. Whether this might also account for the difference between Vmw21 and Vmw22, or whether these may represent different processed forms of the same polypeptide, is unknown.

A 21K polypeptide was found to be both a predominant and strong DNA-binding protein (Bayliss et al., 1975), and Dalziel and Marsden (1984) demonstrated that two late polypeptides of apparent mol. wt. 21K and 22K could specifically interact with the 'a' sequence of HSV-1. Whether these polypeptides are Vmw21 and Vmw22 has yet to be formally proven. However, the high arginine content of the predicted U_S11 gene product renders it a very basic protein, compatible with a DNA-binding function.

XI.2 Aims of the project

The results of Dalziel and Marsden (1984) were the first published account of a sequence-specific interaction between

HSV polypeptides and HSV DNA sequences. The 'a' sequence is involved in a number of important functions - circularisation, site-specific recombination and packaging. Any of these might be expected to involve a sequence-specific DNA-binding protein. It was therefore believed to be important to further characterise this protein and its apparent interaction with the 'a' sequence.

Initially we intended to purify Vmw21, using an immunological approach. Utilising the predicted amino acid sequence of the U_S11 gene product we would synthesise oligopeptides representing various regions of the protein. Antisera against these oligopeptides could then be used in an attempt to purify 21K. Anti-peptide antibodies have the advantage that specifically bound proteins can be eluted from immuno-affinity columns using peptide as a competitor - thereby avoiding the use of harsh and possibly denaturing eluting agents. The hopefully functional 21K polypeptide could then be studied with respect to its DNA-binding properties. For this we would have to develop a more sensitive and convenient assay than the somewhat crude and cumbersome competition assay originally employed (Dalziel and Marsden, 1984) such as, for example, a gel retardation or DNase I footprinting assay.

Additionally, since 21K is a relatively small and possibly sequence-specific DNA-binding protein, it would be interesting (as a long term objective) to purify it in sufficient amounts to crystallize it and determine its 3-dimensional structure by X-ray diffraction analysis. For 21K this would be particularly interesting because of its unusually high (24%) proline content.

One potential drawback to the use of anti-oligopeptide

antibodies, however, is that they may not be of sufficiently high affinity to allow purification of the quantities of protein required for such studies. The generation of higher affinity monoclonal antibodies might allow bulk purification, where recovery of a functionally active protein may not be so important. The immunological reagents generated in the course of this study could then be used to further characterise the products of gene U_S11.

MATERIALS

MATERIALS

Chemicals

Chemicals were standardly obtained from either Sigma Chemical Co. Ltd., Poole, England or BDH Ltd., Glasgow, and were of analytical reagent grade or better. Exceptions are listed below.

acetic acid (glacial)	May and Baker Ltd., Dagenham, England
acetone	May and Baker Ltd., England
acetonitrile (HPLC grade)	Rathburn Chemicals Ltd., Walkerburn, Scotland
acrylamide	Koch-Light Laboratories, Haverhill, England
agarose (for RID) - isogel for isoelectric focussing	FMC Corporation, Rockland, USA
alpha$_2$-macroglobulin	Boehringer Mannheim GmbH., W. Germany
ammonium persulphate	Bio-Rad Laboratories, Richmond, California
ampicillin ('penbritin')	Beecham Research Laboratories, Brentford, England
boric acid	Koch-Light Laboratories, England
bovine serum albumin (BSA), for molecular biology	Boehringer Mannheim GmbH., W. Germany
brain heart infusion agar	Difco Laboratories, Detroit, USA
caesium chloride	Koch-Light Laboratories, England
chloroform	Koch-Light Laboratories, England
deoxyribonuclease I	Worthington Biochemicals Corporation, New Jersey

dimethylsulphate	Fluka AG, Buchs SG, Switzerland
dimethylsulphoxide	Koch-Light Laboratories, England
ethanol - absolute alcohol 100	James Burrough (F.A.D.) Ltd., Witham, England
ether	Koch-Light Laboratories, England
Ficoll	Pharmacia, Uppsalla, Sweden
formamide	Fluka AG, Switzerland
Freund's adjuvant, complete	Gibco, Paisley, Scotland
Freund's adjuvant, incomplete	Gibco, Scotland
glutaraldehyde	Agar Aids, Cambridge, England
glycerol	Koch-Light Laboratories, England
hydrazine	Eastman Kodak, Rochester, USA
hydrochloric acid	May and Baker Ltd., England
Lowicryl K4M resin	Agar Aids, England
methanol (HPLC grade)	Rathburn Chemicals Ltd., Scotland
piperidine	Fluka AG, Switzerland
proteinase K	Boehringer Mannheim GmbH., W. Germany
rabbit anti-mouse immunoglobulin	Cedarlane Laboratories Ltd., Hornby, Canada
'Repelcote' - 2% dimethyl-chlorosiline in 1,1,1-trichloroethane	Hopkins and Williams, Essex England
sodium hydroxide	Koch-Light Laboratories, England
sodium metabisulphite	May and Baker Ltd., England
sodium nitrite	Hopkins and Williams, England
TEMED - N,N,N',N'-tetra-methylethylenediamine	Bio-Rad Laboratories, California
TPCK-treated trypsin (L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK) - treated trypsin)	Worthington Biochemicals Corporation, USA

FIGURE 17

Schematic representation of plasmid constructs pR9, pR10 and pAH_2.

(i) pR9 (Stow et al., 1983) contains an HSV-1 'a' sequence derived from the S terminus plus approximately 150 bp of flanking 'c' sequence, inserted into Hind III/Sma I digested pUC9.

(ii) pR10 (Stow et al., 1983) contains an HSV-1 'a' sequence derived from the L terminus plus approximately 200 bp of flanking 'b' sequence, inserted into Hind III/Sma I digested pUC9.

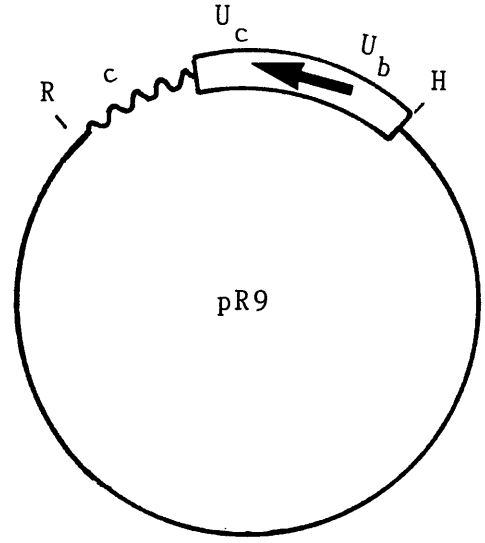
(iii) pAH_2 (constructed by Martin Murray) contains an HSV-2 'a' sequence derived from the L-S junction plus approximately 200 bp of flanking 'b'' sequence, inserted into Hind III/Sma I digested pUC8.

Seed stocks of pR9 and pR10 were supplied by Dr Nigel Stow, while a seed stock of pAH_2 was supplied by Mr Martin Murray.

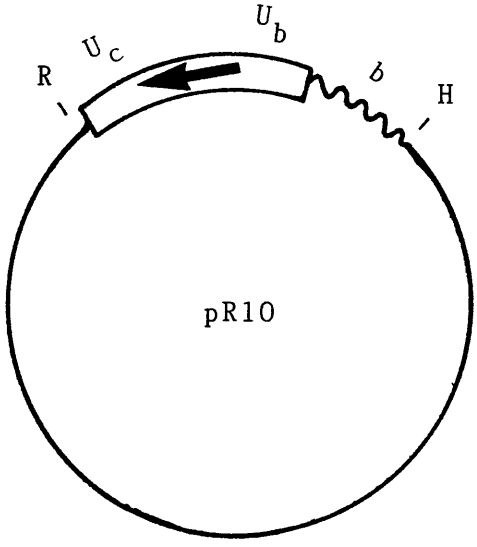
R Eco RI site

H Hind III site

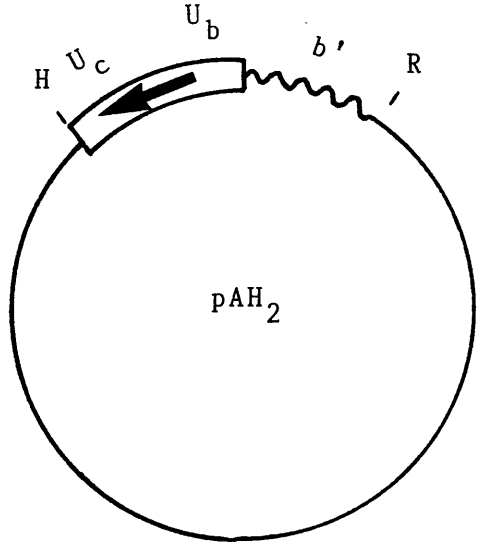
(i)



(ii)



(iii)



(ii) pR10 contains the HSV-1 'a' sequence derived from the long terminus plus approximately 200 bp of flanking 'b' sequence, inserted into Hind III/Sma I digested pUC9 as shown in Figure 17 (Stow et al., 1983).

(iii) pS1 contains a 535 bp Sau 3A fragment, containing the HSV-1 origin of DNA replication from the short repeat region (ori_S), inserted into the Bam HI site of pAT153 (Stow and McMonagle, 1983).

(iv) pAH_2, constructed by Martin Murray, contains an HSV-2 'a' sequence derived from the L-S junction plus approximately 200 bp of flanking 'b'' sequence, inserted into the Sma I/Hind III sites of pUC8 (see Figure 17).

(v) The vectors pAT153 and pUC8 have been described previously (Twigg and Sherratt, 1980; Vieira and Messing, 1982).

Radiochemicals

Radiolabelled compounds were obtained from Amersham International, plc.

	specific activity
(i) NaI^{125}, carrier free	-
(ii) [^{35}S]-L-methionine	>1000 Ci/mmol
(iii) 5'[α-^{32}P]-deoxynucleoside triphosphates	3000 Ci/mmol
(iv) 5'[γ-^{32}P]-adenosine triphosphate	5000 Ci/mmol

Synthetic oligopeptides

Synthetic oligopeptides were obtained from Cambridge Research Biochemicals, Cambridge, England.

Cells

Baby hamster kidney (BHK) 21 clone 13 cells (Macpherson

and Stoker, 1962) were used throughout this study.

Virus

Two wild-type strains, HSV-1 strain 17syn$^+$ (Brown et al., 1973) and HSV-2 strain HG52 (Timbury, 1971), and two mutants, PAA^r-1, a phosphonoacetic acid-resistant mutant derived from HSV-1 strain 17syn$^+$ (Hay and Subak-Sharpe, 1976) containing a mutation within the DNA polymerase gene (Crumpacker et al., 1980) and X12, a deletion mutant derived from HG52 lacking part of the right hand end of the short region of the genome, including gene U_S11 (Brown and Harland, 1987), were used in this study.

Tissue culture medium and solutions

Eagle's medium :

BHK cells were cultured in Glasgow modified Eagle's medium (Busby et al., 1964), supplemented with 50ug/ml gentamycin (Flow Laboratories) and 0.002% phenol red. Tryptose phosphate, calf serum and human serum were added as required (see method section I).

Versene :

6mM EDTA in PBS (see below) containing 0.0015% (wt/vol) phenol red.

Trypsin-versene :

1 volume 0.25% (wt/vol) Difco trypsin in Tris-saline (see below), plus 4 volumes versene.

Giemsa stain :

1.5% (vol/vol) suspension of Giemsa in glycerol, heated at 56^{o}C for 90-100 min and diluted with an equal volume of methanol.

Bacterial culture media

Bacteria were grown in L-broth (170mM NaCl, 10g/l Difco bactopeptone, 5g/l yeast extract) supplemented, unless specified otherwise, with 50ug/ml ampicillin. For agar plates the L-broth contained 1.5% (wt/vol) agar.

Buffer solutions

B2 buffer	20mM Tris.HCl pH 8.0, 50mM NaCl, 1mM EDTA, 1mM 2-mercaptoethanol, 10% glycerol.
PBS-A	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4 pH 7.2.
PBS	PBS-A supplemented with 6.8mM $CaCl_2$ and 4.9mM $MgCl_2$.
TBE[1]	89mM Tris, 38mM boric acid, 1mM EDTA
TBE[2]	89mM Tris, 89mM boric acid, 2mM EDTA
TE, pH 7.4	10mM Tris.HCl pH 7.4, 1mM EDTA
TE, pH 8.0	10mM Tris.HCl pH 8.0, 1mM EDTA
Tris-saline	25mM Tris, 140mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 1mg/ml dextrose, 0.0015% phenol red (pH 7.4), supplemented with 100 units/ml penicillin and 100ug/ml streptomycin.

METHODS

METHODS

SECTION I : (A) GROWTH OF CELLS AND VIRUS STOCKS

I.1 Growth of cells

Cells were grown in 80oz. roller bottles in Eagle's medium containing 5% v/v tryptose phosphate and 10% calf serum (ETC_{10}), in a humidified atmosphere of 95% air, 5% CO_2. Once confluent, the cells were harvested by washing the monolayer briefly twice with about 10ml of versene and once with about 20ml of trypsin/versene (1:4). When the cells began to detatch from the glass or plastic substrate, 10ml ETC_{10} was added to inactivate the trypsin. The cells were finally resuspended at approximately $12x10^6$ cells/ml and stored at 4^oC for up to 5 days. Petri dishes were seeded at $3x10^6$ cells/50mm dish or $8x10^6$ cells/90mm dish for use the next day.

I.2 Growth of virus stocks

Elite stock virus (virus with a low serial passage number) was used to infect approximately 80% confluent BHK cell monolayers at a multiplicity of 1 plaque forming unit (pfu) per 300 cells in 40ml ETC_{10}. Virus was grown at 31^oC and harvested when the cytopathic effect (cpe) was maximal (usually after 4-7 days).

The infected cells were shaken into the medium and pelleted by centrifugation at 2000rpm for 5 min in a Fisons Coolspin. For HSV-1, the supernatant was spun in a Sorvall GSA rotor at 12,000rpm for 2 h and the virus-containing pellet resuspended in 5ml of supernatant and sonicated in a bath sonicator (50W at 40^oC) until homo-

geneous. This was termed the supernatant virus stock. For HSV-2, however, no supernatant virus was collected.

For both HSV-1 and HSV-2 the cell-associated virus in the pellet from the 2000rpm centrifugation was resuspended in 5ml of supernatant, sonicated in a bath sonicator until homogeneous, freeze-thawed, resonicated and then spun at 2000rpm for 10 min. The supernatant was kept and the pellet resuspended in 5ml of fresh ETC_{10} and the above cycle repeated. The two supernatants were pooled and this represented the cell-associated virus stock.

Virus stocks were stored at -70°C and were checked for sterility and titrated before use.

I.3 Sterility checks

Brain heart infusion (BHI) plates were prepared by dissolving 26g of Difco Bacto BHI agar in 500ml deionised water and sterilising by autoclaving for 20 min. This was cooled to around 40°C and poured directly onto 50mm petri dishes (BHI plates); alternatively, 50ml of horse blood (Biocult) was added just before pouring (BHI blood agar plates).

Cell or virus stocks were checked for fungal contamination by streaking on the BHI plates, in duplicate. The plates were sealed with parafilm and then incubated at room temperature for 7 days. Similarly, yeast or bacterial contamination was detected by streaking on the BHI blood agar plates and incubating at 37°C for 7 days. If no contamination became apparent within that time the stocks were considered sterile.

I.4 Titration of virus stocks

Virus stocks were titrated, at both 31°C and 38.5°C, on approximately 80% confluent BHK C13 cells grown on 50mm petri dishes. Virus was serially diluted 10-fold in PBS containing 5% calf serum. Medium was drained from the cells, 0.2ml of each virus dilution was added to each of duplicate plates, and the plates were then incubated at 37°C for 1 h. A 5ml overlay of Eagle's medium containing 4% human serum (a pool from several donors, serving as anti-HSV antiserum) was then added to prevent any extracellular spread of virus, and the plates further incubated at 31°C or 38.5°C for 3 or 2 days, respectively. Finally, the cells were fixed and stained with Giemsa stain at room temperature (RT) for 30 min. Plaques were counted using a dissection microscope.

SECTION I : (B) PREPARATION OF INFECTED CELL EXTRACTS

I.5 Preparation of late extracts

Infected cell extracts were usually prepared using monolayers of BHK C13 cells at about 80% confluency. Stock virus was sonicated and then diluted in ETC_{10} to a concentration of $2x10^8$pfu/ml, and the monolayers were infected with virus at a multiplicity of 20 pfu per cell. Virus was allowed to absorb for 1 h at 37°C and the plates were then washed twice with methionine-labelling medium (Eagle's medium containing one fifth the normal concentration of methionine and 2% calf serum), and further incubated in 2ml (50mm dish) or 5ml (90mm dish) of this medium. If labelled extracts were required, [^{35}S]-methionine was added at 50uCi/ml, usually 3-4 h post-infection (0 h

post-infection (pi) being taken as the end of the 1 h absorption period).

For large scale, roller bottle, preparations of extract, cells were infected with 5-10 pfu/cell in 25ml ETC_{10} and then maintained in 50ml of methionine-labelling medium. For labelling, [^{35}S]-methionine was added at 10uCi/ml.

Infected cells were usually harvested 16-24 h pi.

I.6 Preparation of early extracts

To prepare extracts of proteins produced in the absence of viral DNA replication (loosely termed 'early' extracts) cell monolayers were pre-treated with phosphonoacetic acid (PAA) at 300 ug/ml for 1 h, then infected maintained and labelled essentially as for a normal infection but with the continuous presence of PAA. Cells were harvested at various times pi by the high salt extraction procedure (method section I.7b).

I.7 Harvesting of infected cell extracts

(a) Detergent lysis procedure

This was used for the preferential extraction of glycoproteins, and was adapted from Zweig et al. (1980).

Extraction buffer:
- 100mM Tris.HCl, pH 8.0
- 10% glycerol
- 0.5% NP40
- 0.5% Na deoxycholate
- 500uM PMSF (phenylmethylsulphonyl fluoride)

Infected cells were washed three times with PBS. Extraction buffer - 1ml per 50mm plate -was added, and

the plates left for 60 min at 0°C. The cell extract was then spun in a Beckman microfuge for 5 min and the supernatant stored at -70°C until use.

(b) High salt extraction procedure

This method was used to preferentially extract DNA-binding proteins.

Lysis buffer		B2 buffer	
40mM	Tris.HCl pH 8.2	20mM	Tris.HCl pH 8.0
4M	NaCl	50mM	NaCl
2mM	EDTA	1mM	EDTA
2mM	2-mercaptoethanol	1mM	2-mercaptoethanol
		10%	glycerol

Cells were washed twice with ice-cold PBS and once with ice-cold sterile deionised water. The cells from a 90mm dish were scraped into 1.25ml of sterile deionised water and transferred to a centrifuge tube. An equal volume of lysis buffer was added, with vigorous mixing, and this was then incubated at 0°C for 30 min. Centrifugation in the Sorvall Ti50 rotor at 40,000rpm for 1 h at 4°C was followed by extensive dialysis of the supernatant (S40K) against a 100-fold excess volume of B2 buffer changed at least twice over 24 h. Material which had precipitated in the low salt was then pelleted by centrifugation in a Sorvall SS34 rotor at 8,000rpm for 30 min and the supernatant (S8K) was stored at -70°C until use.

SECTION II : GEL ELECTROPHORESIS

II.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Vertical slab gels - either single concentration acrylamide gels or 5-12.5% gradient acrylamide gels - were used for protein analysis. Fresh stocks of 30%

acrylamide were prepared in water, the ratio of acrylamide to N,N'-methylene bisacrylamide (the cross-linking agent) being 28.5:1.5 and 29.25:0.75 for the gradient and single concentration stocks, respectively. These were then filtered through Whatman N^{o}1 filter paper before use. From these stocks, gel solutions containing the appropriate percentage of acrylamide were prepared in a buffer of final concentration 375mM Tris.HCl, pH 8.9, 0.1% sodium dodecyl sulphate (SDS). In the 5-12.5% gradient gels the higher concentration acrylamide solution also contained 15% glycerol to stabilise the gradient. To polymerise the gels, 200ul of 10% ammonium persulphate and 10ul of TEMED were added per 24ml of gel solution (representing a final concentration of 3.65mM and 2.75mM, respectively).

The gel solutions were poured between two well-washed glass plates separated by 1.5mm spacers and sealed with teflon tape, to within 3-4cm of the top of the glass-plate sandwich. Butan-2-ol was gently layered on top of the acrylamide solution, and following polymerisation a stacking gel, consisting of 5% acrylamide in 122mM Tris.HCl pH 6.7, 0.1% SDS, was added, into which was placed a teflon well-forming comb.

Samples were boiled for 5 min in sample buffer at a final concentration of 50mM Tris.HCl pH 6.7, 2%SDS, 700mM 2-mercaptoethanol (2-ME) and 10% glycerol, with sufficient bromophenol blue (BPB) to visualise the dye front. Electrophoresis was carried out in a buffer containing 52mM Tris, 53mM glycine and 0.1% SDS, at either 60mA for 3-4 h at 4^{o}C or at 10-12mA for 16-24 h at RT.

Following electrophoresis gels were fixed for 1 h in methanol:acetic acid:water (50:7:50) with or without

0.2% Coomassie brilliant blue R250, and then destained several times in 5% methanol, 7% acetic acid. They were then gently agitated in 3 volumes of En^{3}hance (New England Nuclear) for one hour and washed twice with 10 volumes of water over 20-30 min. The gels were dried under vacuum onto Whatman grade 182 filter paper, and exposed to Kodak X-Omat XS-1 film at -70°C.

II.2 Non-denaturing polyacrylamide gel electrophoresis

To analyse or purify DNA fragments slab gels were poured without a stacking gel, using various concentrations of acrylamide in TBE^2. In this case the acrylamide:N,N'-methylene bisacrylamide ratio was 29:1. Four volumes of sample were mixed with one volume of a solution of 5x TBE^2 containing 15% Ficoll, 100mM EDTA and 0.2% BPB, and this was then electrophoresed at 60mA for 2-4 h. Bands were visualised either by autoradiography, if labelled, or by soaking the gel in TBE^2 containing 0.5ug/ml ethidium bromide for 1 h, followed by exposure to uv light.

II.3 Denaturing polyacrylamide gels for DNA sequencing

Sequencing gel mix (6% acrylamide, 8.3M urea in TBE^1) was prepared as follows : 225g urea was dissolved in water with 90ml of 30% acrylamide stock (acrylamide:N,N'-methylene bisacrylamide 29:1) and adjusted to 400ml. This was then deionised by stirring with 10g of amberlite MB.1 for 30 min, and after filtering 45ml of 10x TBE^1 was added. This solution was then stored at 4°C in the dark for up to one month.

To polymerise the gel, 500ul of 10% ammonium persulphate and 55ul TEMED were added to 60ml of sequencing gel mix.

Gels were poured between sealed plates separated by 0.35mm spacers, using a syringe, and once set were pre-electrophoresed at 40W for at least 30 min before loading. Samples were boiled for 2-4 min in the presence of 80% deionised formamide, 0.1% BPB and 0.1% xylene cyanol, rapidly cooled on ice and 2ul loaded using a hamilton syringe. Gels were electrophoresed at RT in TBE[1] at 40W for the required time (usually 1½-2 h). The gels were then covered with cling film and exposed against Kodak X-Omat XS-1 film, at -70°C.

II.4 Agarose gel electrophoresis

Masking tape was applied around all four edges of a 163cm x 264cm glass plate to form a 0.75cm wall. The glass plate was placed on a levelling board and made horizontal. The appropriate concentration of agarose dissolved in TBE[2] and containing 0.5ug/ml ethidium bromide, was then gently poured onto the glass plate and allowed to set. After removing the tape the gel was placed in a horizontal gel kit and TBE[2] added to each reservoir until just level with the surface of the gel. Samples were mixed 4:1 with a solution of 5x TBE[2] containing 15% Ficoll, 100mM EDTA, 0.2% BPB, loaded, and electrophoresed at 100V for 3-6 h or 40V overnight.

For a rapid assessment of plasmid preparations minigels, consisting of 20ml of a 1% agarose solution set in a minigel kit, were run in TBE[2] at 40V for 45-60 min. In both cases bands were visualised by exposure to uv light.

SECTION III : GENERATION AND PURIFICATION OF ANTIBODIES

III.1 Iodination of oligopeptides using NaI^{125}

The iodination reaction is a modification of the method of Hunter and Greenwood (1962). Synthetic oligopeptides (10mg) were resuspended in 100ul of 1M potassium phosphate buffer pH 7.5. Then 100uC of NaI^{125} and 50ul of chloramine T were added, incubated for 20 seconds at RT and the reaction stopped by the addition of 50ul of sodium meta-bisulphite. Removal of the free NaI^{125} involved passage through a Sephadex G10 column preblocked with 0.1% BSA in 0.16M borate pH 9 and equilibrated in 0.16M borate pH 9, 0.13M NaCl (see method section V.1). Iodinated oligopeptide was eluted with the equilibration buffer. Fractions of 0.5ml were collected, assayed in a gamma counter, and fractions containing the iodinated peptide (which eluted in the void volume) were pooled, and either used immediately or stored at $-70^{o}C$.

The iodination reactions were carried out by Dr Howard S Marsden and Mrs Mary Murphy.

III.2 Covalent linkage of synthetic oligopeptides to BSA

(a) Preparation of bis-diazotised benzidine (DAB)

Bis-diazotised benzidine (DAB) was prepared by Dr Howard S Marsden according to the method of Likhite and Sekar (1967). 0.23g of benzidine hydrochloride was dissolved in 45ml of 0.2M HCl. 5ml of 0.5M sodium nitrite was added and the solution stirred slowly at $4^{o}C$ for 1 h. The DAB was then aliquoted into 2ml amounts and stored at $-70^{o}C$.

(b) Linkage reaction

The coupling reaction was carried out according to the method of Bassiri et al. (1979). Briefly, 30mg of BSA was dissolved in 1.5ml of iodinated peptide (usually 10mg) in 0.16M borate pH 9, 0.13M NaCl. 1ml DAB was added and the reaction carried out at 4°C for 1½-2 h. Free and coupled oligopeptides were separated by passage through a Sephadex G25 PD10 column (method section V.1). Samples were eluted using PBS and 10 drop (500ul) fractions were collected and assayed in a gamma counter. Oligopeptide coupled to BSA eluted in the void volume, and the relevant fractions were pooled, dialysed extensively against PBS-A to remove residual DAB, and stored at -70°C. The extent of coupling could be calculated from the formula :

$$\% \text{ coupling} = \frac{\text{cpm coupled}}{\text{total cpm loaded}} \times \frac{100}{1}$$

This was usually in the order of 20-30%.

III.3 Production of antisera to synthetic oligopeptides

Immunisation schedules varied and are outlined in detail in result section I.1. Generally, New Zealand White (NZW) rabbits, about 6 months old, were immunised intramuscularly on several occasions with 500ug of peptide (coupled or uncoupled) and bled 7-14 days after each injection. Pre-immune sera were collected from most rabbits. For the first immunisation the peptides were emulsified in an equal volume of complete Freund's adjuvant, subsequent injections being in incomplete Freund's adjuvant. At least 2 rabbits were used for each antigen.

The sera were tested for reactivity with the relevant peptide by radioimmunoassay (RIA; method section IV.3).

Antibodies to BSA were removed by absorption against BSA coupled to Sepharose before the sera were tested by immunoblotting for reactivity against the relevant protein (method section IV.5). Once good anti-protein responses were detected the rabbits were given a final boost and blood collected by heart puncture.

III.4 Production of antisera to 21K protein

In an attempt to raise monospecific antisera to 21K, partially purified preparations of protein, prepared by DNA cellulose chromatography (see result section III.3) were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose. The nitrocellulose was exposed to X-ray film and aligned with the autoradiograph by means of radioactive ink spots. The strip of nitrocellulose containing 21K was dissolved in dimethylsulphoxide and this solution was emulsified in complete Freund's adjuvant for the first injection, or in incomplete Freund's adjuvant for subsequent injections, and used to immunise 2 NZW rabbits intramuscularly. Again the rabbits were bled 7-14 days after each injection.

III.5 Purification of IgG by ion exchange chromatography

DE-52 (Whatman) is a DEAE-cellulose anion exchange resin. Conditions of pH and ionic strength were chosen such that IgG was among the few proteins that did not have an affinity for the charged resin and therefore passed directly through the column.

Serum was equilibrated in low salt buffer (10mM sodium phosphate pH 7.7, 50mM NaCl) by dialysis against at least a 100-fold excess volume of buffer at $4^{o}C$ over 2 days with

4-5 changes. The DE-52 resin was equilibrated using a batch procedure, washing in a large beaker once with 0.5M sodium phosphate pH 7.6, once with 0.005M sodium phosphate pH 7.7, and then several times over 2 days with 10mM sodium phosphate, 50mM NaCl pH 7.7, decanting the buffer each time the fibres had settled. This was necessary to eliminate fine particles which would block the column. A column of size 5 times the sample volume was poured and washed with several volumes of low salt buffer. The serum was then loaded carefully onto the column and run through with low salt buffer, collecting fractions of 0.5x the original sample volume. Fractions were assessed by reading the optical density at 280nm (OD_{280}), radial immunodiffusion, radioimmunoassay and SDS-PAGE. Peak IgG-containing fractions were pooled and the column discarded after use.

SECTION IV : IMMUNOASSAYS

IV.1 Single radial immunodiffusion (RID)

This method was used to rapidly analyse fractions for the presence of IgG. A 1.5% (wt/v) solution of agarose in PBS was prepared by boiling, and was then allowed to cool to 56^{o}C. Sheep anti-rabbit IgG serum (prepared by Dr C A Moss from hyperimmune sheep immunised with DE-52 purified rabbit IgG) was added to a final dilution of 1:100, and 4ml aliquots carefully poured onto 50mm petri dishes. Once set, holes were punched in the agarose using a pasteur pipette. Fractions to be assayed for IgG were added to fill the well and the plates were incubated in a moist chamber at RT for 48 h.

IV.2 Iodination of protein A

Protein A (Sigma, catalogue number P8143) was reconstituted in PBS at a concentration of 0.5mg/ml, aliquoted into 20ul amounts and stored at -70°C. To 20ul (10ug) of protein A was added, in turn, 10ul of 1M potassium phosphate buffer pH 7.5, 200uCi of I^{125} (NaI^{125}, Amersham International) and 20ul of chloramine T (0.5mg/ml in PBS). This was allowed to react for 20 seconds at RT, then 20ul of sodium metabisulphite (1mg/ml in PBS) was added to stop the iodination reaction. Removal of free NaI^{125} was achieved by passage through a Sephadex G25 PD10 column (method section V.1). The iodinated protein A was eluted using PBS, and 0.5ml fractions were collected and 10ul analysed for radioactivity in a gamma counter. Two peaks were observed, the first containing the protein A and the second the free iodide. Fractions containing the iodinated protein A were pooled and stored at 4°C, in the presence of 0.01% sodium azide.

The iodination reactions were standardly carried out by Dr H S Marsden and Mrs Mary Murphy.

IV.3 Radioimmunoassays (RIA)

The radioimmunoassay for oligopeptides was modified from the method of Green et al. (1982). Peptides were diluted in PBS and added at 25ul/well to round bottomed microtitre plates. The plates were sealed with plate sealers (Titertek, Flow Laboratories) and incubated at 37°C overnight. Excess liquid was shaken off and peptide fixed to the wells by adding 200ul/well of methanol and incubating for 5 min at RT. Remaining non-specific protein binding sites were blocked using 0.5% polyoxyethylenesorbitan

monolaurate (Tween 20) in PBS at 200ul/well for 1 h at 37°C. Plates were washed once with PBS and could then be stored at -20°C until use.

To determine whether rabbit serum contained antibodies against the immunising peptide the rabbit serum/ purified IgG was diluted in PBS, added at 25ul/well and incubated for 1 h at 37°C. Samples were standardly tested in duplicate. The wells were washed three times with 200ul of 0.05% Tween 20 in PBS and then 25ul (20,000 cpm) of iodinated protein A, diluted in 0.05% Tween 20 in PBS, was added to each well. After 30 min incubation at 37°C unbound protein A was washed off by flooding the plates three times with PBS. To elute and quantitate the bound protein A, 200ul of 5M NaOH was added per well, left at least 15 min and then removed and counted in a gamma counter.

Radioimmunoassays to detect antibodies directed against protein antigens (such as BSA) were carried out in an identical manner, except that the methanol fixation step was omitted, since proteins, being much larger than the synthetic peptides, should be capable of efficiently adsorbing to the plastic without fixation.

IV.4 Immunoprecipitation

Immunoprecipitation experiments were carried out in the presence of detergent lysis extraction buffer (method section I.7a). Rabbit antiserum (25-50ul) was incubated for 1 h at 37°C in a shaking water bath with 100-200ul of infected or mock-infected cell extract, in the presence or absence of peptide. Following incubation, 60ul of protein A-Sepharose (Sigma, catalogue number P3391), diluted 1:1 in extraction buffer, was added and further incubated at

0°C for 1-2 h. The protein A-Sepharose pellet was washed 4 times, and antigen-antibody complexes then eluted by boiling for 5 min in denaturing buffer (50mM Tris.HCl pH 6.7, 2% SDS, 700mM 2-ME and 10% glycerol) and analysed by SDS-PAGE.

IV.5 Immunoblotting

The immunoblotting technique was based on the method of Towbin *et al.* (1979), but with several modifications. Cells were harvested into denaturing buffer (method section IV.4) at a concentration of 10^7 cells/ml. After boiling for 5 min samples were loaded onto SDS-polyacrylamide gels in broad wells, at approximately 10^6 cell equivalents per cm. Following electrophoresis proteins were transferred from the gel to a sheet of nitrocellulose using a BioRad 'Transblot' apparatus. Following the manufacturer's protocol, the gel was placed on a sheet of Whatman grade 182 filter paper on top of a foam pad - both presoaked in transfer buffer (25mM Tris.HCl pH 8.3, 192mM glycine, 20% methanol) - and numbered strips of nitrocellulose, also pre-soaked, were placed face down on the gel, being careful to exclude air bubbles. Finally, a further sheet of pre-soaked filter paper and then another foam pad were placed over the gel and the sandwich (held tightly together in a plastic holder) was placed in an electrophoresis tank with the gel towards the cathode and the nitrocellulose towards the anode. Proteins were transferred to the nitrocellulose by electrophoresis in transfer buffer at 250mA for 3 h at RT.

Following transfer the nitrocellulose strips were blocked overnight at either 37°C or 65°C in a 1x solution of wash buffer (1mM Tris.HCl pH 7.4, 15.4mM NaCl, 0.01%

sodium azide) containing 0.05% Tween 20 (Batteiger et al., 1982). The strips were then placed protein side up in shallow (2mm) wells cut in perspex blocks, a piece of Whatman grade 1 chromatography paper placed on top, and 250ul of appropriately diluted antiserum then layered evenly along the strip. This was incubated for 2 h at 37°C (or alternatively at 4°C overnight). The strips were then washed extensively in wash buffer containing 0.05% Tween 20 by gently shaking at 37°C, with at least 3 changes of buffer over 2 h. To detect bound antibody, the strips were again placed protein side up in wells cut in perspex blocks (5mm deep), and were covered with 3ml of a solution of 1x wash buffer containing 3% BSA (Sigma, catalogue number A-4503) and $5x10^5$cpm of iodinated protein A. The blocks were gently shaken at 37°C for 2 h, and the strips then removed and washed twice for one hour in wash buffer containing 1M potassium iodide, followed by a brief (15 min) wash in PBS. The strips were placed on tissues to dry and then taped to cardboard sheets for autoradiography.

To determine with which of the [^{35}S]-methionine labelled proteins the ^{125}I-labelled protein A interacted, the procedure described by Haarr et al. (1985) was used. By this procedure, the strips were simultaneously exposed to 3 films - one film directly on top, followed by a sheet of black paper, 2 further films and an image intensifying screen. Thus, the first film detects the [^{35}S]-methionine label which does not penetrate as far as the second film. The high energy emission from the ^{125}I passes through the three films with little absorption and interacts with the image intensifying screen producing photons which are detected mainly on the third film and partly on the second

film and prevented from reaching the first film by the black paper. Thus the contribution from the ^{125}I to the image on the first film is kept minimal. Radioactive ink (containing ^{14}C and ^{125}I) spotted onto the cardboard allowed the 3 films to be aligned.

IV.6 Immune electron microscopy

Monolayers of BHK C13 cells were either mock-infected or infected with HSV-1 at a multiplicity of 10 pfu/cell, and the cells then harvested at various times post-infection by scraping into PBS. After pelleting into BEEM capsules (Agar Aids Ltd) the cells were fixed for 1 h with 2.5% glutaraldehyde in PBS and washed with PBS. Dehydration was performed through a graded ethanol series. The samples were placed in 30% ethanol at 4°C for 30 min, then in 50% ethanol for 60 min, and shifted to -20°C. The samples were placed sequentially in 70,90 and 100% ethanol for 1 h periods and infiltrated with Lowicryl K4M resin by 1 h exposures to a 1:1 mixture of resin and ethanol followed by two changes of pure resin. Polymerisation was performed using a 360nm wavelength uv lamp (Taab Laboratories Ltd, Reading, England) for 24 h at -20°C and a further 2 days at RT. Sections were cut using a glass knife and collected on uncoated nickel grids. Grids were stored at RT until use.

Preferential staining of ribonucleoproteins was based on the method of Bernhard (1969). Briefly, grids were stained for 1-2 min by submerging in 0.5% aqueous uranyl acetate, destained for 2-10 min in 4,10 or 40mM EDTA and then incubated for 1 min in lead citrate (prepared according to Reynolds, 1963) to enhance the uranium stain.

Unstained grids were submerged in antiserum diluted in PBS containing 0.05% Tween 20 (PBS/Tween 20) for 45 min at RT, in the absence or presence of varying amounts of peptide, and then jet washed with PBS/Tween 20, and further incubated in a 1:50 dilution of goat anti-rabbit/gold (10nm) (Miles Ltd) in PBS/Tween 20 for 45 min. The grids were jet washed as before, with a final wash in deionised water, and then dried. Contrast was enhanced by exposing the sections to OsO_4 vapour for 2 h and the sections were examined using a Jeol 100S electron microscope.

The sections used in these studies were prepared by Dr Frazer J Rixon.

SECTION V : PURIFICATION OF PROTEINS BY CHROMATOGRAPHY

A number of different methods of chromatography have been employed, the specific details of which will be given in the relevant results sections. A general outline for each is given below.

V.1 Sephadex G25 (PD10 columns)

Pre-packed PD10 columns containing a 9ml bed volume of Sephadex G25 were obtained from Pharmacia Laboratories. Non-specific protein binding sites were blocked by washing with 20ml of buffer containing 0.1% BSA, before the columns were equilibrated with the buffer required.

V.2 CNBr-activated Sepharose 4B

The coupling of proteins to cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia Laboratories) was carried out according to the procedure recommended by the manufacturers.

Briefly, 1g of the dried beads was swollen and washed for 15-30 min with 1mM HCl (200ml), on a scintered glass filter, and then rinsed with coupling buffer (0.1M $NaHCO_3$ pH 8.3, 0.5M NaCl). The protein to be coupled (usually at 5-10mg/ml of gel, assuming 1g of gel swells to 3.5ml) was either dissolved in, or dialysed against, the coupling buffer, adjusted to a volume of 5ml and mixed end-over-end with the gel for 2 h at RT. The beads were then washed with 100-200ml of coupling buffer, the wash collected and the OD_{280} read. The coupling efficiency was calculated from the formula :

$$\text{Coupling efficiency} = \frac{(OD_{280} \times \text{volume})_{input} - (OD_{280} \times \text{volume})_{wash}}{(OD_{280} \times \text{volume})_{input}} \times 100$$

This was usually in the order of 90-100%, but fell when coupling around 10mg/ml gel.

Any remaining active groups on the gel were blocked by incubating end-over-end with 15ml of 1M ethanolamine pH 8.0 for 2 h at RT. Non-covalently adsorbed proteins were removed by 3 cycles of a low pH wash followed by a high pH wash, using 0.1M acetate buffer pH 4.0 containing 0.5M NaCl, and 0.1M bicarbonate buffer pH 8.3 containing 0.5M NaCl. Finally the beads were washed in PBS and stored at 4°C in PBS containing 0.01% sodium azide.

V.3 DNA cellulose

(a) Preparation of calf thymus DNA

One gram of calf thymus DNA (Sigma, catalogue number D-1501) was dissolved in 300ml of SSC (150mM NaCl, 15mM trisodium citrate) containing 2% SDS at RT for 1-2 days.

The DNA was then extracted twice with an equal volume of chloroform:isoamylalcohol 40:1 by shaking for 10-15 min followed by centrifugation at 2000rpm for 10 min. The upper phase contained the DNA. Then 2 volumes of ethanol were added and the DNA precipitate was spooled out using a glass rod, and lyophilised in the freeze drier. The dried DNA was again dissolved in 300ml SSC (without SDS), digested with 10ug/ml RNase A (Sigma; pretreated at 90°C for 5 min) for 30 min at 37°C, and then extracted twice with chloroform: isoamylalcohol, precipitated with ethanol and dried, as above. Finally, the DNA was redissolved in 150ml of TE (10mM Tris.HCl pH 7.4, 1mM EDTA) and stored at -20°C. The concentration of DNA, calculated from the OD_{260}, using the formula 1 OD_{260} = 50ug/ml, was usually 1-2mg/ml.

(b) Preparation of cellulose

Munktell's cellulose powder (N°401), obtained from BioRad Laboratories, was purified before use as follows : One volume of powder was washed 3 times with 10 volumes of boiling ethanol, then twice each at RT with 10 volumes of 0.1M HCl, 0.1M NaOH, water and finally 20mM Tris.HCl pH 8.0, 1mM EDTA. Between each wash the cellulose was left to settle and the buffer decanted. The cellulose was then lyophilised and stored in a sealed container at RT until use.

(c) Preparation of DNA cellulose

100ml of calf thymus DNA (at 1-1.5mg/ml) was mixed into a thick paste with 100ml of dry cellulose and spread around the walls of a 5l beaker using a sterile pipette. This was left at 31°C until dry (24-48 h). Fines were removed by

resuspending the beads in a 10-fold excess volume of 20mM Tris.HCl pH 8.0, 1mM EDTA and leaving to settle overnight at 4°C. The buffer was then decanted and the beads again washed as before, for a further 2 h. After decanting the second wash the beads were resuspended in 3 volumes of B2 buffer (20mM Tris.HCl pH 8.0, 50mM NaCl, 1mM EDTA, 1mM 2-ME, 10% glycerol) containing BSA at 500ug/ml total volume, to block any non-specific binding sites, and incubated for at least 2 h.

The DNA cellulose and the column buffers were degassed using a vacuum pump before use. The beads were then poured carefully into a Pharmacia column (diameter 2.5cm) and packed overnight in B2 buffer at 4°C using an LKB peristaltic pump to drive a flow rate of around 0.8ml/min. Before use the column was washed with a low-to-high salt gradient and then re-equilibrated with B2 buffer overnight (details can be found in the relevant result sections). When not in use the column was stored at 4°C in B2 buffer containing 0.01% sodium azide.

V.4 Phosphocellulose

Ten grams of phosphocellulose (Whatman P11 grade) were washed once with 500ml of 0.1M HCl by gently stirring at RT for 30 min, several times with deionised water by filtering until the pH was neutral, once with 500ml of 0.1M NaOH by stirring for 30 min, once with 500ml of 1M EDTA pH 8.0 by stirring for 30 min, and then again with water until the pH was neutral, before being finally equilibrated in B2 buffer. Between washes the beads were allowed to settle and the buffer decanted. Beads were stored at 4°C in B2 buffer containing 0.01% sodium azide. Columns were prepared

as described above (method section V.3c).

V.5 Hydroxylapatite

Hydroxylapatite beads (BDH) were washed by resuspending in 0.2M NaOH, then neutralised by several washes with water, decanting between washes each time. The beads were then equilibrated in B2 buffer by washing several times, and stored at 4°C in B2 buffer containing 0.01% sodium azide until use.

V.6 Heparin-Sepharose

Heparin-Sepharose (Pharmacia Laboratories) was resuspended and washed with B2 buffer on a scintered glass filter, and stored at 4°C in B2 buffer containing 0.01% sodium azide until use.

V.7 ATP agarose

ATP agarose beads (Pharmacia Laboratories) were washed with water and then equilibrated in a buffer containing 20mM Tris.HCl pH 8.0, 50mM NaCl, 5mM $MgCl_2$, 0.1mM EDTA, 1mM 2-ME and 10% glycerol. After use the column was washed with a 50-100-fold excess volume of 1M NaCl and then 6M urea before being finally washed with water and stored at -20°C in 50% glycerol containing 0.02% sodium azide.

V.8 DEAE-cellulose

Whatman DE-52 beads were washed on a scintered glass filter with 0.1M HCl followed by 0.1M NaOH, then deionised water until the pH was neutral. The beads were equilibrated in B2 buffer by resuspending in a beaker and allowing the beads to settle before decanting the buffer, thus removing

the fines which float at the top. The beads were stored at 4°C in B2 buffer containing 0.01% sodium azide and were discarded after use.

V.9 Sephacryl S200

Sephacryl S200 (Pharmacia Laboratories) was equilibrated in the buffer in which chromatography was to be performed. The beads and buffers were degassed using a vacuum pump and a column was carefully poured in a Pharmacia C16 column (dimensions 40cm x 1.6cm) and packed overnight at approximately 0.5ml/min.

V.10 HPLC chromatography

This was carried out on a Waters HPLC system. All buffers were prepared using high quality deionised water (deionised through an Elgastat Spectrum apparatus) and were degassed by filtering before use. Columns were equilibrated in the relevant buffer (see below) and samples, ranging in volume from 200ul to 1ml, and dialysed against the appropriate buffer, were loaded and then eluted with a linear gradient over 20-22 min, while 0.5ml fractions were collected. Samples were analysed by SDS-PAGE.

The following columns were used :

1. Protein Pak SP 5PW - a cation exchange column.

This column was equilibrated in 20mM phosphate buffer pH 7.0 and samples were eluted using a linear gradient over 20 min from 0-0.5M NaCl or 0-1M $MgCl_2$.

2. Protein Pak DEAE 5PW - an anion exchange column.

This column was equilibrated in a buffer containing 20mM Tris.acetate pH 8.0, 1mM dithiothreitol and 10% glycerol. Samples were eluted using linear gradients over

22 min from 0-0.5M NaCl, 0-1M NaCl or 0-1M $MgCl_2$.

3. $C_{18}u$ Bondapak - a hydrophobic, reverse-phase column.

This column was equilibrated in 0.05% trifluoroacetic acid (TFA) in water, and samples were eluted using a linear gradient over 22 min from 20-100% of 0.05% TFA in acetonitrile.

V.11 FPLC chromatography

A mono Q anion exchange column was used on a Pharmacia FPLC system. Buffers were prepared as described for the HPLC system. The column was equilibrated in 20mM Tris.HCl pH 8.0, 50mM NaCl, 1mM EDTA, 1mM 2-ME. Samples were eluted using a linear gradient of 50-400mM NaCl over 20ml, and remaining proteins were then removed using 1M NaCl. The column flow rate was 1.5ml/min and 1ml fractions were collected and analysed by SDS-PAGE.

SECTION VI : TRYPTIC PEPTIDE MAPPING OF POLYPEPTIDES

VI.1 Preparation of tryptic peptides

Polypeptides were separated by SDS-PAGE and the gels dried immediately without fixing and then exposed to X-ray film. Four radio-active ink spots placed at the corners of the gel allowed alignment with the bands on the auto-radiograph. The polypeptide to be digested could then be identified and excised by cutting through the autoradiograph into the underlying gel.

The polypeptides were eluted from the gel slices by the method of Anderson et al. (1973). Briefly, the gel slices were packed into a column containing electrophoresis buffer (25mM Tris, 190mM glycine, 0.1% SDS) and carrier protein (5ug BSA per column), then electrophoresed into a

small dialysis sac attached to the bottom of the column, containing about 300ul of electrophoresis buffer, at 100V for 16 h at 4°C.

The samples were desalted on prepacked PD10 columns (method section V.1) by eluting in water, then lyophilised. The SDS was then removed by the method of Henderson et al. (1979). Briefly, the polypeptides were resuspended in a solution containing acetone, triethylamine, acetic acid and water (85:5:5:5) and incubated at 4°C for 1 h. This should precipitate the polypeptides while solubilising the SDS. Following centrifugation at 13,000rpm for 10 min in a Beckman microfuge, the polypeptide pellet was washed once with the above solution and then twice with acetone alone, by dispersing the pellet by sonication and then repelleting by centrifugation.

For trypsin digestion the polypeptide-containing pellet was resuspended in 100ul of a 1% solution of ammonium bicarbonate containing 5ug of TPCK-treated trypsin, and digested for 16 h at 37°C. An additional 5ug of trypsin was then added and incubated for a further 4 h, after which the sample was lyophilised.

The peptides were then oxidised with performic acid (Hirs, 1967). Performic acid was generated by incubating 0.5ml of hydrogen peroxide (30 volumes) with 10ml of formic acid for 2 h. Then 100ul of performic acid, 23ul of methanol and 40ul of performic acid were added, in turn, to each pellet with thorough mixing. This was incubated at RT for 2.5 h, then diluted with 8ml of water and lyophilised.

VI.2 Separation of tryptic peptides

The peptides were taken up in 5ul of pH 2.1 electro-

phoresis buffer (glacial acetic acid, formic acid, water 8:2:90) and applied, 1ul at a time, to a spot 4cm from each of two adjacent edges of a thin layer cellulose chromatogram (Eastman Kodak). After each application the chromatogram was dried in a stream of cold air. Following electrophoresis at 600V for 45 min, the chromatogram was dried, again in a stream of cold air, and then ascending chromatography was performed, in a direction perpendicular to that of electrophoresis, in 80ml of a buffer containing water, butan-1-ol, pyridine and acetic acid (24:30:20:6). The chromatogram was finally dried in hot air, sprayed with En^3Hance (New England Nuclear) and exposed to X-ray film at -70^oC.

SECTION VII : GROWTH AND PURIFICATION OF PLASMID DNA

The methods described in sections VII, VIII, IX and XII were modified from Maniatis et al. (1982) and references therein.

VII.1 Growth of bacterial cultures

Overnight cultures of bacteria were set up using either 25ul of a bacterial stock (stored in 50% glycerol at -20^oC or -70^oC) or a sterile cocktail stick touched against a single plaque on an agar plate, and were grown in 5ml of L-broth, containing 50ug/ml ampicillin, by shaking overnight at 37^oC. The overnight cultures were then transferred to 2l flanged flasks containing 500ml of L-broth with 50ug/ml ampicillin, and shaken at 37^oC at 220rpm on an orbital shaker for a further 24 h before harvesting.

VII.2 Harvesting of plasmid DNA by alkaline lysis

Bacterial cells were pelleted by centrifugation in a Sorvall GSA rotor at 8000rpm for 5 min, washed once by resuspending the pellet in 10ml of TE (10mM Tris.HCl pH 8.0, 1mM EDTA) and then pelleted again in an SS34 rotor at 10,000rpm for 5 min. Each pellet derived from a single 500ml culture was resuspended in 7ml of a solution containing 50mM glucose, 25mM Tris.HCl pH 8.0 and 10mM EDTA (solution I), then 50mg of lysozyme was added and the mixture vortexed and incubated at RT for 5 min. Freshly prepared solution II, containing 0.2M NaOH, 1% SDS, was then added (14ml/pellet) to lyse the bacteria and the tubes were gently inverted several times and incubated for 10 min on ice. Finally, 10.5ml of ice cold solution III (3M potassium acetate, 11.5% glacial acetic acid) was added and the tubes were mixed by inverting sharply several times and then incubated on ice for 15 min.

Cell debris and DNA was pelleted by centrifugation in an SS34 rotor at 15,000rpm for 30 min, at $4^{o}C$, and the supernatant, containing the plasmid DNA, was extracted 2-3 times with an equal volume of phenol:chloroform (1:1) and once with chloroform alone. Centrifugation at 2000rpm for 5 min caused a sharp interface to form and the upper phase containing the DNA was collected. The DNA was then precipitated by the addition of either 2 volumes of ethanol or 0.6 volumes of isopropanol followed by incubation at RT for 15 min. The DNA precipitate was pelleted by centrifugation in an SS34 rotor at 12,000rpm for 30 min, at RT, and the supernatant discarded. The pellet was then either washed once with 70% ethanol by briefly vortexing, repelleted

by centrifugation at 12,000rpm for 30 min, dried in a vacuum dessicator and then finally resuspended in 0.5ml of TE pH 8.0, or simply dried without a 70% ethanol wash, resuspended in TE pH 8.0 and applied to a caesium chloride gradient (see below).

VII.3 Purification of plasmid DNA by caesium chloride gradient centrifugation

The resuspended DNA was added to 15g of caesium chloride and 10mg of ethidium bromide, and TE added until the mixture weighed 31±0.2g. The caesium chloride was thoroughly mixed until it dissolved and the solution centrifuged in the Sorvall Ti50 rotor at 40,000rpm for 40 h, at 15°C. Bands were visualised under long wave uv light and the lower band, containing the supercoiled plasmid DNA, was collected by puncturing the tube with a syringe needle. Thus, the plasmid DNA was purified away from any host DNA or nicked circular plasmid DNA which would band higher up the gradient, or RNA which pellets to the bottom of the tube.

The ethidium bromide was extracted from the DNA by 3 washes with an equal volume of either caesium chloride-saturated isopropanol or isoamyl alcohol, the DNA this time being in the lower phase. The DNA was then dialysed against TE pH 8.0 at 4°C for 2 h with one change of buffer, and then precipitated overnight at -70°C in the presence of 0.1 volumes of 3M sodium acetate pH 5.5 and 2 volumes of ethanol. The DNA was then pelleted and washed with 70% ethanol, as described above, dried in a vacuum dessicator and finally resuspended in 0.5ml TE. The concentration of DNA was calculated from the OD_{260}, taking 1 OD unit to indicate 50ug/ml, and the purity of the sample judged by agarose

gel electrophoresis.

VII.4 Harvesting of small scale cultures by a modified alkaline lysis procedure

Overnight cultures were grown from isolated plaques as described (method section VII.1), and harvested by a modification of the alkaline lysis method. Briefly, 1.5ml from each culture was pelleted by centrifugation in a Beckman microfuge, then resuspended in 100ul of solution I containing 4-5mg/ml lysozyme. After 5 min at RT, 200ul of solution II was added and the tubes were then inverted 5 times and incubated on ice for 10 min. 150ul of solution III was added and the tubes vortexed in an inverted position and further incubated for 10 min on ice. Following centrifugation for 5 min in the microfuge the supernatant was extracted once with phenol/chloroform and once with ether, then the DNA was precipitated in 2 volumes of ethanol at RT for 2 min and centrifuged for 5 min in the microfuge. The pellet was washed in 70% ethanol with a brief vortex, repelleted and dried in a 'speedivac' vacuum dessicator.

SECTION VIII : PURIFICATION OF DNA FRAGMENTS BY AGAROSE GEL ELECTROPHORESIS

VIII.1 Restriction enzyme digestion of DNA

General restriction enzyme buffers :

	low salt	medium salt	high salt
Tris.HCl	10mM, pH 7.5	10mM, pH 7.5	50mM, pH 7.5
NaCl	-	50mM	100mM
$MgCl_2$	10mM	10mM	10mM
DTT	1mM	1mM	1mM

Specific restriction enzyme buffers :

	Aha II	Eag I
Tris.HCl	10mM, pH 8.0	10mM, pH 8.2
NaCl	100mM	150mM
$MgCl_2$	10mM	10mM
2-ME	6mM	10mM

10x stocks of these buffers were prepared and stored at -20°C.

Some restriction enzymes, their preferred buffers and sites of action :

Enzyme	Buffer	Site
Aha II	Aha II	G Pu↓C G Py C
Bam HI	medium	G↓G A T C C
Bgl II	low	A↓G A T C T
Eag I	Eag I	C↓G G C C G
Eco RI	high	G↓A A T T C
Hind III	medium	A↓A G C T T
Hinf I	medium	G↓A N T C
Pvu II	medium	C↓A G C T G
Sal I	high	G↓T C G A C

Reaction volumes varied from 40ul to 500ul, containing DNA in a solution with final concentrations of 1x reaction buffer, 100ug/ml BSA, the appropriate restriction enzyme at approximately 1 unit per ug DNA, and, if required, 25ug/ml of RNase A (the latter ingredient being used when the plasmid DNA had not been purified by caesium chloride gradient centrifugation). Reactions were carried out at 37°C for 2-24 h. As a rule, when two or more enzymes were used together the higher salt conditions were employed. For electrophoresis, the samples were simply diluted with

0.25 volumes of running buffer (5x TBE^2 containing 15% ficoll, 100mM EDTA, 0.2% BPB) and loaded directly onto the gel.

VIII.2 Purification of insert DNA

To purify insert DNA, the plasmid was cleaved at unique restriction enzyme sites flanking the inserted DNA fragment and then electrophoresed on 1.2% agarose gels as described (method section II.2). The DNA was visualised under long wave uv light and the insert was always well separated from plasmid DNA or RNA, running just above the dye front, and could be easily sliced out without risk of contamination. The gel slice was placed in a boiled dialysis sack with TBE^2, then excess buffer and air bubbles were squeezed out carefully and the bag was immersed in a shallow layer of buffer in an electrophoresis tank. Electrophoresis at 100V for 3-4 h efficiently extracted all detectable insert DNA. The current was briefly reversed (1-2 min) to remove the DNA from the side of the dialysis bag, the gel slices were removed, and the TBE^2 within the sac carefully collected. The sac was then washed with a small volume of buffer. The gel slice could be restained to ensure complete recovery of the insert DNA.

The insert DNA was then extracted 2-3 times with iso-amyl alcohol, to remove any ethidium bromide, precipitated with 2 volumes of ethanol, 0.1 volumes of 3M sodium acetate, rinsed with 70% ethanol, dried and finally resuspended in TE pH 8.0. Again, the concentration of DNA was calculated from the OD_{260}.

SECTION IX : PURIFICATION OF END-LABELLED FRAGMENTS BY POLYACRYLAMIDE GEL ELECTROPHORESIS

To end-label DNA inserts, plasmids were standardly cleaved at a unique restriction enzyme site to one side of the insert, end-labelled at this site, and then cleaved again at a second unique restriction enzyme site to the other side of the insert. The labelled insert is then readily separated from the labelled plasmid by polyacrylamide gel electrophoresis. The DNA must be extracted from the reaction mix between each step.

IX.1 Extraction of DNA

The DNA was (unless otherwise stated) extracted once with an equal volume of saturated phenol and once with an excess of ether, before being precipitated in 2 volumes of ethanol and 0.1 volumes of 3M sodium acetate, by chilling in an ethanol/dry-ice bath for at least 5 min. The DNA was pelleted by centrifugation in a Beckman microfuge for 5 min, the pellet washed once in 70% ethanol by brief vortexing, repelleted as before, and finally dried in the 'speedivac' vacuum dessicator.

IX.2 End-labelling of DNA

(a) End-labelling at the 3' end

Plasmid DNA (approximately 5ug) was cleaved at the relevant site to produce a 5' overhang and extracted as above. The DNA was then end-labelled at the 3' end by re-suspending in NT buffer (50mM Tris.HCl pH 7.9, 5mM $MgCl_2$, 10mM 2-ME) containing a radiolabelled nucleoside triphosphate

(α[^{32}P]-dNTP) complementary to the most 3' nucleotide of the 5' overhang. Reactions were carried out in a volume of 25ul, containing 5ul of the radio-labelled nucleoside triphosphate and 1ul (2 units) of Klenow reagent to catalyse the reaction, at 16^{o}C for 40 min. (Klenow reagent is the large fragment of E. coli DNA polymerase I, and contains the 5'-3' polymerase and the 3'-5' exonuclease activities). The reaction was stopped by extraction and precipitation of the DNA, as described above (method section IX.1).

For some unknown reason Hind III sites did not label well using this method, so a slight modification was used. In this case, the first two positions were filled by cold nucleoside triphosphates (dATP and dGTP, at 0.16mM) while α[^{32}P]-dCTP filled the third. Thus the Hind III site which, when cleaved, produces a 5' overhang 5'-A G C T ----3', is filled in by this procedure to yield 5'-A G C T ----3'
*C G A ----5'

(b) End-labelling at the 5' end

Plasmid DNA (5ug) was cleaved with the appropriate restriction enzyme, extracted and precipitated as described (method section IX.1). The DNA was resuspended in 25ul of a buffer containing 50mM Tris.HCl pH 8.0, 1mM EDTA and incubated at 37^{o}C for 60 min in the presence of 0.5ul (11 units) of calf intestinal phosphatase, to remove the 5' phosphate group. To inactivate this enzyme, 3ul each of proteinase K (500ug/ml) and 2% SDS were added and the reaction continued for a further 60 min at 37^{o}C. The DNA was then extracted twice with phenol and twice with ether before being precipitated (method section IX.1).

The DNA was labelled by resuspending in kinase buffer

(50mM Tris.HCl pH 7.6, 10mM $MgCl_2$, 0.1mM EDTA, 0.1mM spermidine, 5mM DTT) containing 10ul of $\gamma[^{32}P]$-ATP and 0.5ul (5units) T4 polynucleotide kinase, followed by incubation at 37°C for 30 min. The DNA was then extracted and precipitated as usual.

IX.3 Purification of end-labelled inserts

The end-labelled plasmids were cut with a second restriction enzyme to remove the insert and the fragments were separated on polyacrylamide gels as described (method section II.2). Standardly, inserts of 400-500bp were separated on 5% polyacrylamide gels, while smaller fragments of 100-200bp were separated on 10% polyacrylamide gels. One glass plate was removed and the wet gel covered with cling film, wiped with alcohol to remove static, and exposed to Kodak X-Omat XS-1 film, with an intensifying screen, at RT. A strong signal was usually seen within 1-2 min exposure. The gel could then be aligned with the autoradiograph with the aid of a hand-held minimonitor (type S·10E), and the relevant band was excised using a scalpel, and the gel re-exposed to film to ensure that the correct band had been completely removed without contamination.

The acrylamide slice was then placed in a small ampoule and mashed gently using a glass rod, 400-500ul of elution buffer (500mM ammonium acetate, 1mM EDTA, 0.1% SDS) was added and the DNA eluted overnight at 42°C. The mixture was then filtered through siliconised glass wool in a small (0.5ml) eppendorf tube punctured 3-4 times with a medium gauge needle, and the flow-through was collected into larger (1.5ml) eppendorf tubes. The gel pieces were then washed with 1-1.5ml of elution buffer, until less than 10% of the

total counts were retained on the column.

Following elution, 2 volumes of ethanol were added (without any additional salt) and the DNA precipitated by chilling. The pellet was washed by resuspending in 100ul of water and then precipitated with 200ul of ethanol without salt, as before. Finally, the DNA was rinsed with 95% ethanol and dried. The dried DNA was stored at -20°C and usually used within 2 weeks of preparation.

SECTION X : DNA SEQUENCING AND DNase I FOOTPRINT REACTIONS

X.1 Preparation of carrier calf thymus DNA

Calf thymus (CT) DNA, 100mg, was dissolved in 10ml sterile distilled water, at 37°C, then sonicated using a soniprobe for 3x30 seconds, in an ice bath. The DNA was extracted 3 times with an equal volume of saturated phenol, centrifuging at 2000rpm for 5 min at RT, to achieve a sharp interface between the two phases, then extracted a further 3 times with ether, before being dialysed overnight against sterile distilled water. Following a further 3 h dialysis against fresh distilled water the concentration of DNA was calculated from the OD_{260} and aliquots adjusted to 1mg/ml and stored at -20°C.

X.2 DNA sequencing reactions

The sequencing reactions were based on the method of Maxam and Gilbert (1980). This involved the modification of bases in reactions that are relatively base-specific, and then cleavage of these modified bases by piperidine.

G+A reaction buffer

0.1g	diphenylamine
5 ml	66% formic acid/ 1mM EDTA

G reaction buffer

50mM	sodium cacodylate pH 8.0
10mM	$MgCl_2$
1mM	EDTA

DMS stop solution

1.5M	sodium acetate pH 7.0
1M	2-ME
100ug/ml	tRNA

hydrazine stop solution

0.3M	sodium acetate pH 5.2
0.1mM	EDTA
25ug/ml	tRNA

formamide dye mix

1ml	deionised formamide
100ul	1% BPB
100ul	1% xylene cyanol

(a) G+A reaction

Dried DNA (usually 200cps) was resuspended by vortexing in 1ul CT DNA (1mg/ml) and 15ul G+A reaction buffer, and then incubated at 25°C for 5 min. The reaction was stopped by the addition of 45ul of deionised water, then extracted 3 times with an excess of ether, frozen on dry-ice and lyophilised. The DNA was then treated with piperidine, as described below.

(b) G reaction

Dried DNA (usually 100cps) was resuspended by vortexing in 5ul of water, 1ul of CT DNA (1mg/ml) and 200ul of G reaction buffer. This was then incubated with 1ul of dimethylsulphate (DMS) at 25°C for 2-3.5 min (for example, 500bp fragments were incubated for 2 min, whereas 150bp fragments were incubated for 3.5 min). The reaction was stopped by the addition of 50ul of DMS stop solution.

The DNA was then precipitated by the addition of 750ul of 95% ethanol, chilled in a dry-ice/ethanol bath and microfuged for 5 min to pellet the DNA, then resuspended in 250ul of 0.3M sodium acetate followed by the addition of 750ul of 95% ethanol and again chilled and pelleted. The DNA was rinsed in 95% ethanol by vortexing, microfuged and dried. The sample was then treated with piperidine, as described below.

(c) C+T reaction

Dried DNA (usually 200cps) was resuspended in 20ul water, 1ul CT DNA (1mg/ml) and 30ul hydrazine, and then incubated at 25°C for 5 min. The reaction was stopped by the addition of 200ul of hydrazine stop solution. The DNA was then precipitated and washed exactly as described for the G reaction protocol, and then treated with piperidine as described below.

(d) C reaction

Dried DNA (usually 100cps) was resuspended in 5ul water, 1ul CT DNA (1mg/ml), 15ul 5M NaCl and 30ul hydrazine, then incubated at 25°C for 5 min. The reaction was stopped by the addition of 200ul of hydrazine stop solution. The DNA was then precipitated and washed exactly as described for the G reaction protocol, and then treated with piperidine as described below.

(e) Piperidine reaction

The dried DNA was resuspended in 100ul of 1M piperidine (prepared by diluting a 10M stock solution in water), and

incubated at 90°C for 30 min. Then 100ul of water, 20ul 3M sodium acetate, 5ul CT DNA (1mg/ml) and 500ul 95% ethanol were added and the mixture chilled in a dry-ice/ethanol bath for 15-20 min, then microfuged for 5 min. The pellet was rinsed with 70% ethanol, microfuged and dried. The dried DNA was washed twice by resuspending in 10ul of water then lyophilising.

Samples were finally resuspended in formamide-dye mix at 10-15cps/ul, for the single base reactions (G,C) and 20-30cps/ul for the double base reactions (G+A,C+T) and analysed on sequencing gels as described (method section II.3).

X.3 DNase I footprinting assay

The DNase I footprinting assay was modified from that originally described by Galas and Schmitz (1978). A standard procedure is outlined below although the reaction conditions were occasionally varied, as described in the relevant result sections.

DNA binding reactions were carried out in a 50ul volume containing 10-20ng end-labelled DNA fragment (100cps), 1ug unlabelled competitor CT DNA and variable amounts of protein in a buffer of final concentration 20mM Tris.HCl pH 8.0, 50mM NaCl, 1mM EDTA, 1mM 2-ME, 10% glycerol, 2mM $MgCl_2$, 0.1mg/ml BSA. Samples were kept on ice, the protein being added only immediately prior to incubation at 37°C for 5 min, after which the reactions were stopped by cooling rapidly on ice. The samples were then warmed to RT and digested for 60 seconds by the addition of 1ul of DNase I (10ug/ml). The DNase digestion was terminated by the addition of 12.5ul of a DNase stop solution (3M sodium

acetate, 250mM EDTA, 1mg/ml CT DNA) and the samples were immediately phenol extracted. The DNA was precipitated in 10ul 3M sodium acetate/200ul ethanol and pelleted (method section IX.1). The pellet was resuspended in 100ul water, reprecipitated as before and then finally rinsed with 70% ethanol and dried. Pellets were resuspended in formamide-dye mix at 10-15cps/ul (in a minimum of 3ul) and analysed on sequencing gels (method section II.3).

SECTION XI : SYNTHESIS AND PURIFICATION OF SYNTHETIC OLIGONUCLEOTIDES

Synthetic oligonucleotides were synthesised on a Biosearch 8600 DNA synthesiser by either a macroscale or microscale synthesis procedure. The DNA was then eluted from the column by resuspending the beads in 1ml of ammonia and incubating at 55°C for 5 h. The ammonia was removed by lyophilisation in the 'speedivac' vacuum dessicator, and the dried samples were purified by denaturing polyacrylamide gel electrophoresis.

75ml of 16% sequencing gel mix, polymerised with 400ul of 10% ammonium persulphate and 40ul TEMED, was poured between two 20x22cm glass plates separated by 1.5mm spacers. Wells of approximately 1cm in width were formed using an 8-tooth teflon comb. The DNA samples were resuspended in 50ul of water by vortexing, then microfuged for 3 min. The supernatant was transferred to 50ul of sample buffer (28ul 10x TBE[1], 117ul water, 800ul deionised formamide) and boiled for 10 min then quenched on ice and loaded immediately.

2ul of formamide-dye mix was loaded in a separate

well to act as a molecular weight marker. The gels were electrophoresed slowly, at 3.5-4mA overnight in TBE[1].

To visualise the DNA the gel was removed, wrapped in cling film and viewed against a white chromatograph plate by angled long-wave uv light. If the synthesis had been successful then a predominant, strong top band with possibly a few minor lower mol. wt. bands was seen. The top band was cut out with a scalpel and mashed and eluted exactly as described in method section IX.3, except that chilling in dry-ice/ethanol was always for at least 20 min. Finally, the recovered DNA was resuspended in water and the OD_{260} determined. For synthetic oligonucleotides the conversion factor is taken as 1 OD unit = 20ug/ml.

SECTION XII : DNA CLONING

XII.1 Preparation of competent bacteria

1ml of an overnight culture of DH1 cells (a strain of Escherichia coli K12, Hanahan (1983)) was transferred to 22ml L-broth without ampicillin in a medium-sized (250ml) tissue culture flask and shaken slowly for approximately 90 min, until the OD_{630} of the culture was about 0.2 (equivalent to mid-log phase). The cells were then cooled on ice for at least 10 min, pelleted in an SS34 rotor at 8000rpm for 5 min, resuspended in 10ml ice-cold 50mM $CaCl_2$ and incubated on ice for a further 20-30 min. The cells were again pelleted and resuspended in 1ml 50mM $CaCl_2$, divided into 100ul aliquots and stored on ice overnight before use.

XII.2 Preparation of ampicillin plates

L-broth in agar was melted and then allowed to cool until the flask containing it was comfortable to hold. Ampicillin was added to a concentration of 100ug/ml, and the agar poured into 90mm sterile petri dishes and allowed to set. The plates were dried in an inverted position before being stored at $4^{o}C$ and were used within 3 weeks.

XII.3 Cloning of DNA fragments

Insert DNA was cloned into the Bam HI site of the vector pUC8. The plasmid DNA was cleaved with Bam HI at $37^{o}C$ for 6 h in the presence of 0.5ul (11 units) of calf intestinal phosphatase, then further incubated for 60 min in the presence of 50ug/ml proteinase K and 0.2% SDS, to inactivate the phosphatase. The DNA was then extracted twice each with phenol and ether and precipitated as described (method section IX.1). The DNA fragments to be inserted all had 5' overhangs equivalent to a Bam HI site and therefore should anneal with the ends of the Bam HI digested plasmid. Removal of the 5' phosphate groups on the plasmid should minimise religation of plasmid molecules without an insert.

For ligation, a DNA concentration of approximately 50ug/ml was used, in a volume of 20ul, and with a ratio of approximately 10:1 wt/wt insert:plasmid DNA. Thus, approximately 0.1ug of pUC8 DNA was incubated overnight at RT in the presence of 0.9ug of insert DNA in ligase buffer (50mM Tris.HCl pH 7.5, 10mM $MgCl_2$, 1mM ATP, 20mM DTT, 50ug/ml BSA) containing 0.5ul T4 DNA ligase. Following ligation the DNA was extracted and precipitated as described.

XII.4 Transformation of bacteria

Dried DNA was resuspended in 20ul of water or TE. Varying amounts (0.1-0.5ug in 5-10ul) of DNA were mixed with 50ul of competent DH1 cells and 25ul of 50mM $CaCl_2$ and incubated on ice for 45 min. The cells were then heat shocked at 42°C for 90 seconds, placed on ice for 60 seconds, then 400ul of L-broth without ampicillin was added and the cells incubated at 37°C for 60-90 min. 100ul of cells were then spread with an L-shaped glass rod onto ampicillin plates, at various dilutions (usually neat, 1/10, 1/100) and incubated at 37°C for 16-24 h.

Individual plaques were analysed by growing overnight cultures, isolating the plasmid DNA and testing for the presence of insert DNA by restriction enzyme analysis. Cultures were grown from positive clones and stocks were then stored in 50% glycerol at both -20°C and -70°C.

RESULTS

RESULTS

SECTION I : GENERATION AND CHARACTERISATION OF ANTISERA

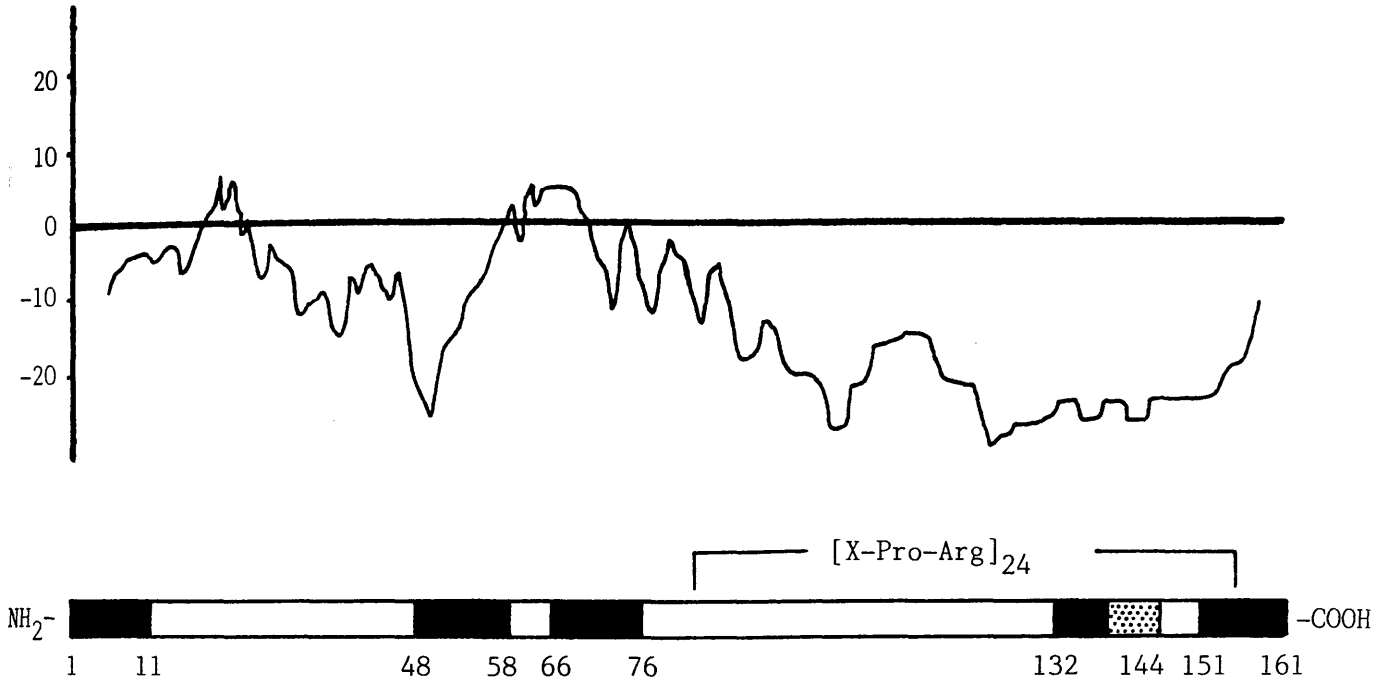
I.1 Generation of anti-peptide sera

The original aim of this project was to purify and characterise the 21K DNA-binding protein, believed to be the product of gene U_S11 (see introduction section XI). The available DNA sequence information (Rixon and McGeoch, 1984) was used to obtain synthetic oligopeptides corresponding to 5 regions within the predicted U_S11 gene product. These are shown in Figure 18. A number of studies have suggested that the best results in inducing anti-peptide sera which cross-react with the native protein are obtained by using amino- or carboxy-terminal peptides or peptides representing hydrophilic regions of the protein (reviewed in Palfreyman et al., 1984). Thus, peptides corresponding to amino acids 1-11, 151-161 and 155-161 were chosen, since they represent the amino- and carboxy-termini of the predicted polypeptide, respectively, while peptides corresponding to amino acids 48-58 and 132-138 were chosen on the basis of their hydrophilicity (see Figure 18). The amino acid sequence predicted for the U_S11 gene product contains a region which shares limited homology with a consensus sequence for α-helix 3 of known sequence-specific DNA-binding proteins (Gicquel-Sanzey and Cossart, 1982; Takeda et al., 1983). Amino acids 66-76 lie within this region (Figure 19).

The peptides used in this study have been named simply on the basis of the amino acid sequence they represent, ie. peptide 1-11, 48-58 et cetera. In addition, for simplicity in the figures, the terminal peptides have been designated

FIGURE 18

Schematic diagram of the U_S11 gene product. The predicted product of gene U_S11 is a 161 amino acid protein with a predicted mol. wt. of 17,756 (Rixon and McGeoch, 1984; McGeoch *et al.*, 1985), and has the unusual feature of containing 24 tandem repetitions of the sequence X-Pro-Arg towards the carboxy-terminus. The hydropathicity profile of this putative polypeptide, obtained using the program SOAP, based on the method of Kyte and Doolittle (1982), is shown above the schematic diagram of the predicted gene product. Negative hydropathicity values indicate hydrophilic regions of the protein. Synthetic oligopeptides, corresponding to amino acids 1-11, 48-58, 66-76, 132-138, 151-161 and 155-161, respectively (▬), were used to generate antisera. The amino acid sequences of these oligopeptides are shown below. An additional amino-terminal tyrosine residue was added to each sequence to facilitate coupling to BSA.



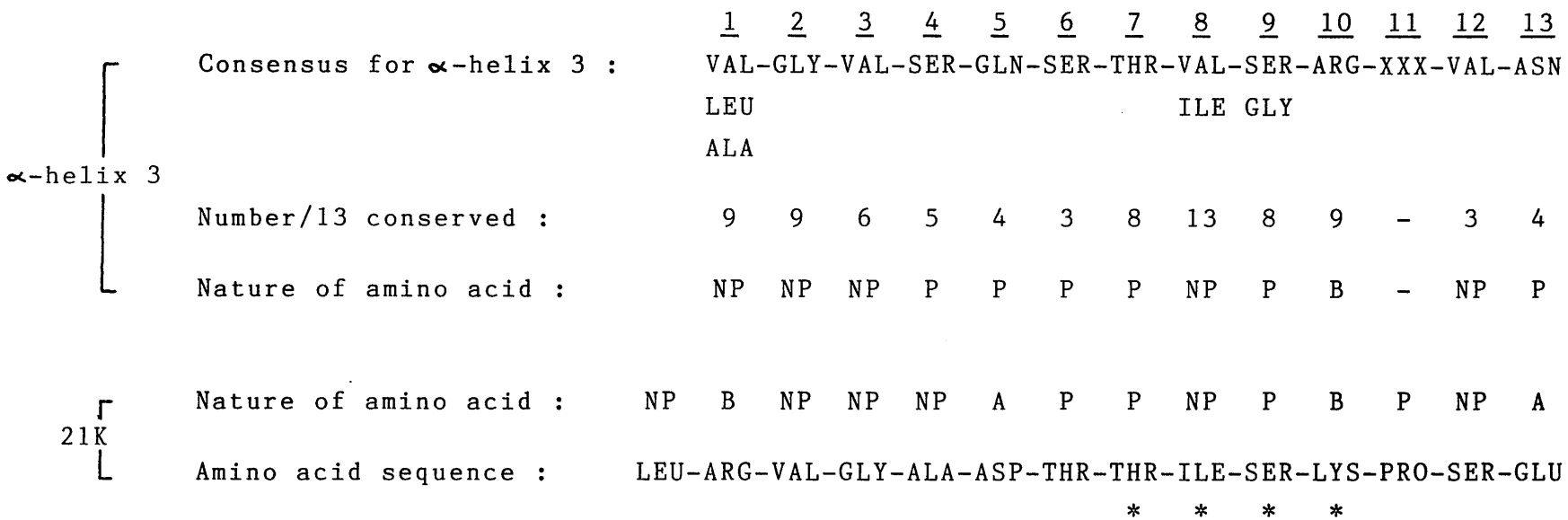
Amino acid sequence of the synthetic oligopeptides

1-11	:	(Tyr)-Met-Ser-Gln-Thr-Gln-Pro-Pro-Ala-Pro-Val-Gly
48-58	:	(Tyr)-Arg-Gly-Asp-Asn-Asp-Gln-Ala-Ala-Gly-Gln-Cys
66-76	:	(Tyr)-Val-Gly-Ala-Asp-Thr-Thr-Ile-Ser-Lys-Pro-Ser
132-138 138-144	:	(Tyr)-Glu-Pro-Arg-Ser-Pro-Arg-Glu
151-161	:	(Tyr)-Pro-Arg-Glu-Pro-Arg-Thr-Ala-Arg-Gly-Ser-Val
155-161	:	(Tyr)-Arg-Thr-Ala-Arg-Gly-Ser-Val

FIGURE 19

Homology between the U_S11 gene product and known sequence-specific DNA-binding proteins. The consensus sequence derived for α-helix 3 of known sequence-specific DNA-binding proteins (Gicquel-Sanzey and Cossart, 1982; Takeda et al., 1983) is shown. Thirteen different sequence-specific DNA-binding proteins were compared by the former authors, and the number of these which are conserved at a given amino acid is indicated. The nature of the amino acid - ie. polar (P), non-polar (NP), acidic (A) or basic (B) - is also shown for comparison. The region within the predicted U_S11 gene product (21K) from amino acids 64-77, inclusive, is shown below. This has limited homology with the consensus sequence, and in particular shares a highly conserved region found between positions 7 and 10 (*).

XXX no conserved amino acid


1 2 3 4 5 6 7 8 9 10 11 12 13
Consensus for α-helix 3 :
VAL-GLY-VAL-SER-GLN-SER-THR-VAL-SER-ARG-XXX-VAL-ASN
LEU
ALA
ILE GLY
α-helix 3
Number/13 conserved :
9 9 6 5 4 3 8 13 8 9 - 3 4
Nature of amino acid :
NP NP NP P P P P NP P B - NP P
Nature of amino acid :
NP B NP NP NP A P P NP P B P NP A
21K
Amino acid sequence :
LEU-ARG-VAL-GLY-ALA-ASP-THR-THR-ILE-SER-LYS-PRO-SER-GLU
* * * *

N_{11}, C_{11} and C_7, indicating that they represent the amino-terminal 11 amino acids, the carboxy-terminal 11 amino acids and the carboxy-terminal 7 amino acids, respectively. Peptide 132-138 represents not only amino acids 132-138, but also amino acids 138-144 : the amino acid sequence of this peptide (excluding the amino-terminal tyrosine residue, see below) forms part of a trimeric repeat sequence [Ser-Pro-Arg-Glu-Pro-Arg] found between amino acids 129 and 146.

The synthetic peptides were obtained from Cambridge Research Biochemicals (CRB) Limited. They all contained an additional amino-terminal tyrosine residue, to facilitate coupling to carrier protein (Bassiri et al., 1979). It has long been believed that small molecules, such as synthetic peptides, are poorly immunogenic, and that coupling to a carrier protein greatly enhances their immunogenicity. Bovine serum albumin (BSA) is an efficient carrier molecule (Palfreyman et al., 1984b), readily available and relatively inexpensive and was therefore used throughout.

The peptides were iodinated, via the tyrosine residue, in order to monitor the efficiency of the coupling reaction. The iodination reactions were carried out by Dr Howard S Marsden or Mrs Mary Murphy and are therefore not shown. Coupling to BSA was carried out as described in method section III.2, using bis diazobenzidine as the cross-linking reagent. Since this procedure has been fully described (Dalziel, 1984) results will not be shown. A brief outline is, however, given below.

Peptide-BSA conjugates were separated from uncoupled peptide by passage through a Sephadex G25 column. Collected fractions were analysed for radioactivity in a gamma counter. Two peaks were observed - the first peak representing coupled

peptide and the second free peptide. Fractions from peak 1 were then pooled and the coupling efficiency calculated (method section III.2b). Over the 12 coupling reactions that were carried out in total, coupling efficiencies ranged from 18% to 40%, with an average coupling efficiency of 28.7%. This represents 3-10 moles of peptide per mole of BSA. Coupled peptides were dialysed against PBS-A before use.

To monitor the preparation of the BSA-peptide conjugates, samples taken before and after coupling were subjected to SDS-PAGE and analysed by both Coomassie blue staining and auto-radiography. Prior to coupling no label was seen comigrating with the BSA, whereas after coupling this protein appeared strongly labelled. By subjecting samples at various stages of the procedure to SDS-PAGE, it was established that neither passage through Sephadex G25 nor the subsequent dialysis resulted in significant loss of the BSA-peptide conjugate (results not shown).

New Zealand White rabbits were immunised according to the schedule shown in Table 3 (see method section III.3). For each immunisation 400-500ug of either free, uncoupled peptide, or peptide coupled to BSA, immulsified in complete or incomplete Freund's adjuvant, was injected intramuscularly, into both rear legs. The rabbits were bled 7-14 days after each injection. Pre-immune sera were obtained from all rabbits, except numbers 14059 and 14060.

I.2 Establishment of a radioimmunoassay for anti-peptide antibodies

(a) Optimal concentration of peptide

Immune sera were assessed for reactivity against the immunising peptide by a radioimmunoassay (RIA). This is

Table 3 : Immunisation schedule used to generate anti-peptide sera

New Zealand White rabbits were immunised with peptide, either alone (free) or conjugated to BSA. For simplicity, the rabbits have been designated numbers 1-17, but the full rabbit number is also given.

ϕ The day upon which the rabbits were either immunised or bled. Day 0 is defined as the date of the first immunisation of a given rabbit. All subsequent dates are relative to this.

* Serum 17 (12848) was raised by R.G. Dalziel and H.S. Marsden, and has been described previously (Dalziel, 1984).

Table 3 : Immunisation schedule used to generate anti-peptide sera

Rabbit N°	Immunogen	Immunisation									Bleed								
		1	2	3	4	5	6	7	8	9+	1	2	3	4	5	6	7	8	9+
1. (14473)	1-11, BSA	0	23	34	86	163	-	-	-	-	9	33	44	97	127	175	-	-	-
2. (14474)	1-11, BSA	0	23	34	86	163	-	-	-	-	9	33	44	97	127	175	-	-	-
3. (14471)	1-11, free	0	23	34	204	-	-	-	-	-	9	33	44	212	-	-	-	-	-
4. (14472)	1-11, free	0	23	34	204	-	-	-	-	-	9	33	44	212	-	-	-	-	-
5. (14059)	48-58, BSA	0	21	35	-	-	-	-	-	-	44	-	-	-	-	-	-	-	-
6. (14060)	48-58, BSA	0	21	35	51	91	-	-	-	-	44	62	99	-	-	-	-	-	-
7. (18244)	48-58, BSA	0	21	46	75	-	-	-	-	-	11	32	56	84	-	-	-	-	-
8. (18245)	48-58, BSA	0	21	46	75	-	-	-	-	-	11	32	56	84	-	-	-	-	-
9. (18246)	66-76, BSA	0	50	119	125	-	-	-	-	-	9	61	135	-	-	-	-	-	-
10. (18416)	66-76, BSA	0	50	119	125	-	-	-	-	-	9	61	135	-	-	-	-	-	-
11. (12850)	132-138, BSA	0	11	36	-	-	-	-	-	-	46	-	-	-	-	-	-	-	-
12. (12851)	132-138, BSA	0	11	36	157	174	434	452	508	-	46	186	431	444	462	518	531	545	559/642
13. (14327)	151-161, BSA	0	22	35	50	108	185	-	-	-	10	31	45	59	118	149	197	-	-
14. (14328)	151-161, BSA	0	22	35	50	108	185	-	-	-	10	31	45	59	119	149	197	-	-
15. (14469)	151-161, free	0	23	34	-	-	-	-	-	-	9	33	44	203	-	-	-	-	-
16. (14470)	151-161, free	0	23	34	-	-	-	-	-	-	9	33	44	-	-	-	-	-	-
17. (12848)*	155-161, BSA	0	7	21	35	49	70	98	119	166/189/361	32	46	89	109	130	178	202	278	371/475

outlined in method section IV.3. To establish the optimal concentration of peptide to use as antigen, doubling dilutions of peptide in PBS were adsorbed onto microtitre plates, and then tested against doubling dilutions of antibody. In theory, the antigen dilution giving the highest antibody titre for a given serum is the most sensitive assay condition to use. A positive reaction was taken as $\geqslant$3 times the normal rabbit or pre-immune serum control value, a control serum being present on each microtitre test plate. Examples of antigen dilution curves are shown in Figures 20A and 20B.

For some peptides there was an obvious reduction in sensitivity as the antigen concentration fell. For example, for peptide 155-161 (Figure 20A) there was a rapid fall in the antibody titre measurable after an antigen dilution of 1/4 (4ug/ml). However, for other peptides, for example peptide 132-138 (Figure 20B), there was no obvious loss of sensitivity with decreasing concentrations of antigen. Therefore, in these cases consideration was also taken of the absolute counts measured. The higher the counts the more reliable the assay, as the percentage error in the measurement of radioactivity would be less. In Figure 20C, a constant dilution of antiserum against peptide 132-138 (anti-(132-138)) was reacted against doubling dilutions of peptide. The counts measured fell sharply after an antigen dilution of 1/4 (4ug/ml). In comparison, the normal rabbit serum control values were approximately 300-400 cpm for each antigen dilution. Therefore, for both the peptides shown, a concentration of 4ug/ml was considered sufficiently sensitive for our purpose. In a similar manner, the optimal concentration of each of the other peptides was calculated (see Table 4).

FIGURE 20

To determine the optimal concentration of peptide antigen for RIA. Assays were carried out as described in method section IV.3.

A,B Doubling dilutions of peptide 155-161 (A) or peptide 132-138 (B), from 16 ug/ml, were adsorbed onto microtitre plates and reacted against serial dilutions of either (A) anti-(155-161) serum 12848, or (B) anti-(132-138) serum 12851. Reactions $\geq$3x the NRS control were considered positive. The antibody titre represents the highest dilution of antiserum considered positive.

C Doubling dilutions of peptide 132-138, from 16 ug/ml, were adsorbed onto microtitre plates and reacted against anti-(132-138) serum 12851, diluted 1/10. Bound antibody was detected using ^{125}I-labelled protein A, and the absolute level of counts measured is shown. The NRS control bound between 290-414 cpm (results not shown).

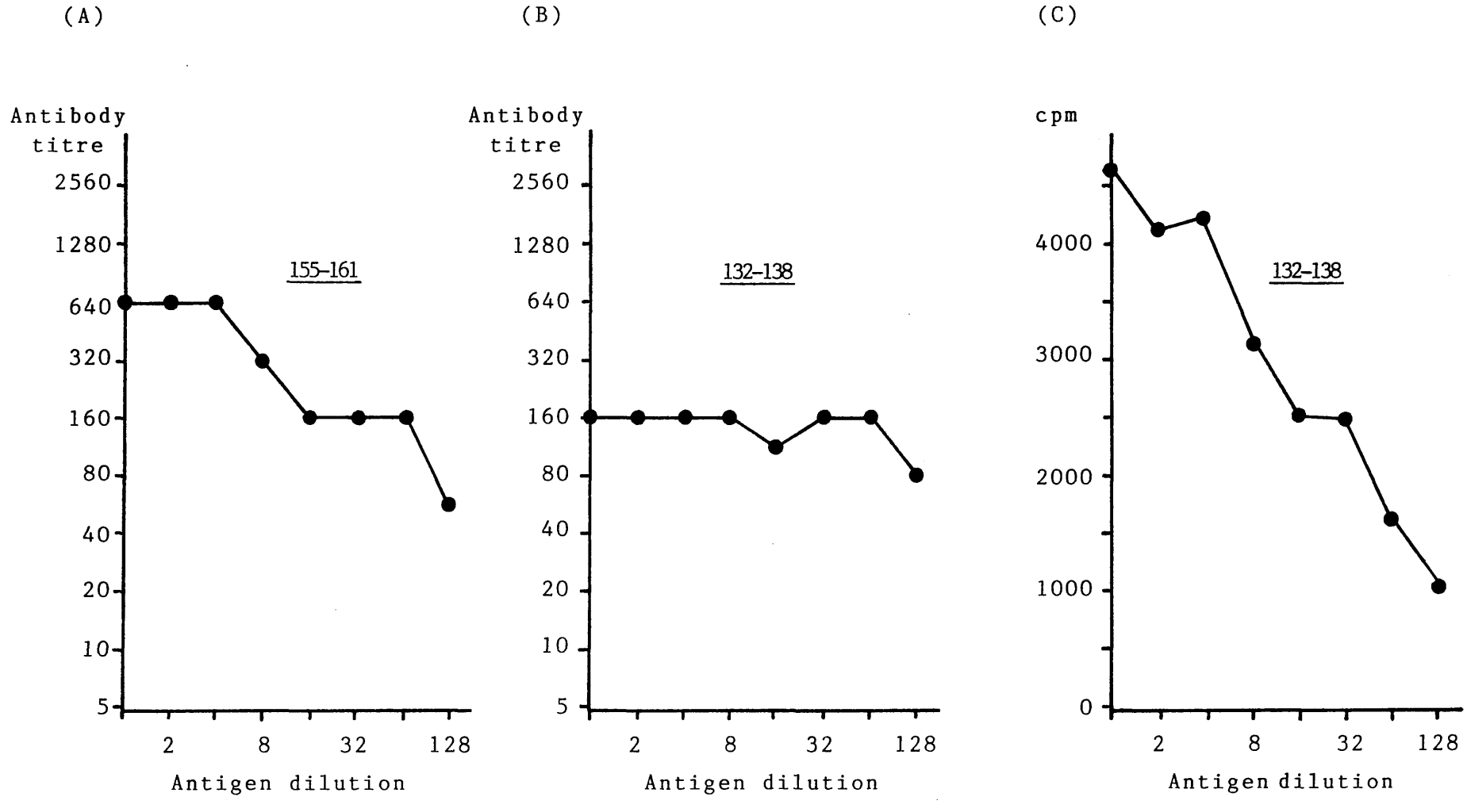

(A)
Antibody titre
2560
1280
640
320
160
80
40
20
10
5
155–161
2
8
32
128
Antigen dilution
(B)
Antibody titre
2560
1280
640
320
160
80
40
20
10
5
132–138
2
8
32
128
Antigen dilution
(C)
cpm
4000
3000
2000
1000
0
132–138
2
8
32
128
Antigen dilution

Table 4 : Concentration of peptides used in the RIA

The concentration of peptide subsequently used in the RIA was determined as described in the text. 25ul of peptide, at the concentration indicated, was added per well of a microtitre plate. From the estimated mol. wt. of each peptide, the approximate number of picomoles of peptide added was calculated.

Table 4 : Concentration of peptides used in the RIA

Peptide	Concentration	pMoles/well
1-11	10 ug/ml	196
48-58	20 ug/ml	385
66-76	25 ug/ml	504
132-138	4 ug/ml	96.8
151-161	10 ug/ml	180
155-161	4 ug/ml	110

(b) Specificity of the radioimmunoassay

In order to confirm the specificity of the assay, sera were tested against several different antigens. The anti-peptide sera only reacted against specific peptide and not against unrelated peptides or proteins. An example of one such test is shown in Table 5A. Using an antiserum against the BSA-conjugated peptide 132-138, a strong reaction is detected against BSA, the related protein ovalbumin, and the peptide 132-138, but not against an unrelated peptide (peptide 155-161), the amino acid glycine or the unrelated protein lysozyme. No reaction is seen in the absence of antigen, indicating that non-specific binding sites are efficiently blocked by the Tween 20 blocking reagent.

(c) Removal of anti-BSA antibodies from anti-peptide sera

To obtain truly mono-specific antisera against the peptides, antibodies to BSA were removed. This was achieved by covalently coupling BSA to Sepharose 4B (method section V.2), incubating the anti-peptide serum on the Sepharose:BSA column for 1 h at RT and then eluting with PBS. Material was collected from the first drop of reddish-brown colour to the last drop of colour, resulting in a 2-3-fold dilution of the antiserum. The degree of colour, which probably corresponds to the concentration of serum, was shown to correlate with the presence of antibody, as assessed by RIA (Mary Murphy, personal communication), and this procedure therefore safely included all significant antibody-containing fractions. The sera were then tested for the presence of antibodies to BSA and peptide. Sera which had not been absorbed with Sepharose:BSA, and therefore still contained anti-BSA antibodies, were termed nonabsorbed (NA) sera, while those which

Table 5 : Specificity of the RIA, and removal of anti-BSA antibodies

A. Peptide, at the concentration shown in Table 4, glycine (1M), lysozyme, ovalbumin, BSA (all 0.1% in PBS) or PBS alone (nil), were adsorbed onto microtitre plates, fixed and blocked as described (method section IV.3). The plates were then reacted with anti-(132-138) serum 12851, either absorbed against BSA (PC), or not absorbed (NA), or normal rabbit serum (not shown). Results are expressed as a ratio of the ^{125}I-labelled protein A (cpm) bound by the antiserum compared to the control serum.

B. Peptide 155-161 (4 ug/ml) or BSA (0.1%) were adsorbed onto microtitre plates and reacted against anti-(155-161) serum 12848 either non-absorbed (NA) or absorbed against Sepharose: BSA (PC), the antibodies eluted from the Sepharose:BSA column by 3M sodium thiocyanate and dialysed against PBS (EL), or normal rabbit serum (NRS), diluted as indicated. The level of bound ^{125}I-labelled protein A, in cpm, is given.

Table 5 : Specificity of the RIA, and removal of anti-BSA antibodies

A.

Antigen	Anti-(132-138) NA	PC
132-138	28.6	22.2
155-161	1.1	1.0
Glycine	1.5	1.2
Lysozyme	2.4	1.0
Ovalbumin	10.9	2.4
BSA	11.4	0.9
nil	1.1	1.1

B.

Dilution	Anti-(155-161) NA	PC	EL	NRS	Antigen
1	5329	1001	3560	2744	BSA
10	4838	118	1714	255	
100	2499	81	573	67	
1000	688	99	91	42	
1	2625	2539	109	160	155-161
10	1416	835	110	101	
100	325	144	93	112	
1000	136	103	56	110	

had been absorbed against Sepharose:BSA were termed post-column (PC) sera.

Tables 5A and 5B show the effect of absorption of antisera with BSA. In Table 5A, removal of anti-BSA antibodies significantly reduces the reaction with both BSA and ovalbumin without significantly affecting the response to peptide 132-138. Thus, within the limits of sensitivity of the assay, the antiserum is now mono-specific. In Table 5B, antiserum against peptide 155-161 was absorbed with Sepharose:BSA. After extensive washing of the column, specifically retained antibodies were eluted using 3M sodium thiocyanate, and then dialysed against PBS. This material was termed the eluate (EL). In the non-absorbed serum a high titre of anti-BSA antibodies was present ($\geqslant$1000), whereas following absorption the titre was not detectable (ie. was not significantly different from the normal rabbit serum (NRS) control). Radioimmunoassays for BSA antibodies always had a high background with undiluted serum, and the results for these samples must be interpreted with caution. In addition, it should be mentioned that during chromatography the post-column and eluate samples were diluted 2.5-fold in volume compared to the non-absorbed serum. Neither the non-absorbed serum nor the NRS control were similarly diluted to take this into account. Bearing these comments in mind, there would still appear to be at least a 100-fold (probably 1000-fold) reduction in the anti-BSA titre, while in comparison the anti-peptide response was diminished less than 10-fold. Titration experiments have shown that, in general, absorption against Sepharose:BSA results in a 2-4-fold reduction in anti-peptide titre. Since absorption also causes a 2-3-fold increase in volume this represents overall a very small loss of anti-peptide antibodies. Material eluted

from the Sepharose:BSA specifically reacted with BSA antigen, to a titre of about 100, while it did not react at all with peptide antigen (Table 5B). This would represent a recovery of approximately 10% of the anti-BSA activity. Full recovery would not be expected since 3M sodium thiocyanate is a rather harsh and denaturing treatment.

Thus, it can be concluded that absorption against BSA specifically removes antibodies to BSA without a significant loss of anti-peptide antibodies.

I.3 Titration of anti-peptide sera by radioimmunoassay

Sera were assessed for response to peptide by RIA prior to removal of the BSA antibodies. The results are shown in Table 6. It was interesting to note that in a number of cases the anti-peptide titres fell with time, despite further immunisation. No attempts were made to boost using different carrier proteins. Four rabbits were immunised with free peptide, but produced only very low or undetectable titres of anti-peptide antibodies. However, when coupled to BSA these peptides induced high levels of antibody (compare rabbits 1,2 with 3,4 and 13,14 with 15,16). Therefore, at least for these two peptides, using this immunisation protocol, the coupling to carrier protein was essential for a good anti-peptide response.

I.4 Interaction of anti-peptide antibodies with HSV polypeptides

(a) Establishment of the immunoblotting assay

The antisera were screened for reactivity against protein by immunoblotting. The immunoblotting procedure is described fully in method section IV.5. Very briefly, [^{35}S]-methionine

Table 6 : Anti-peptide response of immunised animals

Sera were assessed for response to peptide by RIA prior to removal of anti-BSA antibodies. Rabbit numbers and immunogens are designated as in Table 3. Consecutive bleeds from each rabbit were assessed simultaneously. The titres represent the highest dilution of a given serum considered positive (ie. $\geq$3x the pre-immune or NRS control).

PI pre-immune

nd not determined

- no such bleed exists

Table 6 : Anti-peptide response of immunised animals

Rabbit/Immunogen	Bleed							
	PI	1	2	3	4	5	6	7	8+
1. 1-11, BSA	0	256	512	≥1024	512	512	256	-	-
2. 1-11, BSA	0	32	≥1024	≥1024	≥1024	256	512	-	-
3. 1-11, free	nd.	0	0	4	2	-	-	-	-
4. 1-11, free	nd.	0	0	0	0	-	-	-	-
5. 48-58, BSA	nd.	320	-	-	-	-	-	-	-
6. 48-58, BSA	nd.	320	160	160	-	-	-	-	-
7. 48-58, BSA	0	nd.	1280	640	320	-	-	-	-
8. 48-58, BSA	0	nd.	640	640	640	-	-	-	-
9. 66-76, BSA	0	10	1280	640	-	-	-	-	-
10. 66-76, BSA	0	160	1280	640	-	-	-	-	-
11. 132-138, BSA	nd.	40	-	-	-	-	-	-	-
12. 132-138, BSA	nd.	80	80	40	160	-	-	-	-
13. 151-161, BSA	0	32	256	256	512	128	128	64	-
14. 151-161, BSA	0	64	≥1024	≥1024	≥1024	256	128	≥1024	-
15. 151-161, free	nd.	0	0	0	2	-	-	-	-
16. 151-161, free	nd.	0	0	0	nd,	-	-	-	-
17. 155-161, BSA	0	nd.	nd.	1280	1280	640	80	160	80/80/80/80

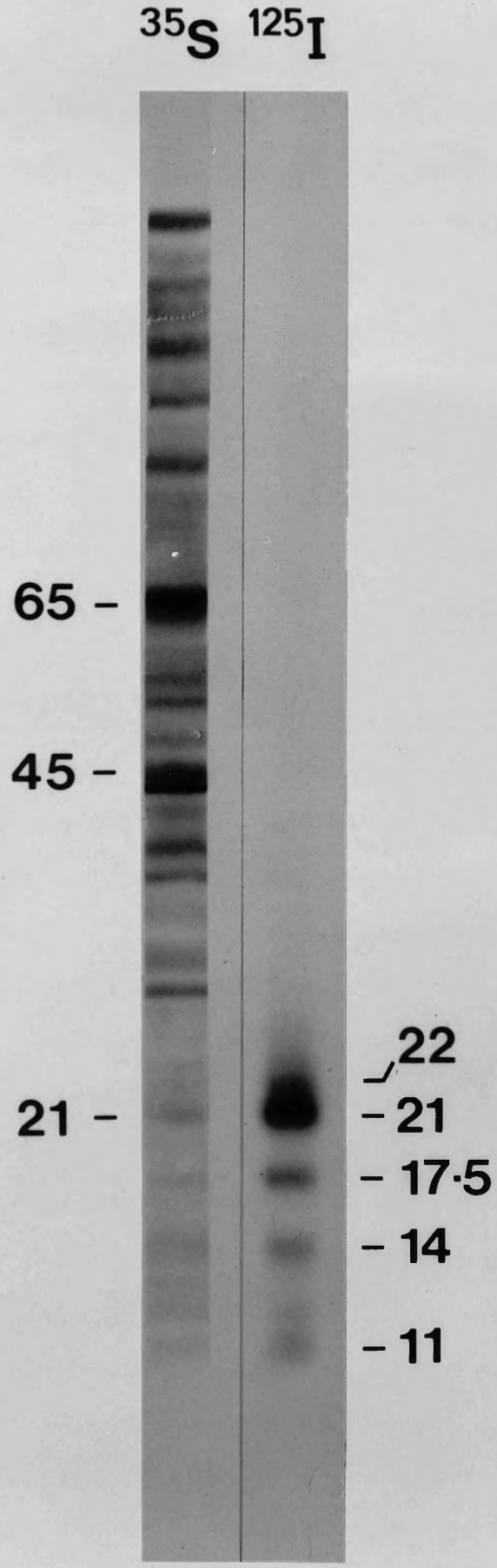
labelled polypeptides, electrophoretically separated by SDS-PAGE and transferred to nitrocellulose, were reacted with antiserum and the bound antibody molecules then detected using ^{125}I-labelled protein A. The nitrocellulose strips were set up for autoradiography in such a way that the signals from the ^{35}S and ^{125}I were detected on separate X-ray films. The films were then aligned by means of marker spots of radioactive ink, allowing identification of the proteins recognised by the antisera. This is illustrated in Figure 21.

The HSV polypeptides themselves serve as mol. wt. markers. They produce a characteristic banding pattern on SDS-PAGE, and apparent mol. wt. values have previously been very carefully determined for each of these bands (Marsden et al., 1976). In Figure 21, antiserum against peptide 151-161 is seen to react with a number of low mol. wt. polypeptides. By alignment with the corresponding ^{35}S-labelled protein profile, these can be identified as having apparent mol. wt. 22K, 21K, 17.5K, 14K and 11K. A faint band can also be seen in the region of 12K, but for reasons which will be discussed later, this is thought to represent a non-specific interaction.

Anti-peptide antibodies react in immunoblotting experiments to varying extents depending on the temperature at which the nitrocellulose strips are blocked (Howard S Marsden, personal communication). The reason for this behaviour is not known, but it is possible that heat denaturation of the protein may lead to some epitopes becoming more (or in some cases, less) accessible to the relevant antibody. Thus, two blocking temperatures were originally used, 37°C (the 'non-denaturing' temperature) and 65°C (the 'denaturing' temperature). For all our antisera, the immunoblotting reactions were as good or better at 65°C when compared to 37°C, and for this reason the

FIGURE 21

Immunoblotting with anti-C_{11} serum 14328. Immunoblotting was carried out as described in method section IV.5. The first track shows ^{35}S-methionine labelled HSV-1-infected cell proteins transferred to nitrocellulose. The second track shows the polypeptides on the nitrocellulose that are detected by the anti-C_{11} serum. Alignment of the two lanes allows the apparent mol. wt. of the polypeptides to be identified (Haarr *et al.*, 1985).


35S
125I
65
45
21
22
21
17·5
14
11

blocking reactions were subsequently carried out at 65°C.

In immunoblotting experiments it was necessary to use antisera after the removal of BSA antibodies. Non-absorbed sera reacted with a 68K polypeptide, presumably corresponding to BSA, and also showed a high level of background staining on the immunoblots (Figure 22), possibly due to the presence of multiple breakdown products of BSA (Dalziel, 1984) or cross-reaction with cell or viral proteins. In contrast, the post-column antisera showed a relatively low level of background staining. Pre-immune sera varied in their level of background staining, but never reacted with a 21K/22K band, suggesting that this represents a specific interaction.

(b) Reactivity of anti-peptide sera by immunoblotting

A typical example of an immunoblotting reaction is shown for each serum in Figure 23. The arrow represents the position occupied by the 21K polypeptide, determined by comparison with the corresponding [^{35}S]-labelled polypeptide profile (not shown). In some cases, the 21K and 22K bands are clearly resolved (for example, rabbits 7, 10, 14; see also Figure 21), indicating that both 21K and 22K polypeptides are being recognised. However, often these bands run very close together and cannot be clearly distinguished on immunoblots.

The sera raised against the BSA-conjugated peptides (ie. all sera excluding 3, 4, 15 and 16), all reacted with a protein of apparent mol. wt. 21K, with the single exception of the antiserum against peptide 132-138 (rabbit 12). This serum consistently failed to recognise any apparently specific band on immunoblots, and showed a high level of background staining. A very faint band in the region of 21K was, however, among the many bands present.

FIGURE 22

Effect of removal of anti-BSA antibodies on the immuno-blotting assay. HSV-1-infected cell polypeptides, separated on 10% SDS-polyacrylamide gels and transferred to nitro-cellulose, were reacted against anti-C_{11} serum 14328. PI represents pre-immune serum from rabbit 14328; PC represents 'post-column' serum - ie. anti-C_{11} serum 14328 absorbed against Sepharose:BSA; NA represents non-absorbed anti-C_{11} serum 14328.

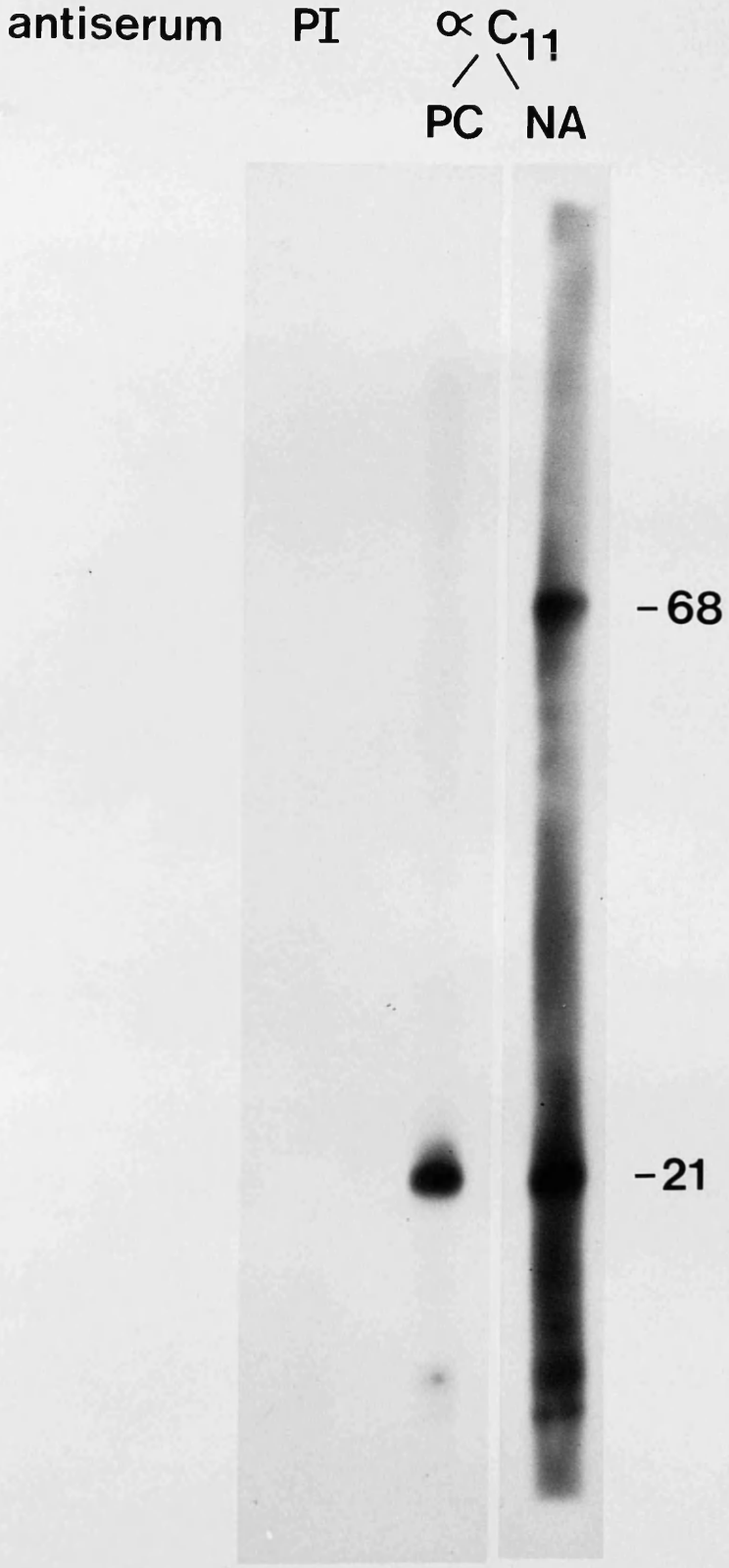

antiserum
PI
∝ C11
PC
NA
-68
-21

FIGURE 23

Immunoblotting of HSV-1-infected cell polypeptides with the anti-peptide sera. Rabbit numbers are designated as in Table 3 :

1-4 : anti-N_{11} sera 14473, 14474, 14471 and 14472, respectively. The latter two rabbits were immunised with free oligopeptide.

5-8 : anti-(48-58) sera 14059, 14060, 18244 and 18245, respectively.

9-10 : anti-(66-76) sera 18246 and 18416, respectively.

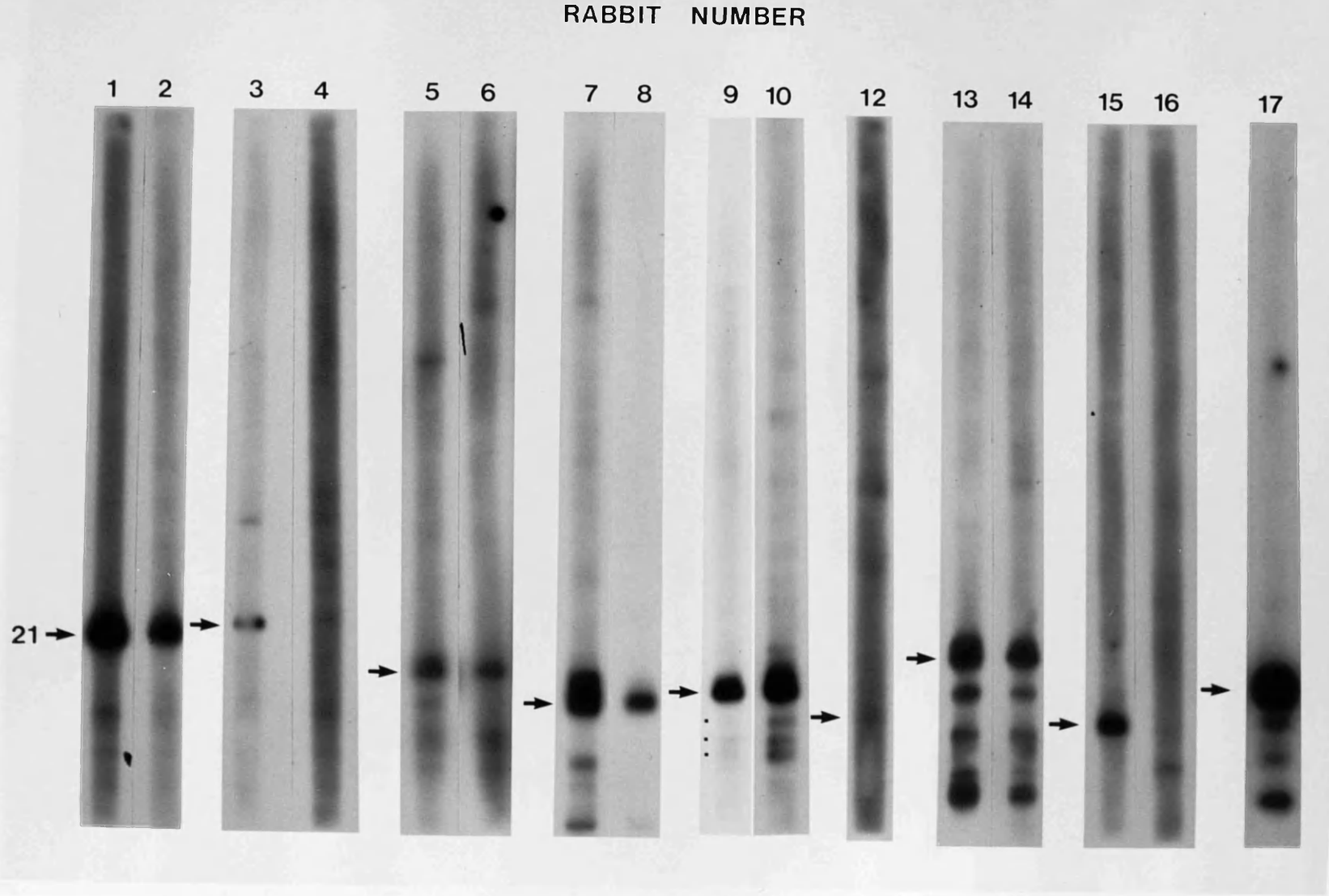
12 : anti-(132-138) serum 12851.

13-16 : anti-C_{11} sera 14327, 14328, 14469 and 14470, respectively. The latter two rabbits were immunised with free oligopeptide.

17 : anti-C_7 serum 12848, raised by R.G.Dalziel and H.S. Marsden.

The position of the 21K polypeptide, identified by alignment with the corresponding ^{35}S-methionine profile, is indicated (➔).

⁝ indicates 3 bands of apparent mol. wt. 17.5K, 15K and 14K recognised by the anti-(66-76) sera. Their specificity is uncertain.


RABBIT NUMBER
1
2
3
4
5
6
7
8
9
10
12
13
14
15
16
17
21

Free, uncoupled peptides were less successful in inducing antisera which reacted against the 21K polypeptide. Only one serum from each pair reacted with 21K (rabbits 3 and 15, respectively), and the reaction with the anti-(1-11) serum (rabbit 3) was only barely detectable. Some sera appeared to recognise not only 21K/22K but also additional lower mol. wt. polypeptides. These are discussed in more detail below.

(c) Specificity of the immunoblotting reaction

Specificity of the immunoblotting reaction was suggested by a number of findings. Firstly, as already mentioned, pre-immune sera did not recognise the 21K and 22K polypeptides (Figure 22, and results not shown). Secondly, immune sera recognised bands of apparent mol. wt. 21K/22K in HSV-1-infected but not mock-infected cell extracts (Figure 24: compare lanes 1 and 3, and lanes 4 and 6 in (A); compare lanes 1 and 3, and lanes 5 and 7 in (C)). And thirdly, incubation of anti-(1-11) serum in the presence of homologous but not heterologous peptide specifically inhibited the interaction with the 21K and 22K bands (Figure 24B). In contrast, lower mol. wt. bands in the region of 12K and 14K were not inhibited by the homologous peptide, suggesting that these represent non-specific interactions.

A number of additional lower mol. wt. polypeptides appeared to be specifically recognised in that they were also present only in HSV-1-infected cell extracts, and only with immune sera. A 17.5K polypeptide was recognised by antisera against peptides 48-58 (Figure 24C, lanes 1-3), 151-161 (Figure 24A) and 155-161 (Figure 24C, lane 4). A 15K poly-peptide was very weakly detected by antiserum against peptide 1-11 (Figure 24A) and more strongly detected by antiserum

FIGURE 24

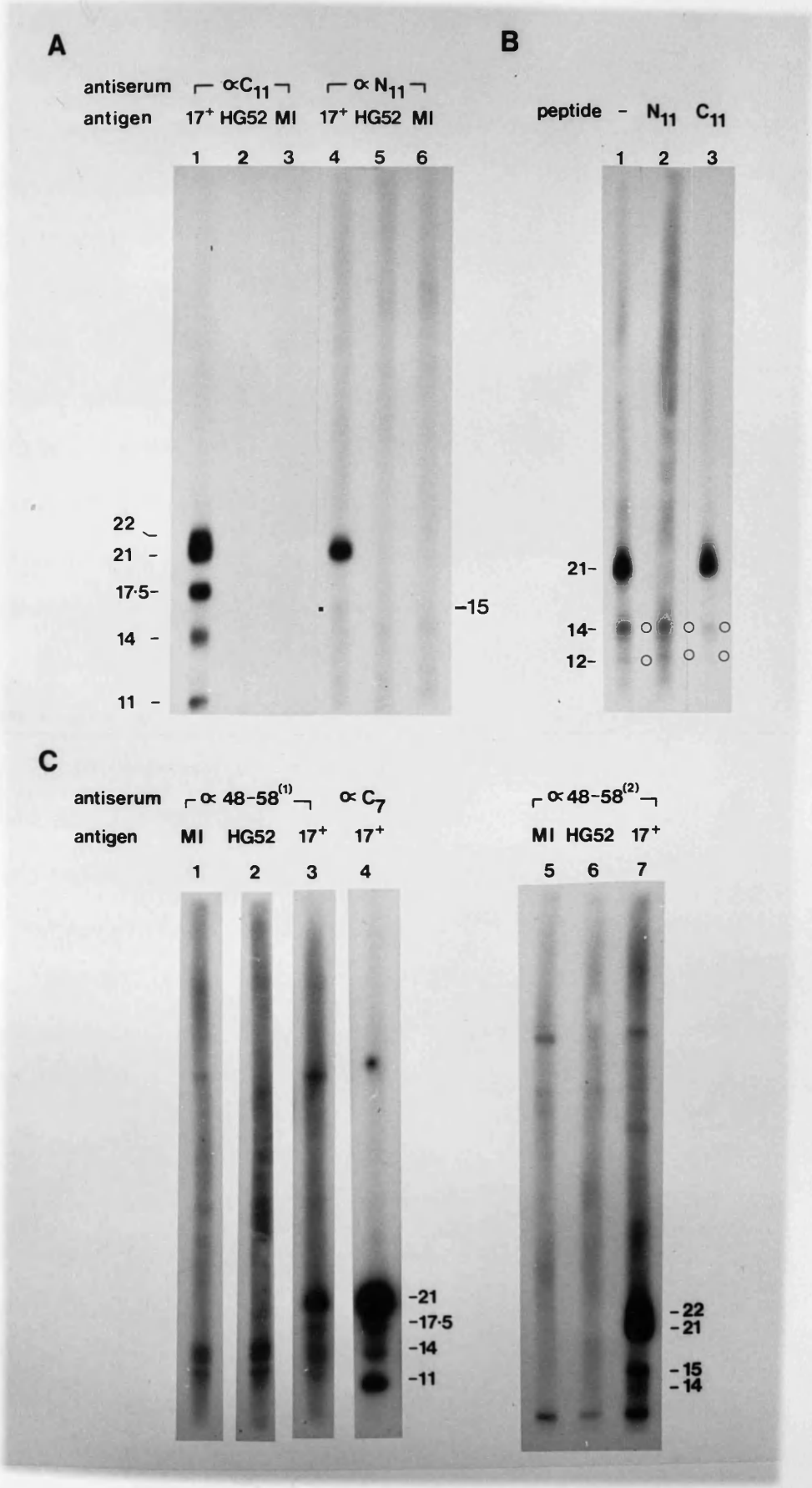
Specificity of the immunoblotting reaction. Polypeptides were separated on 5-12.5% SDS-polyacrylamide gels, transferred to nitrocellulose and reacted with antisera as described (method section IV.5).

A. anti-C_{11} serum 14327 (lanes 1-3) and anti-N_{11} serum 14473 (lanes 4-6) were reacted in immunoblotting experiments against extracts from HSV-1-infected cells (lanes 1 and 4), HSV-2-infected cells (lanes 2 and 5) and mock-infected cells (lanes 3 and 6).

B. anti-N_{11} serum 14473 was reacted against HSV-1-infected cell extracts either alone (lane 1) or in the presence of 100 ug of N_{11} peptide (lane 2) or of C_{11} peptide (lane 3).

C. anti-(48-58) serum 14060 (lanes 1-3) and serum 18244 (lanes 5-7) were reacted against extracts from mock-infected cells (lanes 1 and 5), HSV-2-infected cells (lanes 2 and 6) and HSV-1-infected cells (lanes 3 and 7). Anti-C_7 serum 12848 reacted against HSV-1-infected cell extract is shown for comparison (lane 4).

Bands detected by the anti-N_{11} serum in all cell extracts, and not inhibited by peptide, are indicated in (B) (o).


A
antiserum
antigen
17+ HG52 MI
17+ HG52 MI
1 2 3 4 5 6
22
21
17·5
14
11
15
B
peptide
N11
C11
1 2 3
21
14
12
C
antiserum
antigen
MI HG52 17+ 17+
1 2 3 4
21
17·5
14
11
MI HG52 17+
5 6 7
22
21
15
14

against peptide 48-58 (Figure 24C, lanes 5-7). Both these polypeptides appeared to be recognised by the antisera against peptide 66-76 (Figure 23, and results not shown), but these latter sera were obtained towards the end of this project and have not been well characterised. A 14K polypeptide, recognised by antisera to all peptides, except 1-11 and 132-138, was often strongly detected (Figure 24A, lanes 1-3; Figure 24C, lanes 5-7). However, a number of sera non-specifically recognised bands in the region of 12-14K (for example, see Figure 24C, lanes 1-3), and thus the significance of these reactions is not clear. Finally, an 11K polypeptide was detected, often strongly, by antisera raised against the carboxy-terminal peptides, 151-161 and 155-161 (Figure 24A, lanes 1-3; Figure 24C, lane 4). The possible relationship between these polypeptides will be discussed later.

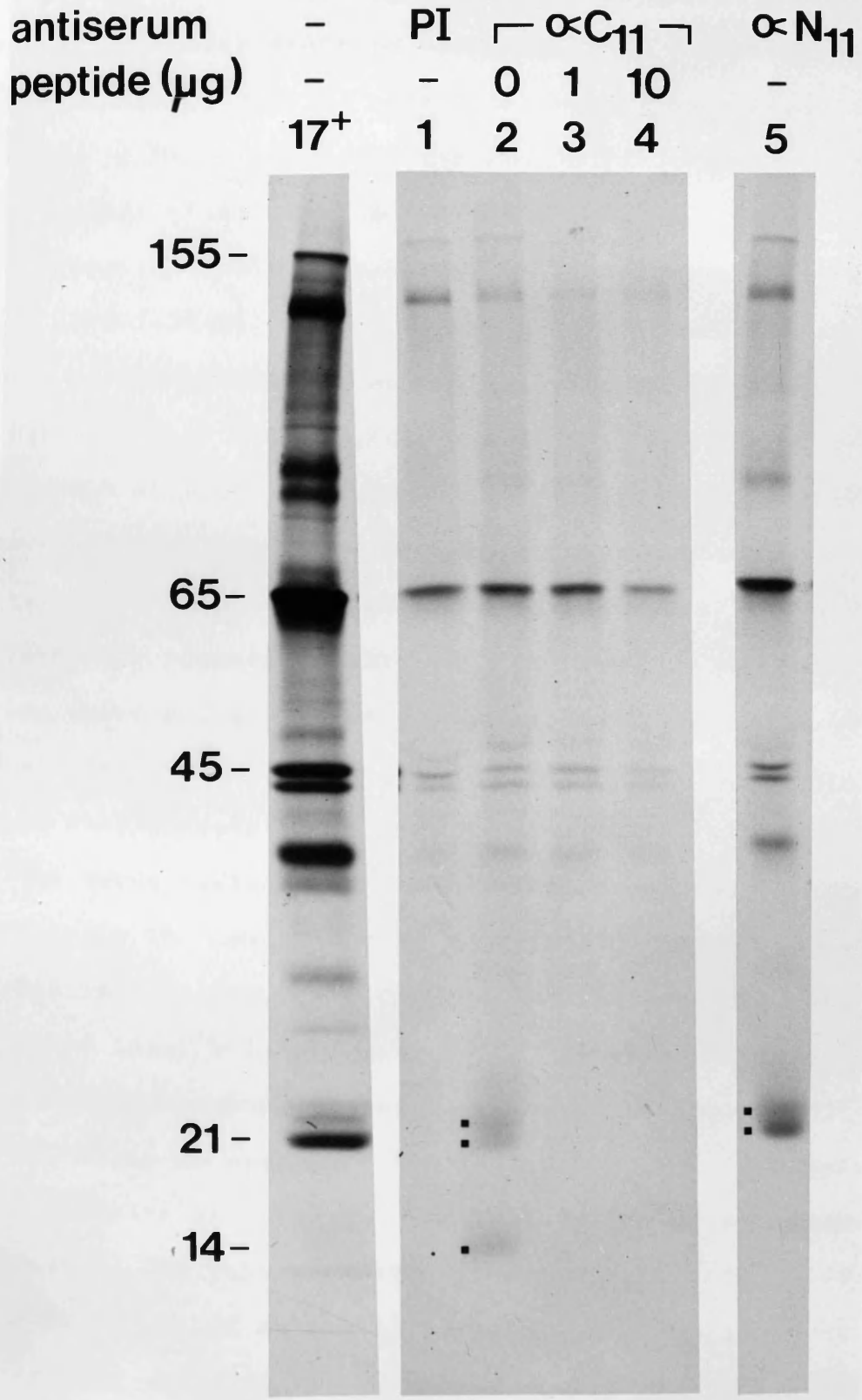
None of the sera recognised the polypeptide equivalent to Vmw21 in HSV-2-infected cell extracts (Figures 24A,C). However, since HSV-2 strain HG52 greatly underproduces the 20.5K polypeptide relative to its HSV-1 counterpart (Marsden et al., 1978), this may simply reflect a lack of sensitivity in the assay rather than a lack of homology between these two proteins.

(d) Immunoprecipitation of infected cell polypeptides by anti-peptide sera

Immunoprecipitation experiments were also used to investigate the reactivity of the anti-peptide sera. In Figure 25, antisera to peptide 1-11 (lane 5) and 151-161 (lane 2) clearly precipitate proteins of apparent mol. wt. 21K and 22K which are not precipitated by the pre-immune serum (lane 1). The specificity of the reaction was confirmed by incubation of

FIGURE 25

Immunoprecipitation with anti-peptide serum. Proteins labelled with ^{35}S-methionine were precipitated from an HSV-1-infected cell extract (17^{+}) by either pre-immune serum from rabbit 14327 (lane 1), anti-C_{11} serum 14327 incubated in the presence of 0, 1 or 10 ug of C_{11} peptide (lanes 2-4, respectively) or anti-N_{11} serum 14473 (lane 5). Bands which appear to be specifically precipitated are marked (■).

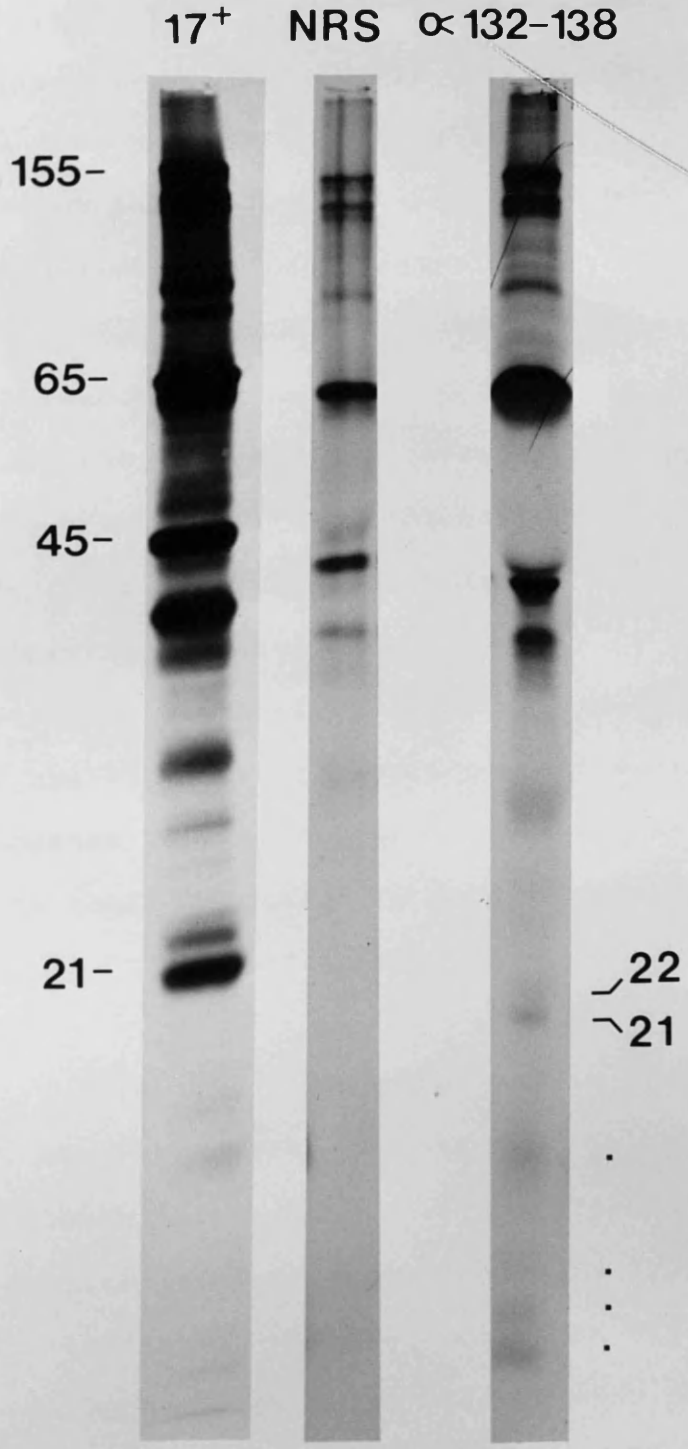

antiserum
peptide (µg)
-
PI
∝C11
∝N11
0
1
10
17+
1
2
3
4
5
155-
65-
45-
21-
14-

the anti-(151-161) serum in the presence of the relevant peptide. This peptide inhibited precipitation of the 21K and 22K polypeptides without significantly affecting the precipitation of other proteins (compare lanes 2 and 3). High concentrations of peptide caused an overall reduction in the intensity of bands (lane 4). The reason for this is unclear, but a similar effect can be seen in Figure 24B, lane 3. A 14K polypeptide would also appear to be specifically precipitated by the anti-(151-161) serum (compare lanes 2 and 3). This finding is interesting as no strong 14K band is evident in the HSV-1 extract and suggests either that the antiserum has a very high affinity for the 14K polypeptide, or that 14K is generated during the immunoprecipitation reaction. Co-precipitation of polypeptides could arise either if the polypeptides were present as part of a complex, or alternatively, if they share antigenic determinants. The interaction with a polypeptide of similar mol. wt. in immunoblotting experiments (Figure 24A) favours the latter explanation.

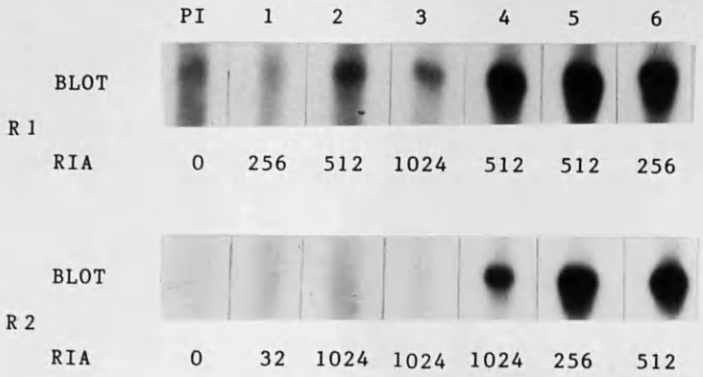
The serum against peptide 132-138, which failed to react convincingly in immunoblotting experiments, may very weakly precipitate 21K, since the 21K and 22K polypeptides, and additional lower mol. wt. polypeptides precipitated, were absent from the normal rabbit serum control (Figure 26). However, since no peptide inhibition control was attempted, the specificity of these interactions is not unambiguously established. The background in immunoprecipitation experiments was often high, and in general specific precipitation by the anti-peptide sera was rather weak (as, for example, in Figure 26). For this reason immunoprecipitation reactions were not carried out on all sera.

FIGURE 26

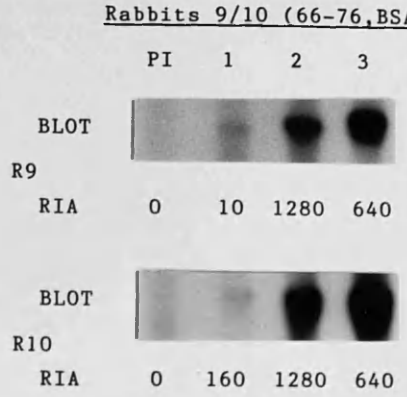
Immunoprecipitation with anti-(132-138) serum. Proteins labelled with ^{35}S-methionine were precipitated from an HSV-1-infected cell extract (17^{+}) by either normal rabbit serum (NRS) or anti-(132-138) serum 12851 (α132-138). The 21K and 22K polypeptides precipitated by the immune serum are indicated. ■ represents additional lower mol. wt. polypeptides precipitated only by the immune serum.


17+
NRS
∝132-138
155-
65-
45-
21-
22
21

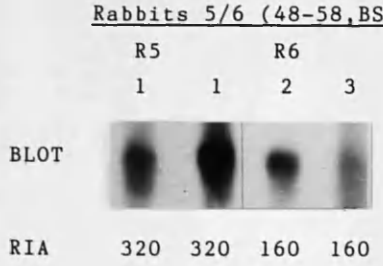
Rabbits 1/2 (1-11,BSA)



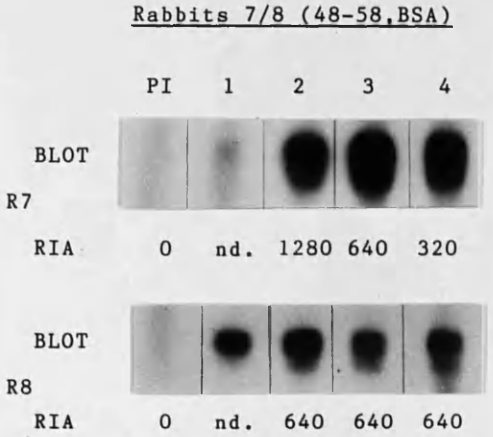
Rabbits 9/10 (66-76,BSA)



Rabbits 5/6 (48-58,BSA)



Rabbits 7/8 (48-58,BSA)



Rabbits 13/14 (151-161,BSA)

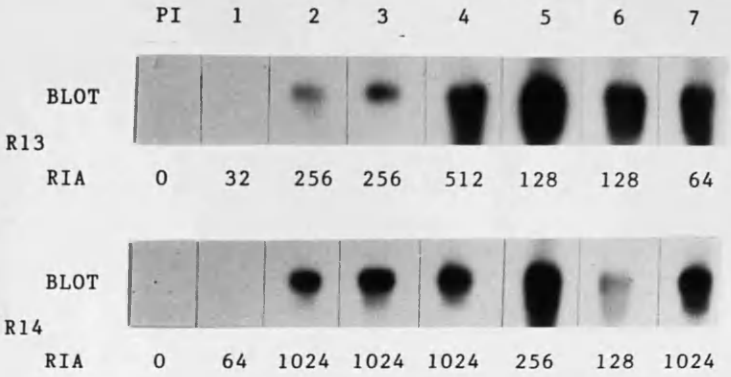


FIGURE 27

Comparison between anti-peptide RIA and anti-protein immunoblotting responses of the oligopeptide-induced antisera. The rabbit numbers and immunogen are indicated. Rabbit numbers are designated as in Table 3. The immunoblots show only the region of the gel encompassing the 21K polypeptide. Above each immunoblot is shown the bleed number, and below is shown the anti-peptide titre, as assessed by RIA.

R1, R2.... : rabbit number 1, 2....

PI : pre-immune

nd : not done

immunoaffinity chromatography (see later) unsuccessful. Even in immunoblotting experiments, where proteins are presumed to be at least partially denatured, and sequential epitopes more accessible to antibodies, the antisera could be used at best at a 1/4 dilution. Only one anti-peptide serum which had a high affinity for 21K on immunoblots was obtained, bleed 4 from rabbit 17, prepared by R. Dalziel. This serum worked in immunoblotting experiments at a 1/1024 dilution (Mary Murphy, personal communication), but was unfortunately available in very limited quantity, and even it failed to react strongly by immunoprecipitation or immunoaffinity chromatography with 21K.

I.6 Further attempts to raise antibodies to 21K

Due to the poor affinity of the anti-peptide sera for 21K, attempts were made to raise antibodies to this protein by two other methods : firstly, to raise mouse monoclonal antibodies against both peptides and purified 21K, and secondly, to raise monospecific antisera in rabbits against purified 21K. Neither of these approaches met with success, and they are therefore outlined only briefly below.

(a) Monoclonal antibodies

Sera from mice immunised with either partially-purified 21K, prepared by DNA cellulose chromatography (result section III.3), or with synthetic oligopeptides conjugated to BSA, were analysed by immunoprecipitation, immunoblotting and RIA. Only the RIA provided evidence for a response to the immunogen. Clones, resulting from fusion between spleen cells from the immunised mice and a mouse myeloma cell line, were screened for antibody production in a similar manner, and again only

the RIA provided evidence for a positive response - and only from the peptide-immunised animals. However, we subsequently found that mouse monoclonal antibodies gave a high level of false positive reactions in our RIA procedure, and no true positive cultures were obtained. Therefore, lacking confidence in our screening procedure, we made no further attempts to pursue this approach.

(b) Monospecific anti-21K sera

The 21K/22K polypeptides were partially-purified by DNA cellulose chromatography (result section III.3), then separated by SDS-PAGE and transferred to nitrocellulose. The region of the nitrocellulose containing 21K/22K was isolated, dissolved in DMSO, and two rabbits then immunised with this material on several occasions. Sera from these animals were screened by immunoprecipitation and immunoblotting, but no convincing reaction was observed with the 21K/22K polypeptides. This approach was therefore discontinued.

SECTION II : CHARACTERISATION OF THE U_S11 GENE PRODUCTS

In the preceding section the specificity and reactivity of the anti-peptide sera were established. Here, they have been used to characterise some of the properties of the U_S11 gene products.

II.1 Temporal regulation of the U_S11 gene products

(a) Appearance and accumulation of the 21K polypeptide during infection

To determine the temporal regulation of 21K, protein levels were monitored at different times following infection. A detailed timecourse was carried out, harvesting samples (as described in method section IV.5) at one hour intervals from 0-8 h, then at 10, 12, 16, 20 and 24 h pi. The immunoblotting procedure was slightly modified for these experiments. Briefly, proteins were electrophoretically separated by SDS-PAGE and transferred to a single large sheet of nitrocellulose. Free protein-binding sites were blocked at 65°C and then the sheet was incubated with 10ml of a strongly reactive antiserum, raised against peptide 155-161 (rabbit 12848, bleed 4) and probed with $20x10^6$ cpm of ^{125}I-labelled protein A in 120ml of the relevant buffer.

Using this procedure the 21K polypeptide was first detected at 4 h pi (Figure 28). The intensity of the reaction rose steadily until 12-16 h pi, when it appeared to plateau. This is shown quantitatively in Figure 29. The relative intensities of the bands were estimated by densitometric analysis of autoradiographs from various exposures of the gel (selected to give a linear response between absorbance

FIGURE 28

Timecourse of appearance of the U_S11 gene products. Immunoblotting was carried out essentially as described in method section IV.5, with the following modifications : Mock-infected (mi) or HSV-1-infected cell extracts, harvested at the times indicated (hours pi), were separated on 5-12.5% SDS-polyacrylamide gels and then transferred to a single large sheet of nitrocellulose. The nitrocellulose sheet was incubated with 10 ml of anti-C_7 serum 12848, diluted 1/20, and after washing was further incubated with 100 ml of ^{125}I-labelled protein A at $2x10^5$ cpm/ml. The apparent mol. wt. of the polypeptides recognised were determined by alignment with the ^{35}S-labelled polypeptide profile (not shown).

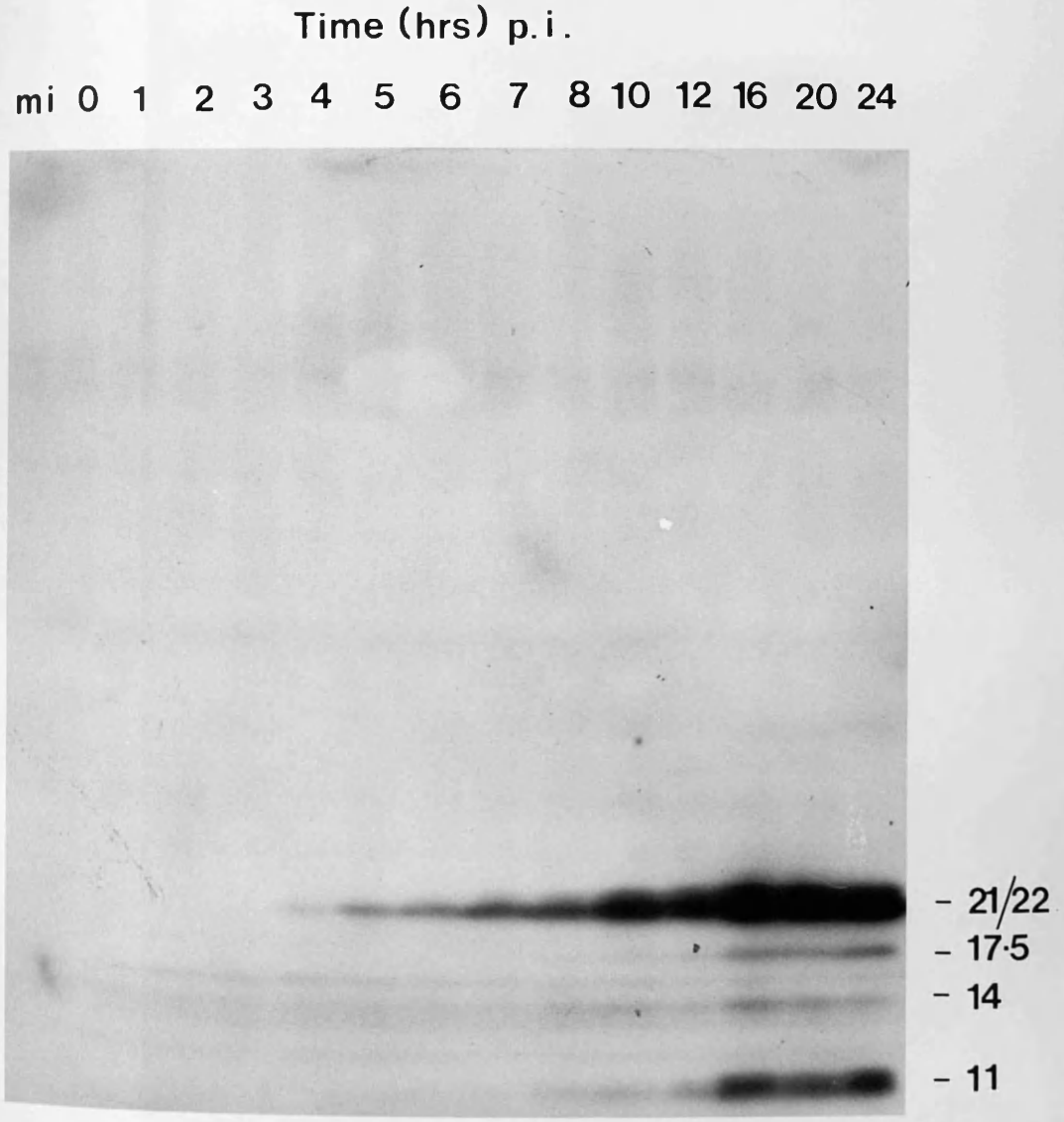
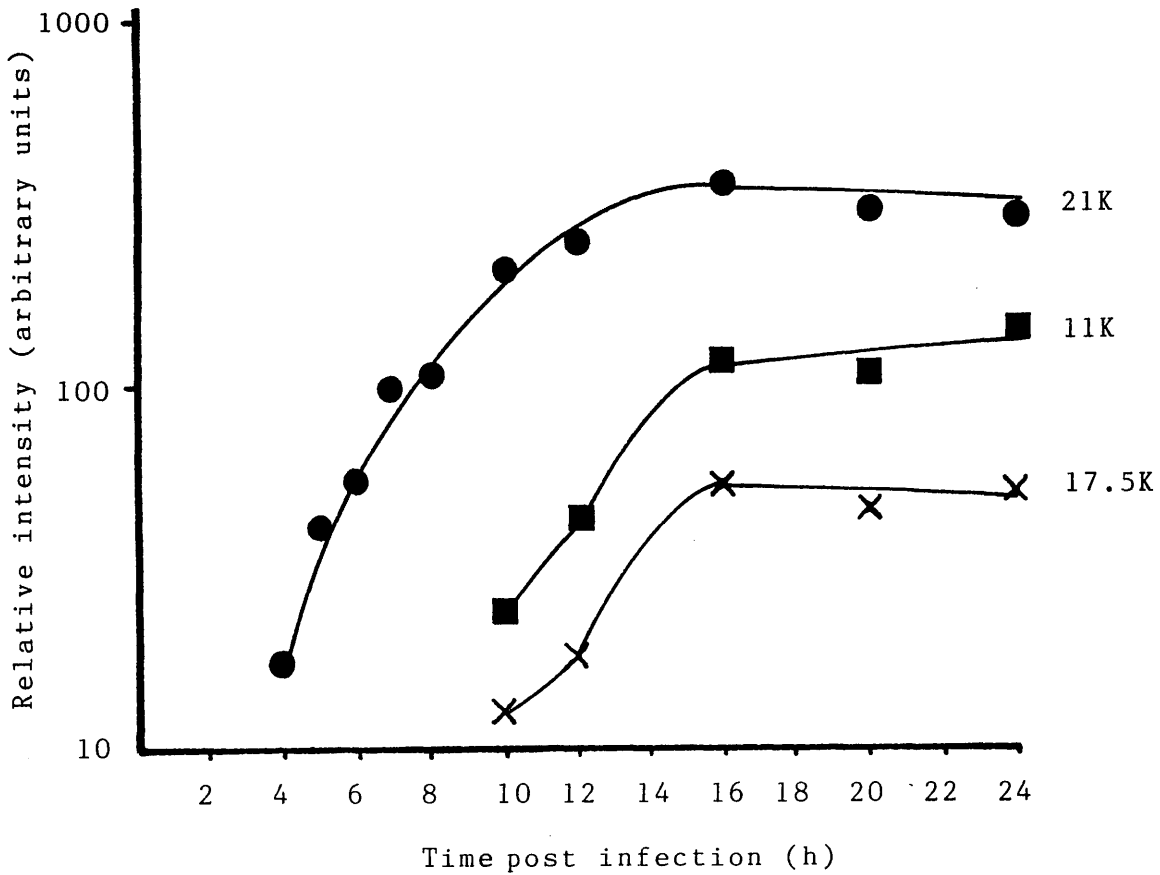

Time (hrs) p.i.
mi 0 1 2 3 4 5 6 7 8 10 12 16 20 24
- 21/22
- 17·5
- 14
- 11

FIGURE 29

Timecourse of appearance of the U_S11 gene products - densitometric analysis. Various exposures of the gel shown in Figure 28 (selected to give a linear response between absorbance and time of exposure) were subjected to densitometric analysis, using a Joyce-Lœbl scanning densitometer and measuring the area under the appropriate peaks using a program, DENS, written by Dr P Taylor for a DEC PDP 11/44 computer linked to a digitizing two-dimensional tablet. The relative intensities of the bands at different time points are plotted in arbitrary units.


1000
100
10
Relative intensity (arbitrary units)
2
4
6
8
10
12
14
16
18
20
22
24
Time post infection (h)
21K
11K
17.5K

and time of exposure), and plotted in arbitrary units. The plateau after 12-16 h pi reflects the upper limit of sensitivity of the assay, and no longer reflects the absolute level of protein (see later, result section II.1c).

As shown previously, additional bands are also recognised by this serum. Polypeptides in the region of 12-16K were recognised in all samples, including the mock-infected control, and are therefore considered non-specific. However, a band of apparent mol. wt. 17.5K and a doublet in the region of 11K appeared approximately 8-10 h pi and increased in intensity in parallel to the 21K band, until about 16 h pi (Figure 28). Their intensity was quantitated and plotted in Figure 29. In addition a band of apparent mol. wt. 14K appeared to increase in intensity late in infection. However, because of the high background from the underlying non-specific bands it was not possible to quantitate this reaction. The timecourse of appearance of these additional bands (17.5K, 14K and 11K) strengthens the previous suggestions that they are related to 21K and are breakdown products or processed forms of the U_S11 gene product.

In conclusion, within the sensitivity of the assay, the 21K U_S11 gene product would appear to be a late viral polypeptide.

(b) Is the 21K polypeptide a true late gene product?

To determine whether the 21K polypeptide is regulated as a true late gene product - ie. one whose synthesis is highly dependent on viral DNA replication - similar timecourse experiments were carried out, in the presence and absence of phosphonoacetic acid (PAA), an inhibitor of viral DNA replication. As a control, the level of gD, a delayed-

early ($\beta\delta$) protein, was measured. This work was carried out in conjunction with Paul Johnson, who analysed the levels of U_S11 and gD RNA from parallel cultures, and who also measured the level of viral DNA synthesis (Johnson et al., 1986).

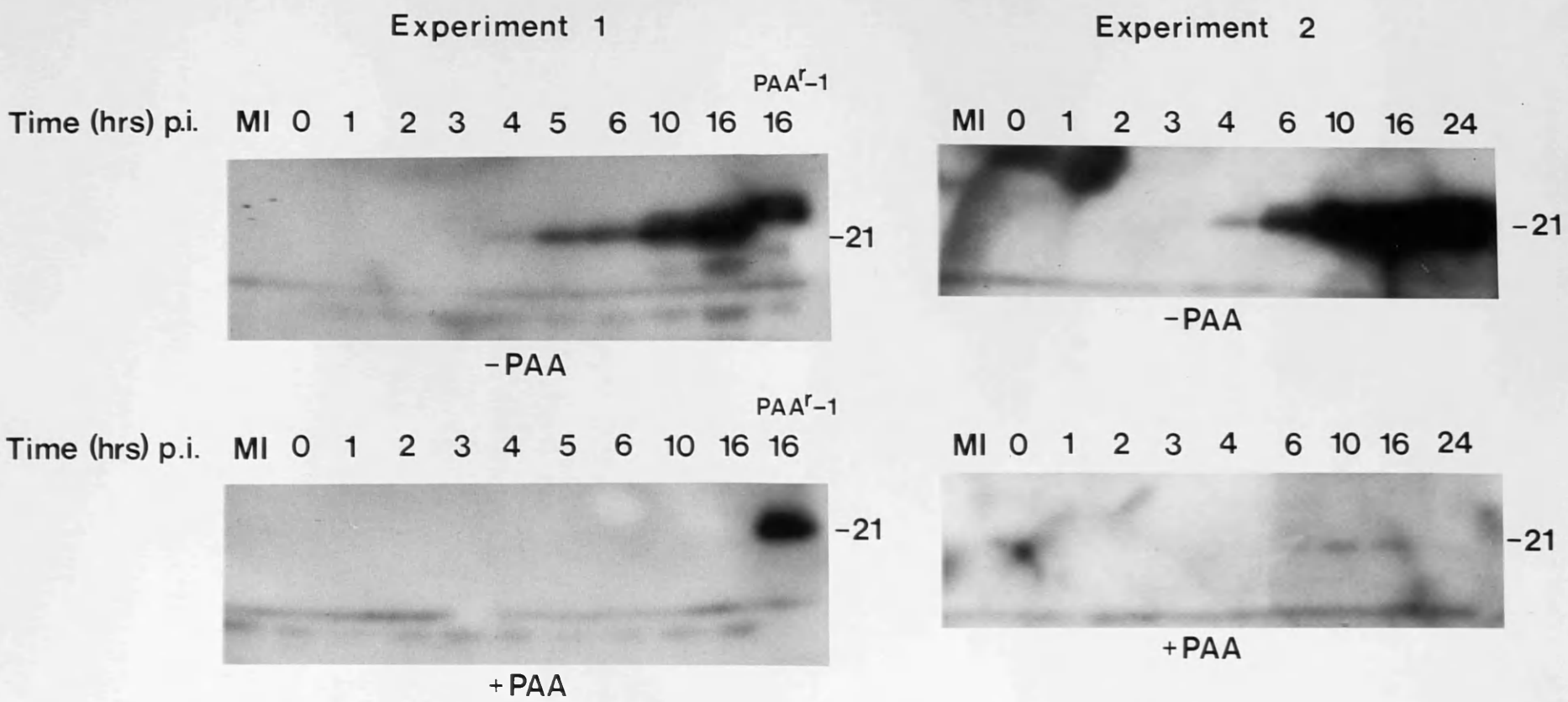
The results of two separate experiments are presented in Figure 30. Samples were harvested at the times shown and probed as before with the antiserum to peptide 155-161. A PAA-resistant mutant, PAA^r-1 (Hay and Subak-Sharpe, 1976), known to have a mutation mapping to the DNA polymerase gene (Crumpacker et al., 1980), was used as a control to confirm that any effect of PAA on protein synthesis was via its effects on viral DNA replication.

The control cultures grown in the absence of PAA behaved as before, 21K accumulating steadily from 4 h pi (3 h in experiment 2) to 12-16 h pi, when the reaction begins to plateau. In the presence of PAA there was a significant reduction in the level of 21K which was undetectable in the first experiment and only barely detectable in the second (Figure 30). There was no obvious reduction in 21K under these conditions with the PAA-resistant mutant. However, since this assay is not sensitive at high levels of antigen concentration (see later) a small reduction would not have been detected. Interestingly, the '21K,22K' polypeptides of the PAA-resistant mutant show an altered mobility in comparison to the HSV-1 wild-type virus 21K,22K polypeptides - a feature previously described for the viral polypeptides Vmw21, Vmw22 (Lonsdale et al., 1979).

For comparison, the appearance and accumulation of gD was measured in the same cultures by immunoprecipitation of [^{35}S]-methionine labelled gD with a gD-specific monoclonal antibody. This antibody precipitates two bands, of apparent mol. wt. 57,000 (gD) and 51,000 (pgD), which are absent from

FIGURE 30

Effect of PAA on synthesis of the U_S11 gene products. Immunoblotting was carried out as described in the legend to Figure 28. Results from two separate experiments are shown. Extracts from mock-infected or HSV-1-infected cells, cultured in the absence or presence of PAA (-PAA, +PAA, respectively), were harvested at the times indicated (hours pi). In experiment 1, extracts from cells infected with the PAA-resistant mutant, PAA^r-1, were harvested at 16 h pi. The PAA^r-1 21K polypeptide, labelled in experiment 1/+PAA, migrates with a slightly higher apparent mol. wt. than wild-type 21K (compare experiment 1/-PAA).


Experiment 1
Experiment 2
Time (hrs) p.i.
MI 0 1 2 3 4 5 6 10 16 16
PAA^r-1
-21
-PAA
Time (hrs) p.i.
MI 0 1 2 3 4 5 6 10 16 16
PAA^r-1
-21
+PAA
MI 0 1 2 3 4 6 10 16 24
-21
-PAA
MI 0 1 2 3 4 6 10 16 24
-21
+PAA

the ascites control (Figure 31). In both the presence and absence of PAA, gD is first detected at low levels at 2 h pi (just visible in the exposure shown in Figure 31) and continues to accumulate throughout infection. Densitometric analysis (Figure 32) shows that gD accumulates very rapidly from 2-6 h pi, then continues to accumulate at a reduced rate until at least 16 h pi. Treatment with PAA reduces the level of gD by approximately 5-fold, but the overall pattern of accumulation is not changed. In comparison, the level of gD at 16 h pi with the PAA-resistant mutant is reduced about 2-fold by treatment with PAA (not shown). The level of gD at 2 h pi is very similar in the presence and absence of PAA, consistent with the effect being due to inhibition of viral DNA replication, which begins around 2-3 h pi (Johnson et al., 1986). From this series of experiments it was concluded that 21K is regulated as a true late gene.

(c) Relationship between antigen concentration and intensity of the immunoblotting reaction

To establish the relationship between the intensity of the immunoblotting reaction and the antigen concentration, antigen dilution experiments were carried out. The 16 h sample from experiment 1 (Figure 30) was serially diluted two-fold and tested by immunoblotting for 21K and by immunoprecipitation for gD (Figure 33). The reactions were again analysed by densitometry (Figure 34).

There is an approximately linear relationship between the intensity of the bands precipitated by the anti-gD monoclonal antibody and the level of antigen, with a gradient close to 1 (1.2) - ie. a two-fold increase in intensity is approximately equivalent to a two-fold increase in antigen

FIGURE 31

Effect of PAA on synthesis of gD, an early polypeptide. Mock-infected and HSV-1-infected cells, cultured in the presence (+) or absence (-) of PAA, were harvested by the detergent lysis procedure (method section I.7a) at the times indicated. Immunoprecipitation was carried out essentially as described by Zweig et al. (1980). Forty ul of cell extract, 50 ul of a 1/100 dilution of either monoclonal antibody 1892 or an ascites control and 5 ul of a rabbit anti-mouse Ig, were incubated for 3 h at 4°C, before being further incubated with protein A-Sepharose and analysed as described (method section IV.4). gD (57K) and pgD (51K) are indicated (●).

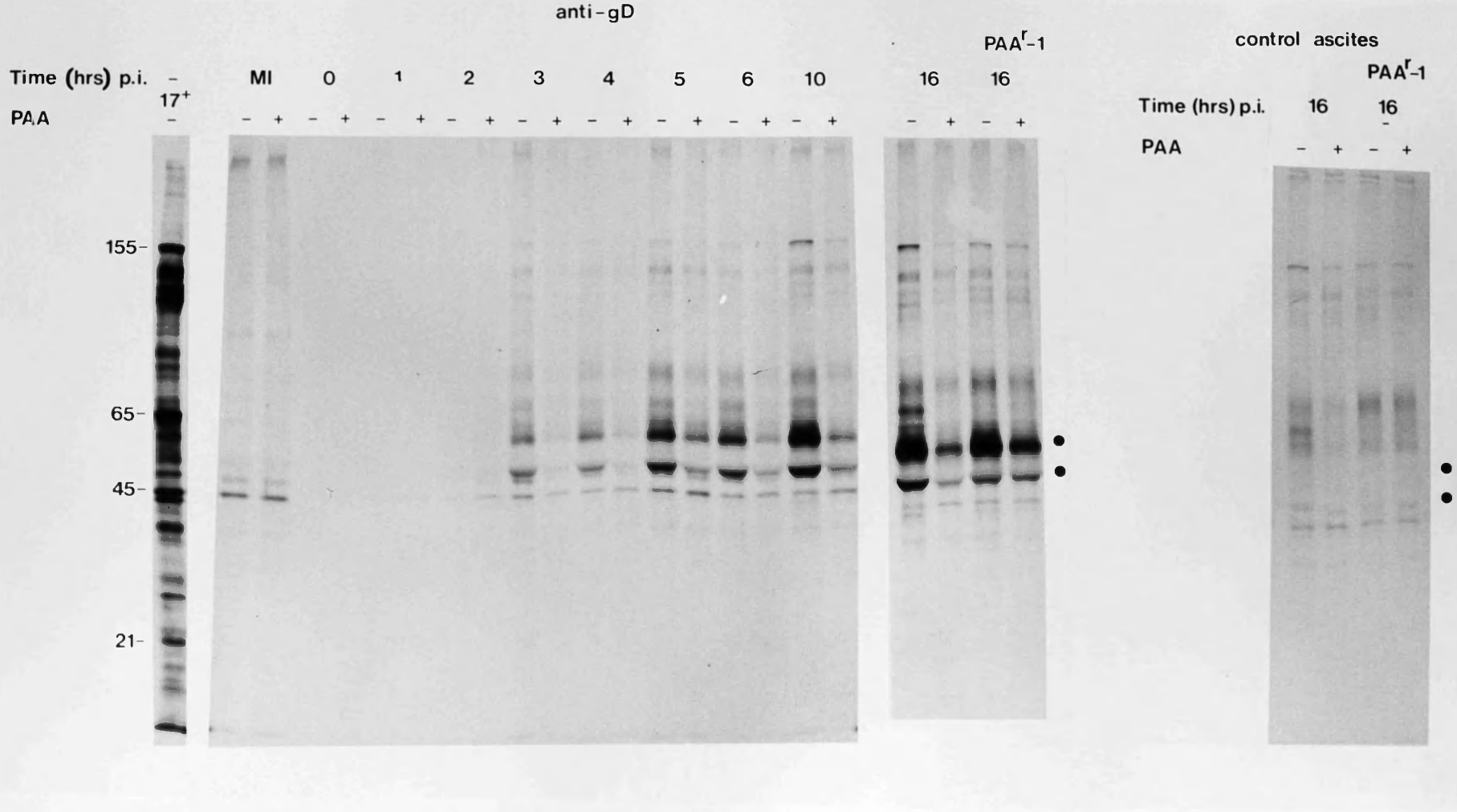

anti-gD
PAA^r-1
Time (hrs) p.i.
–
MI
0
1
2
3
4
5
6
10
16
16
17$^+$
PAA
155
65
45
21
control ascites
PAA^r-1
Time (hrs) p.i.
16
16
PAA

FIGURE 32

Effect of PAA on the synthesis of 21K and gD. Various exposures of the gels shown in Figures 30 and 31 were subjected to densitometric analysis, as described in the legend to Figure 29.

○ 21K +PAA

● 21K -PAA

□ gD +PAA

■ gD -PAA

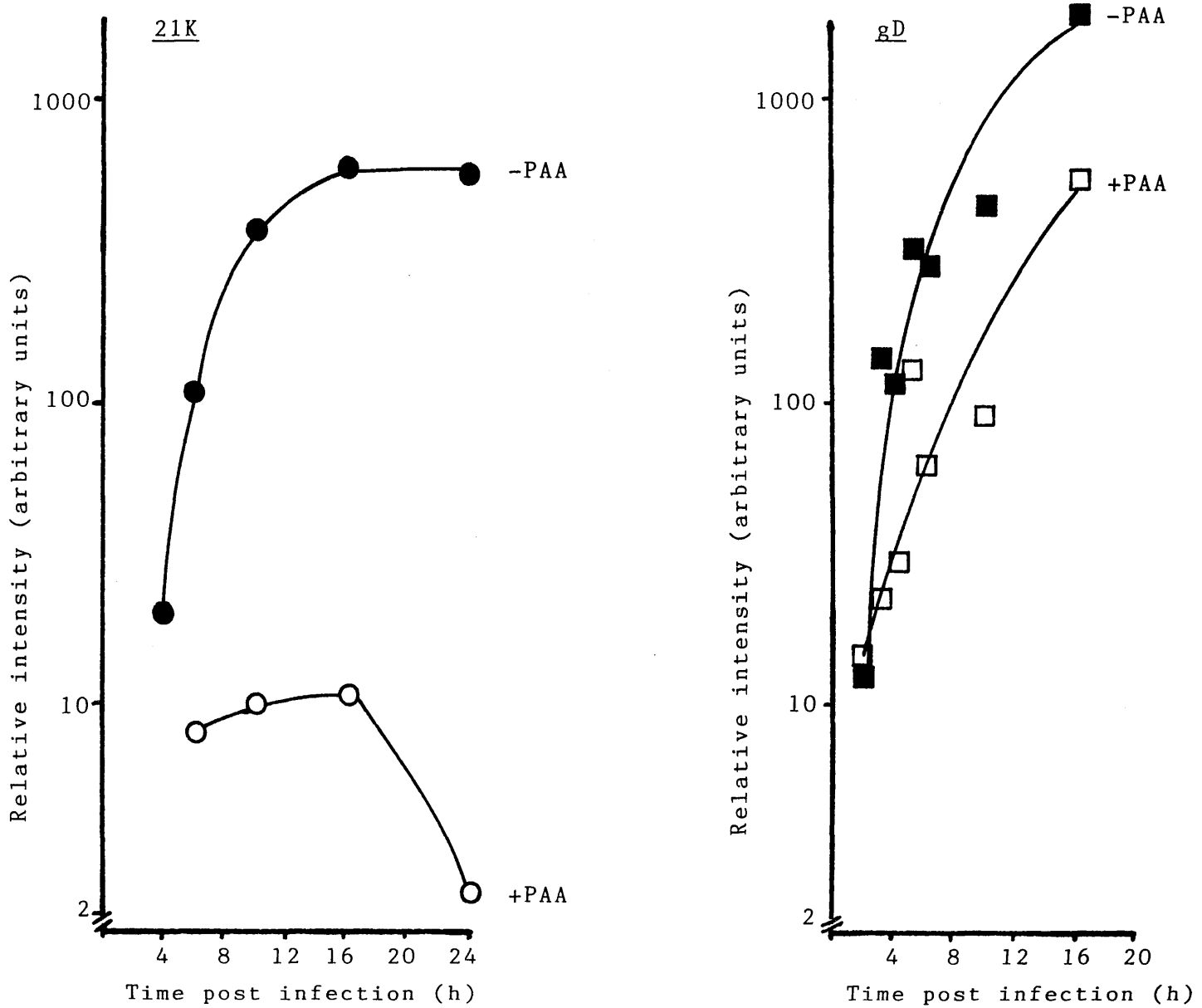

21K
1000
100
10
2
Relative intensity (arbitrary units)
-PAA
+PAA
4
8
12
16
20
24
Time post infection (h)
gD
1000
100
10
2
Relative intensity (arbitrary units)
-PAA
+PAA
4
8
12
16
20
Time post infection (h)

FIGURE 33

Relationship between antigen concentration and the intensity of the immunoblotting and immunoprecipitation reactions. The 16 h samples from Figure 31 (A) and Figure 30, experiment 1 (B) were serially diluted two-fold and a constant volume (40 ul) tested in immunoprecipitation and immunoblotting experiments, respectively, as before. The antigen dilution factor is indicated above the relevant lane, 1 representing the neat 16 h sample.

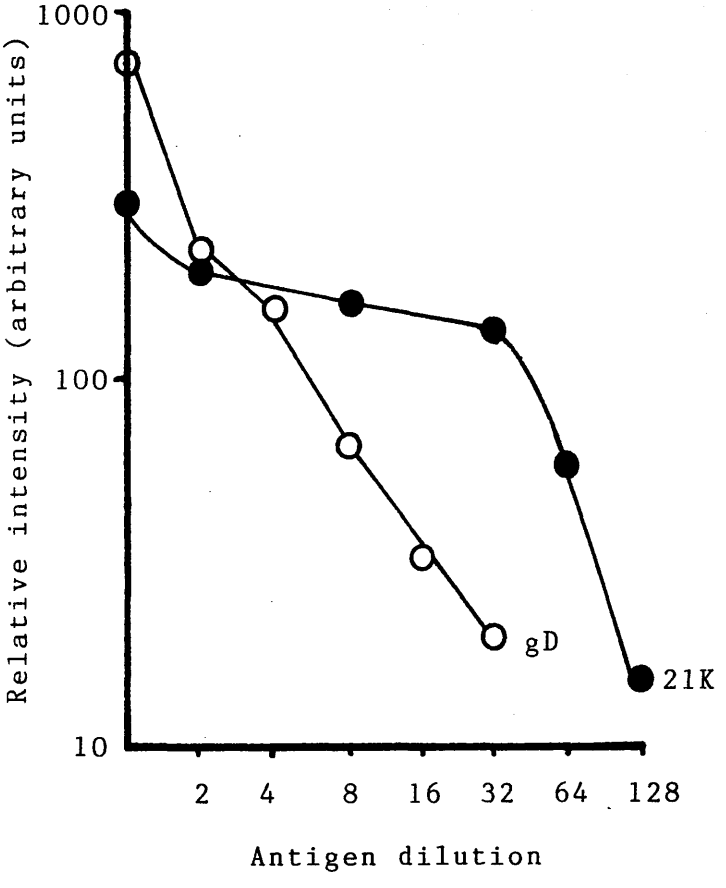
A
gD−
pgD−
1
2
4
8
16
32
64
128
256
512
B
21−
1
2
4
8
16
32
64
128
256
512

FIGURE 34

Relationship between antigen concentration and the intensity of the immunoblotting and immunoprecipitation reactions - densitometric analysis. Various exposures of the gels shown in Figure 33 were subjected to densitometric analysis as described in the legend to Figure 29.

● 21K

○ gD (gD + pgD)


1000
100
10
Relative intensity (arbitrary units)
2
4
8
16
32
64
128
Antigen dilution
gD
21K

concentration. This would confirm the interpretation from Figures 31 and 32, that gD levels are reduced about 5-fold in the presence of PAA.

In contrast, in the immunoblotting assay there was initially a very shallow gradient, where the intensity of the reaction changed very little over a 32-fold dilution of antigen. This was followed by a very sharp gradient, the intensity of the reaction falling from a near-maximal level to an undetectable level over an 8-fold dilution. Thus, there is a very short (4-8x) range of antigen concentration over which this assay is very sensitive. The assay is not sensitive at high concentrations of antigen. Therefore, little can be concluded about the concentration of antigen in the plateau region observed in the timecourse experiments (Figures 29 and 32).

This experiment also demonstrates that the immunoblotting procedure used is capable of detecting at least a 1/128 dilution of the antigen concentration present in the 16 h sample from experiment 1. Therefore, the absence of detectable 21K in the corresponding time point from the PAA-treated samples suggests that there is at least a 100-fold reduction in the level of 21K. Similarly, in experiment 2 the reaction with 21K in the PAA-treated samples is only barely detectable, and even less intense than in any of the untreated 4 h samples, suggesting that the level of 21K synthesised in the presence of PAA is on the borderline of sensitivity of the assay - and therefore about 100-fold reduced in relation to a normal 16 h sample.

In conclusion, 21K, under normal conditions is first detected at 3-4 h pi, while gD is detected earlier at 2 h pi. Both proteins accumulate rapidly until at least 16 h pi. In

the presence of PAA only about 1% of the normal levels of 21K protein, but around 20% of the normal levels of gD protein, are synthesised. Thus the synthesis of 21K is highly dependent on viral DNA replication, and 21K can therefore be defined as a true late gene product.

II.2 The lower mol. wt. polypeptides and the 21K/22K products of gene U_S11 have common amino acid sequences

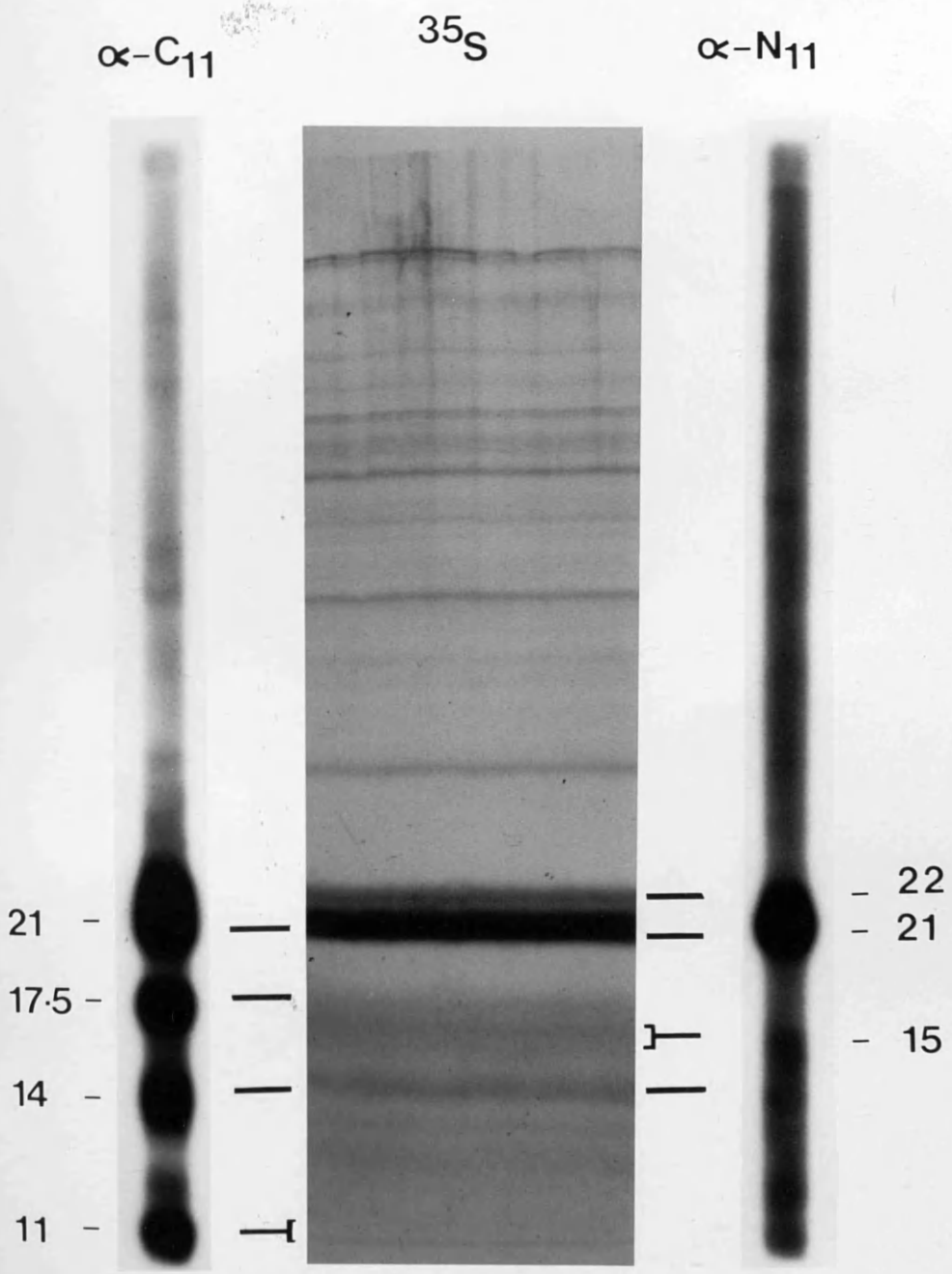
From the previous experiments it is apparent that the anti-peptide sera specifically recognise the 17.5K and 11K, and possibly the 14K, polypeptides and that these accumulate late in infection in parallel with 21K. The 15K polypeptide, however, was not recognised by the antiserum used in the timecourse experiments. To investigate further the relationship between the lower mol. wt. polypeptides and the 21K/22K products of gene U_S11 they were individually subjected to tryptic peptide analysis.

To do this, 21K was partially purified by phosphocellulose chromatography (result section III.5, Figure 53), concentrated and then separated by SDS-PAGE. A portion of the gel was removed for immunoblotting and the rest dried down and exposed for autoradiography. Immunoblots from this gel and from previous experiments were then used to identify the bands on the autoradiograph, as shown in Figure 35. The proteins were then isolated and subjected to tryptic peptide analysis, as described in method section VI.

Tryptic peptide digests were subjected to chromatography both individually and after mixing 1:1 with the tryptic peptide digest of 21K, to confirm suspected identities of apparently comigrating spots. The results (Figure 36) clearly demonstrate

FIGURE 35

Separation and identification of polypeptides for tryptic peptide analysis. Partially-purified 21K, labelled with ^{35}S-methionine, was prepared by phosphocellulose chromatography. Material (a pool of the second, third and fourth fractions seen in Figure 53, result section III.5b) was separated on a 10% SDS-polyacrylamide gel and exposed for autoradiography. A small portion of this gel is shown (^{35}S). Bands to be excised were identified by alignment with immunoblots carried out on HSV-1-infected cell extracts, also separated on 10% SDS-polyacrylamide gels, using anti-C_{11} serum 14327 and anti-N_{11} serum 14473.


α-C11
35S
α-N11
21
17·5
14
11
22
21
15

that the 22K, 21K, 17.5K, 15K, 14K and 11K polypeptides have common amino acid sequences. The 21K and 22K polypeptides differ mainly in the presence of an additional strong spot in 22K (spot 4). The lower mol. wt. polypeptides are strikingly similar to 21K, all the major spots being present, although their relative intensities may be altered. The 14K sample also contains a number of spots apparently unrelated to 21K (for example, spots a-d), but the region of the gel from which this polypeptide was excised contains mock-infected cell polypeptides (compare Figures 35 and 24A,B), and it is likely that the additional spots derive from these.

II.3 The 21K/22K U_S11 gene products are DNA-binding proteins

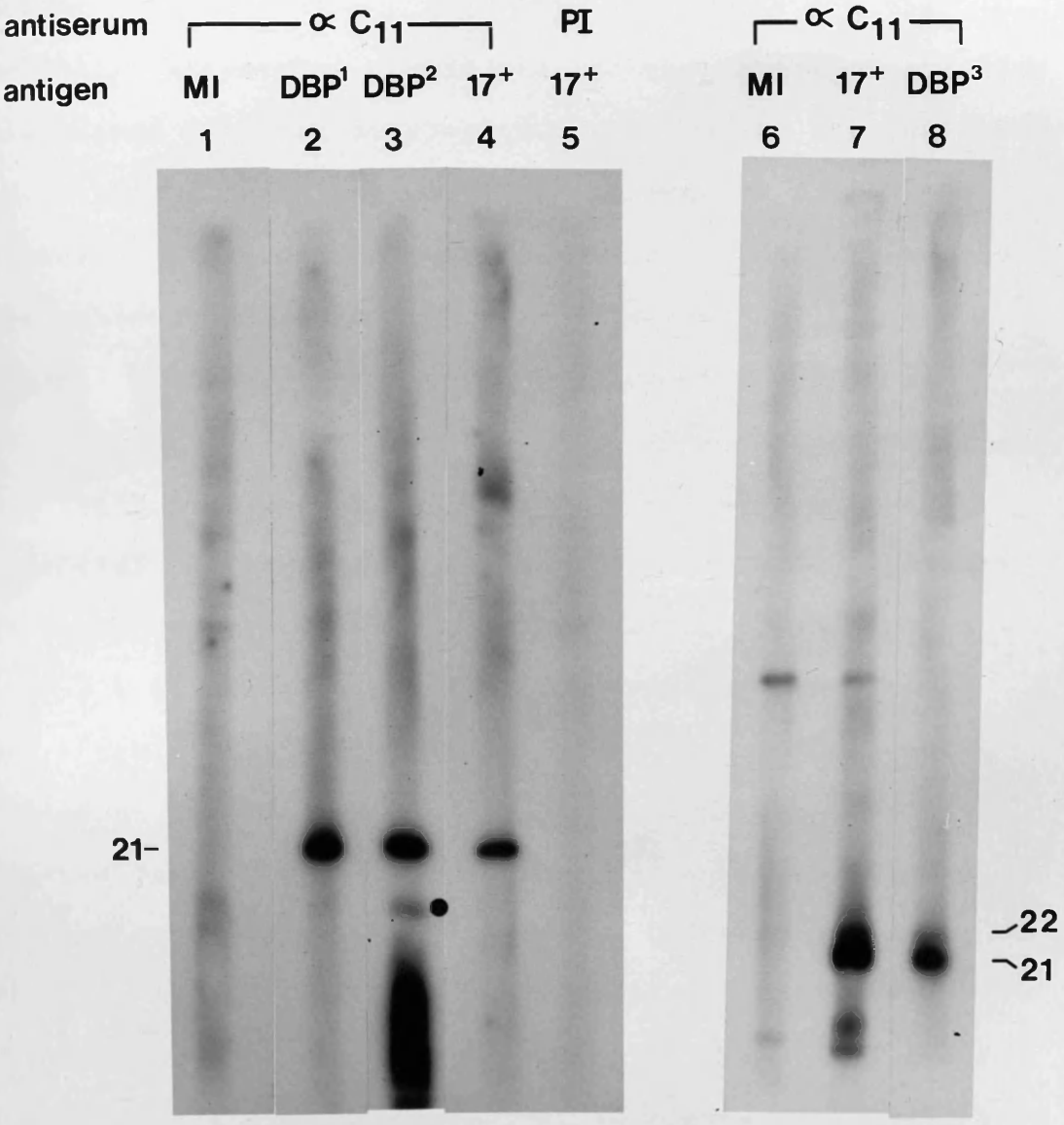
The 21K/22K polypeptides encoded by gene U_S11 are regulated as true late gene products (result section II.1). To determine whether the late 21K/22K U_S11 gene products are related to the late 21K/22K DNA-binding proteins, we purified the latter by DNA cellulose chromatography (result section III.3) and tested them for reaction with the anti-peptide sera. Immunoblotting experiments (Figure 37) confirm that the 21K and 22K products of gene U_S11 are indeed DNA-binding proteins. A band in the region of 17.5K was detected on one occasion (lane 3), but faint bands in this region were also present in the mock-infected control (lane 1) and so the specificity of this reaction is uncertain. The inability to detect the lower mol. wt. polypeptides does not necessarily imply that they do not bind to DNA, since they were not detected in the high salt extract originally applied to the DNA cellulose columns (lane 4).

FIGURE 37

The 21K and 22K U_S11 gene products are DNA-binding proteins. Pre-immune serum 14328 (lane 5) or anti-C_{11} serum 14328 (lanes 1-4, 6-8) were reacted in immunoblotting experiments against mock-infected (lanes 1 and 6) and HSV-1-infected (lanes 4, 5 and 7) cell extracts, prepared by the high salt extraction procedure (method section I.7b), and against proteins eluted by high salt from a DNA cellulose column (lanes 2, 3 and 8).

DBP^1, DBP^2, DBP^3 represent three separate preparations of DNA-binding proteins.

● represents a 17.5K band of uncertain origin.


antiserum
∝ C11
PI
∝ C11
antigen
MI
DBP1
DBP2
17+
17+
MI
17+
DBP3
1
2
3
4
5
6
7
8
21
22
21

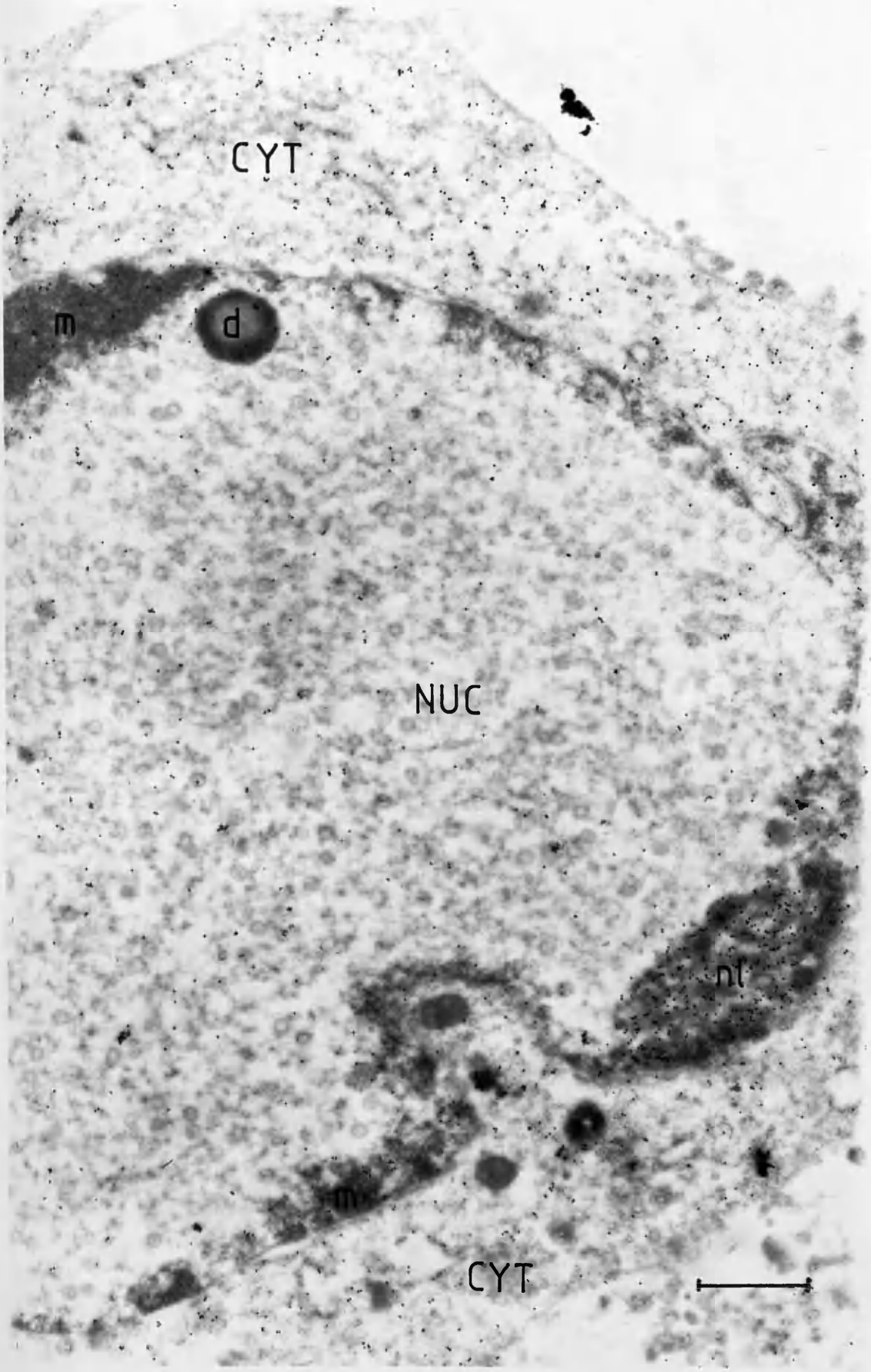

CYT
m
d
NUC
nl
m
CYT

FIGURE 38

Immunolocalisation of the U_S11 gene products. Electron micrograph of a thin section through HSV-1-infected BHK C13 cells harvested at 12 h pi. The section was incubated with anti-C_{11} serum 14327, in the presence of 1 ug of N_{11} peptide. Bound antibody was visualised using colloidal gold-conjugated goat anti-rabbit Ig. The bar marker represents 1 um.

CYT cytoplasm

NUC nucleus

d dense body

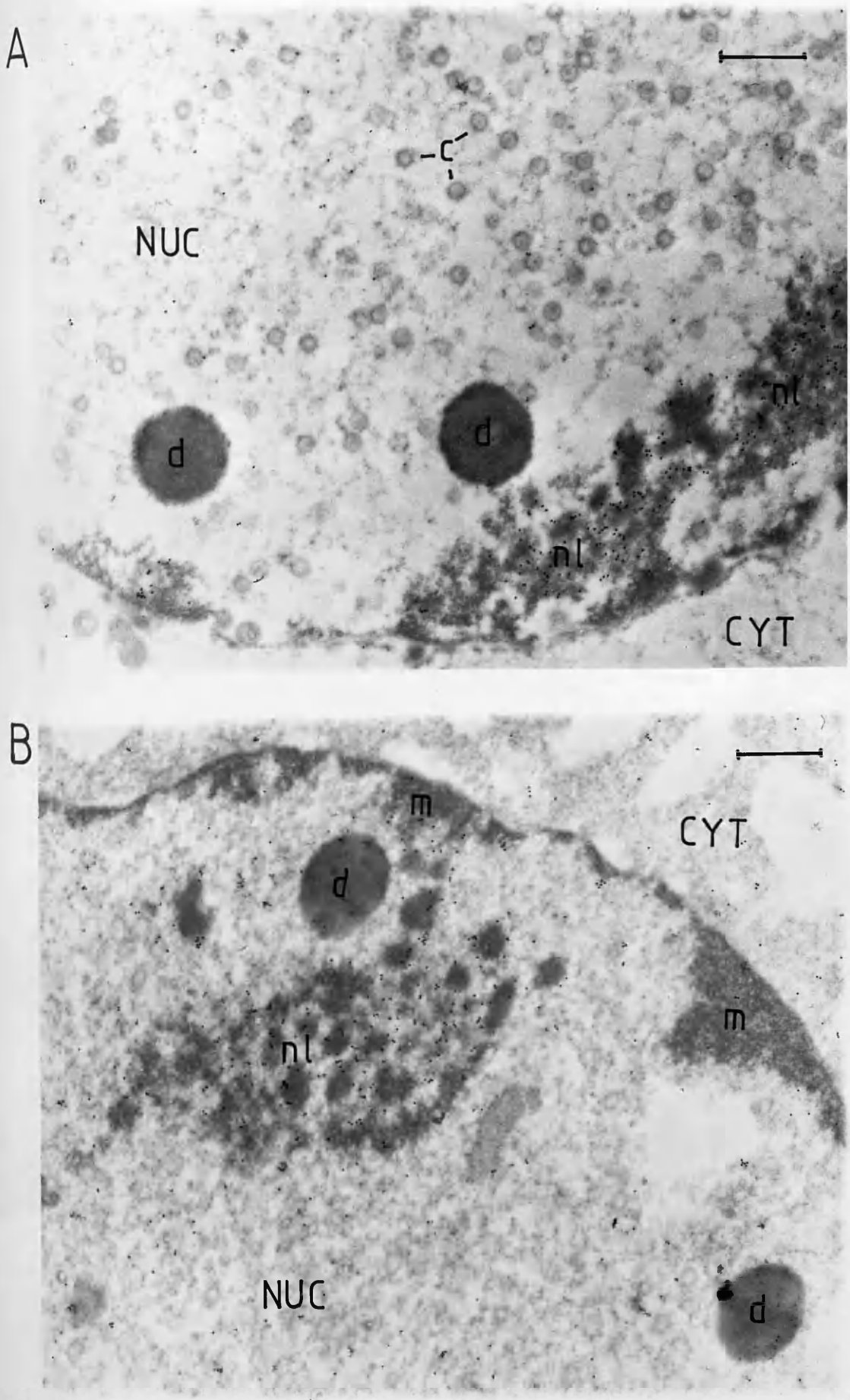
m marginated chromatin

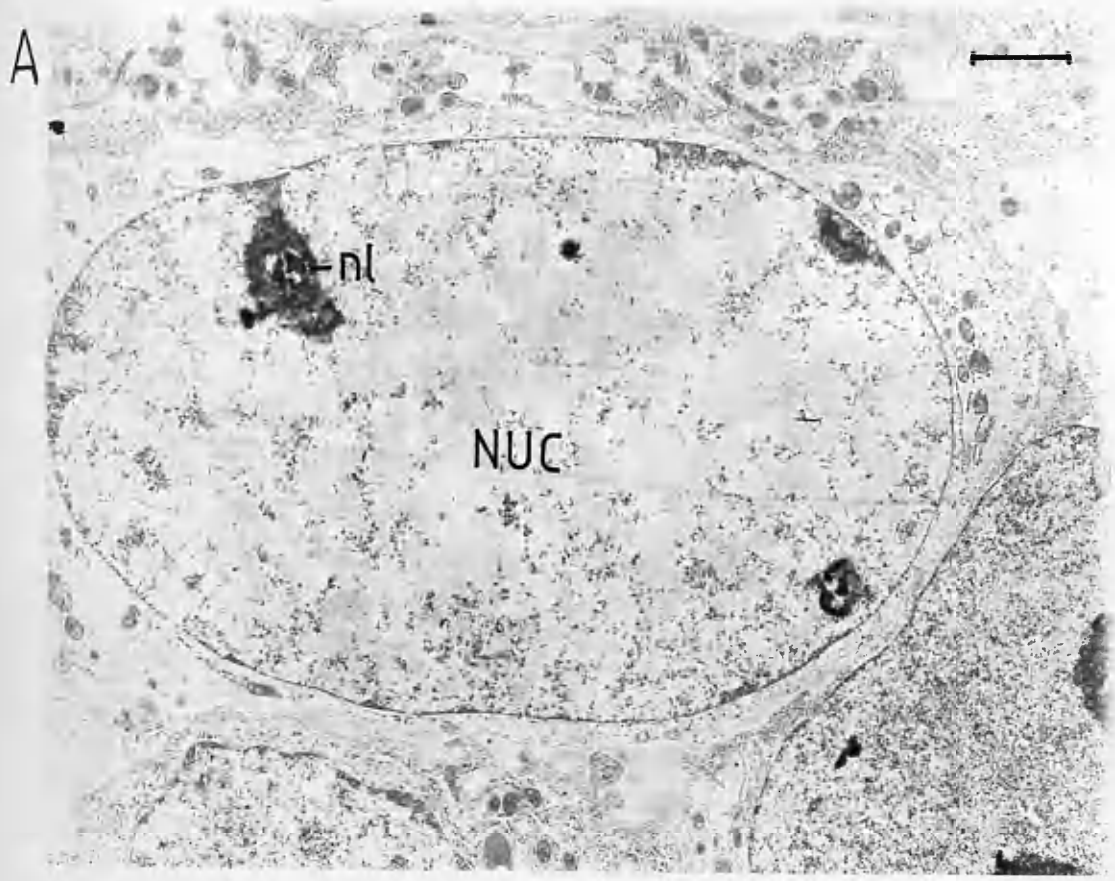
nl nucleolus

FIGURE 39

Immunolocalisation of the U_S11 gene products. Electron micrographs of thin sections through HSV-1-infected BHK C13 cells harvested at 12 h pi. The sections were incubated with anti-C_{11} serum 14327, either alone (B) or in the presence of 1 ug of N_{11} peptide (A). Bound antibody was visualised using colloidal gold-conjugated goat anti-rabbit Ig. The bar marker represents 0.5 um.

CYT	cytoplasm
NUC	nucleus
c	capsids
d	dense body
m	marginated chromatin
nl	nucleolus


A
C
NUC
d
d
nl
nl
CYT
B
m
CYT
d
m
nl
NUC
d


A
nl
NUC

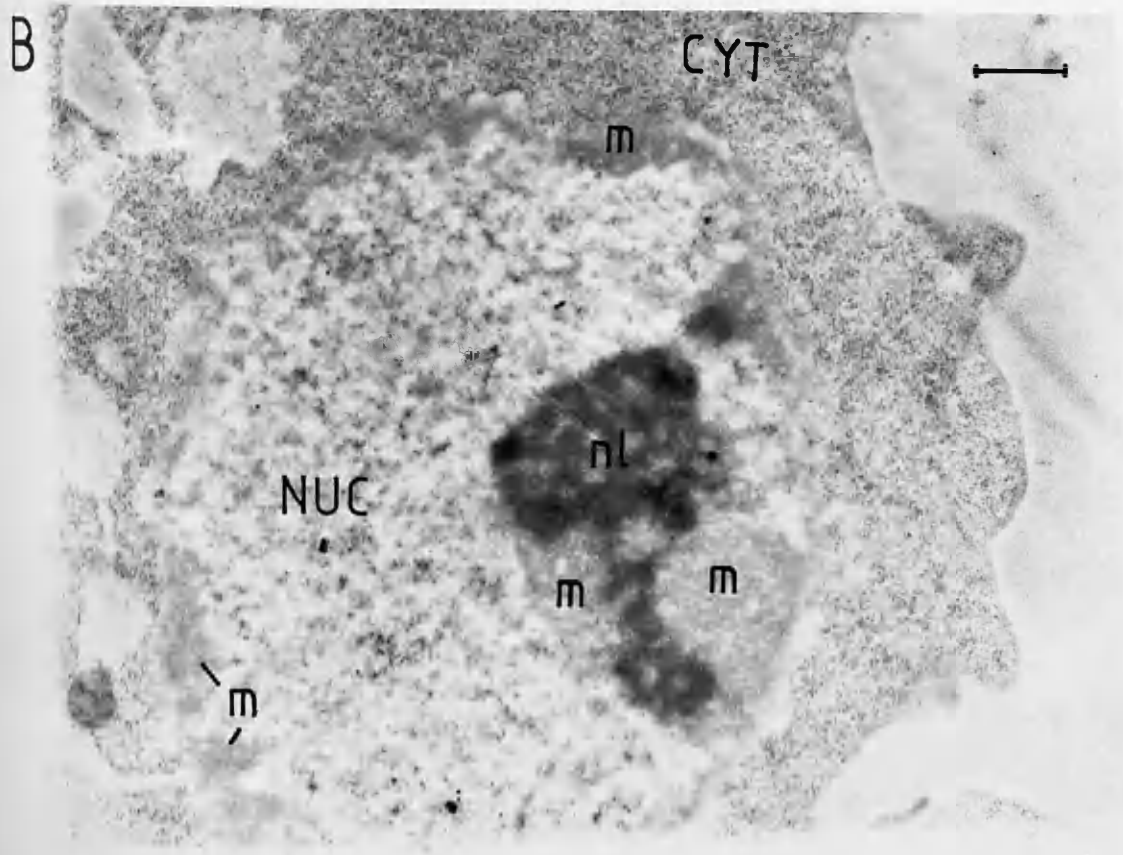

B
CYT
m
nl
NUC
m
m
m

FIGURE 40

Electron micrographs of thin sections through HSV-1-infected BHK C13 cells, harvested at 2 h pi, stained to show preferentially ribonuclear material. Sections were stained with uranyl acetate, and then either post-stained with lead citrate (A) or destained with 40 mM EDTA before post-staining (B). The bar markers represent 2 um (A) and 0.5 um (B).

CYT cytoplasm

NUC nucleus

m marginated (condensed) chromatin

nl nucleolus

FIGURE 41

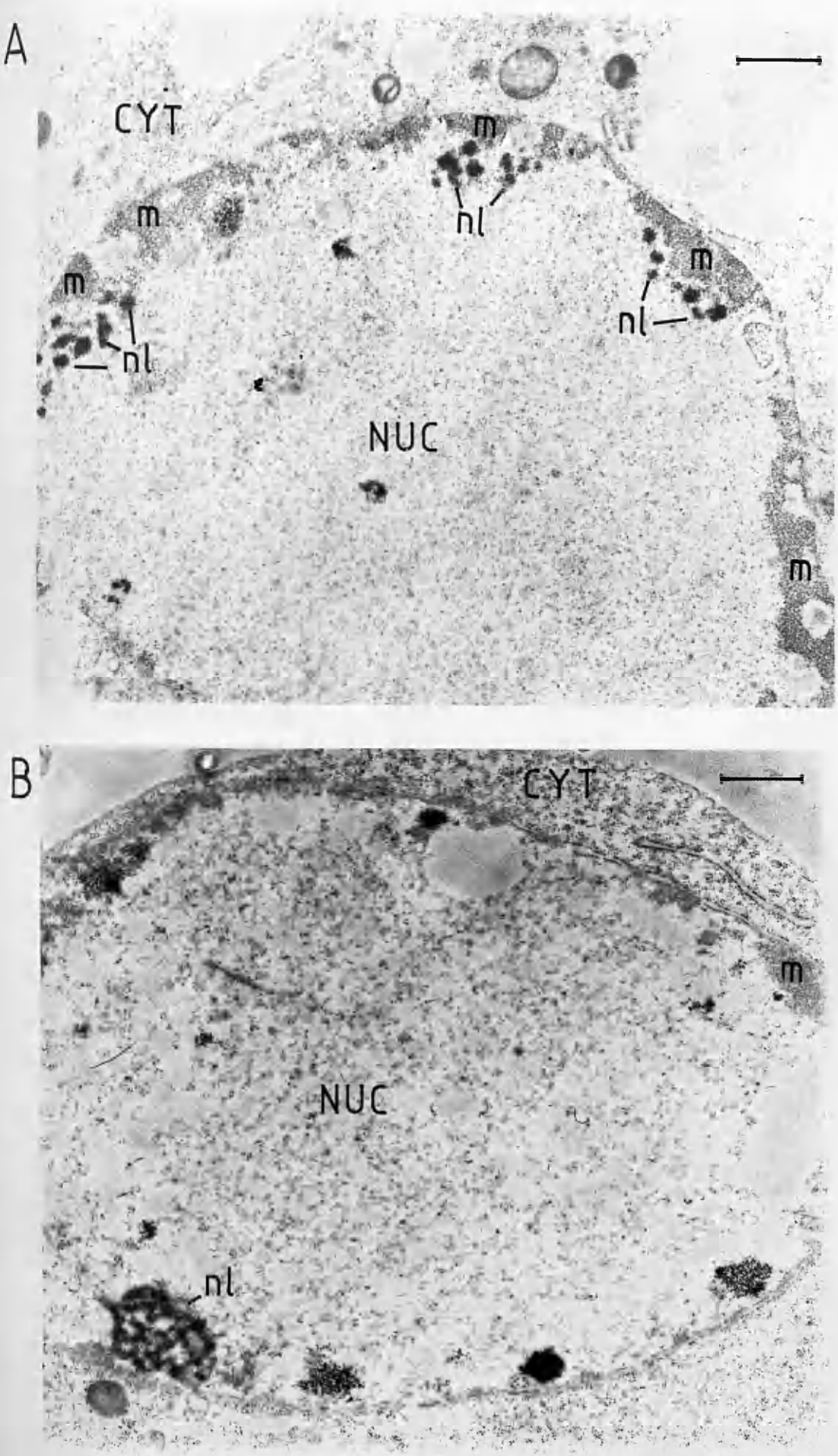
Electron micrographs of thin sections through HSV-1-infected BHK C13 cells, harvested at 12 h pi, stained to show preferentially ribonuclear material. Sections were stained with uranyl acetate and destained with 40 mM EDTA (A) or 10 mM EDTA (B). The bar markers represent 1 um.

CYT cytoplasm

NUC nucleus

m marginated chromatin

nl nucleolus


A
CYT
m
nl
m
m
nl
m
nl
NUC
m
B
CYT
m
NUC
nl

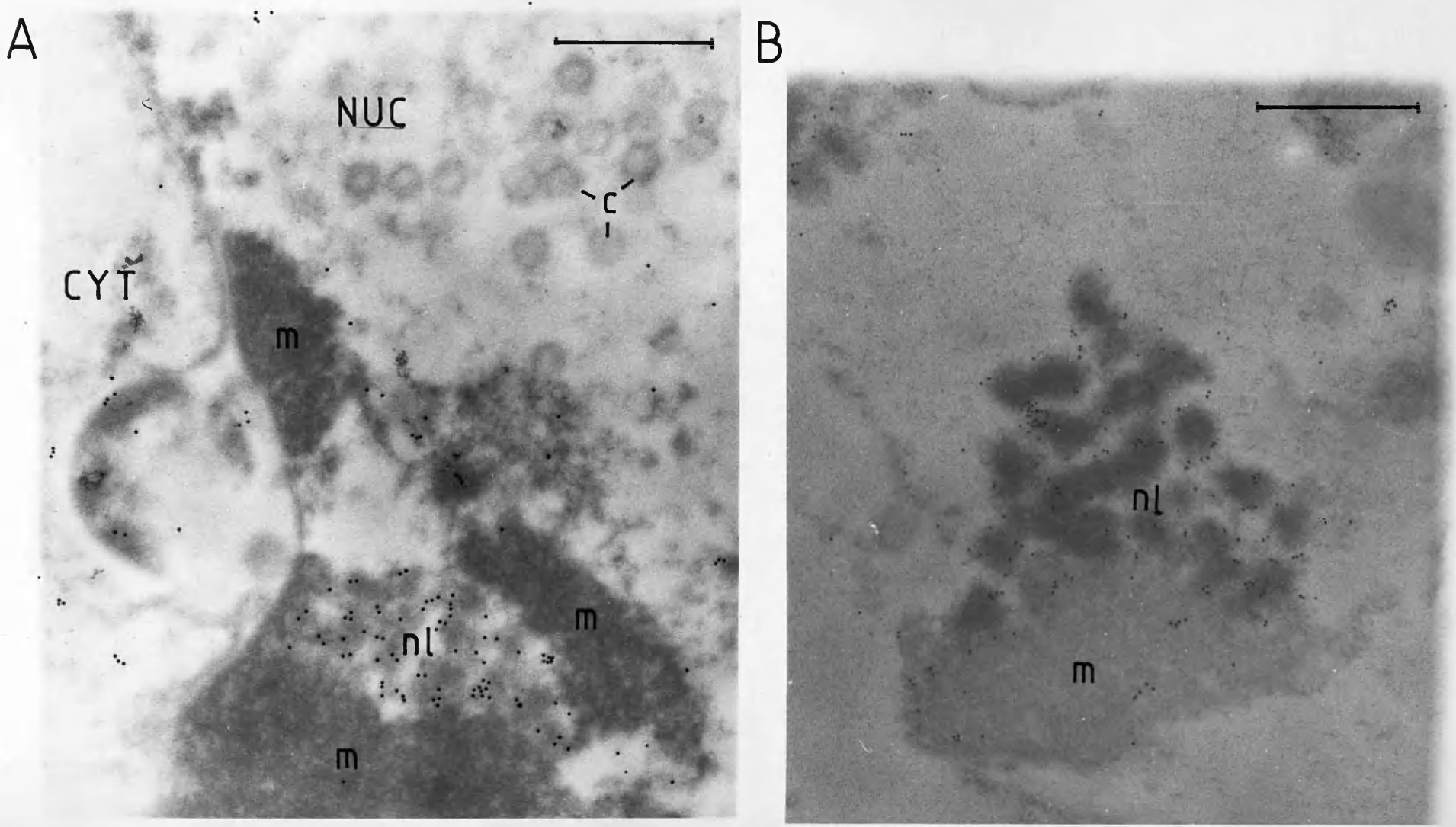

A
NUC
C
CYT
m
nl
m
m
B
nl
m

FIGURE 42

Immunolocalisation of the U_S11 gene products. Electron micrographs of thin sections through HSV-1-infected BHK C13 cells harvested at 12 h pi. Sections were incubated with anti-C_{11} serum 14327, and bound antibody detected using colloidal gold-conjugated goat anti-rabbit Ig. (A) An unstained section, osmicated after incubation with the antiserum. (B) A uranyl acetate stained section, destained with 80 mM EDTA prior to incubation with the antiserum. The bar markers represent 0.5 um.

c capsids

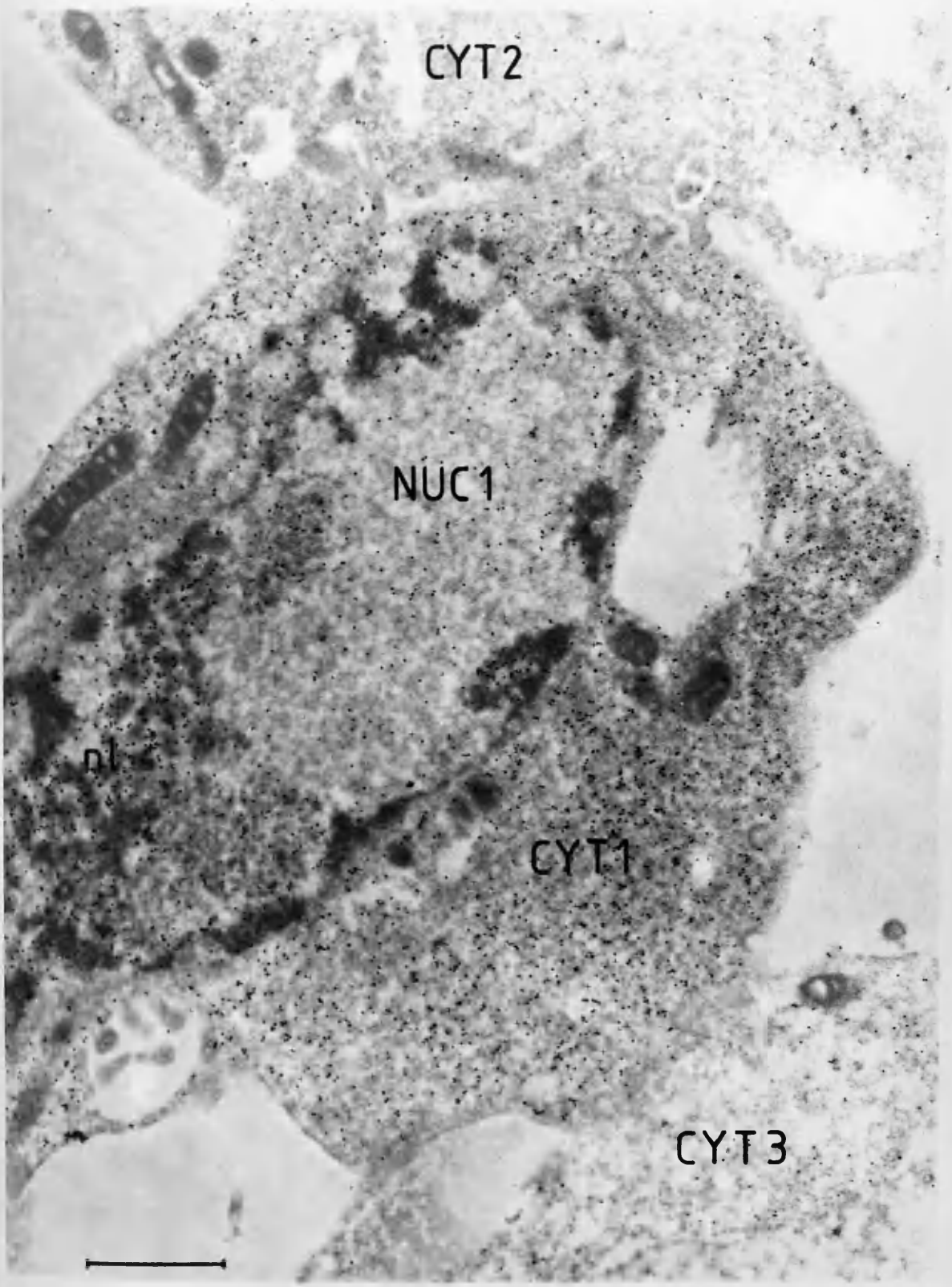
m marginated chromatin

nl nucleolus

FIGURE 43

Immunolocalisation of the U_S11 gene products. Electron micrograph of a thin section through HSV-1-infected BHK C13 cells harvested at 12 h pi. The section was incubated with anti-C_{11} serum 14327, and bound antibody detected using colloidal gold-conjugated goat anti-rabbit Ig. The bar marker represents 1 um.

CYT1, CYT2, CYT3	cytoplasm of three adjacent cells
NUC1	nucleus of cell 1
n1	nucleolus


CYT2
NUC1
CYT1
CYT3

localisation of antibody was seen with pre-immune sera or with immune sera reacted with mock-infected cells (results not shown). These results would suggest that the localisation pattern observed accurately reflects the distribution of the U_S11 gene products and is not the result of non-specific interactions or chance cross-reaction of the antibodies with an unrelated cellular protein. Thus, the U_S11 gene products appear to localise strongly to nucleoli within infected cells, although in some cells it is found in the cytoplasm.

SECTION III : PURIFICATION OF THE 21K POLYPEPTIDE

III.1 Immunoaffinity chromatography

Immunoaffinity chromatography is a widely used method of protein purification, but one disadvantage is the harsh conditions under which proteins are often eluted. The use of anti-peptide antibodies has the advantage that protein absorbed onto the column can be eluted by competition with the peptide against which the antibody was raised, increasing the probability of recovering functionally active protein.

To prepare these columns, IgG was first purified from the relevant antisera by ion-exchange chromatography on DEAE-cellulose (DE-52). The resin DE-52 was used in preference to the newer synthetic resin, Trisacryl M, since higher yields of IgG were obtained (results not shown).

The conditions used for chromatography are intended to allow IgG to pass unhindered through the column while most other proteins remain bound. Fractions were tested for protein by reading the optical density at 280nm (OD_{280}), for IgG by single radial immunodiffusion (RID) against sheep anti-rabbit IgG, and for specific anti-peptide antibodies by RIA (method sections IV.1 and IV.3). Figure 45 shows the purification of IgG from one antiserum through DE-52. A relatively small peak of protein coincident with a peak of both IgG and specific antibody is seen in the flow-through. High salt buffer added to the column then elutes a very large protein peak which contains only a low level of IgG. These fractions were analysed by SDS-PAGE (Figure 46) and the peak antibody fractions then pooled. The IgG recovered in the low salt fractions is very pure, containing only a few minor contaminating bands and one major contaminant, presumed to be haemoglobin on the basis

FIGURE 45

Purification of IgG by ion-exchange chromatography. IgG was purified from anti-C_7 serum 12848 by passage through DE-52 cellulose, as described in method section III.5. Protein which failed to bind to the column eluted in the first few fractions collected (approximately, fractions 3-10). Protein bound to the resin was subsequently eluted by high salt buffer, added after fraction 21. Protein levels of collected fractions were measured by reading the OD_{280} (□), IgG levels were measured by RID (....) and specific anti-peptide antibody levels were measured by RIA (●). The OD_{280} readings for the five fractions off scale in this diagram (fractions 27-31, respectively) were, in order, 0.695, >2, >2, 2, 0.740.

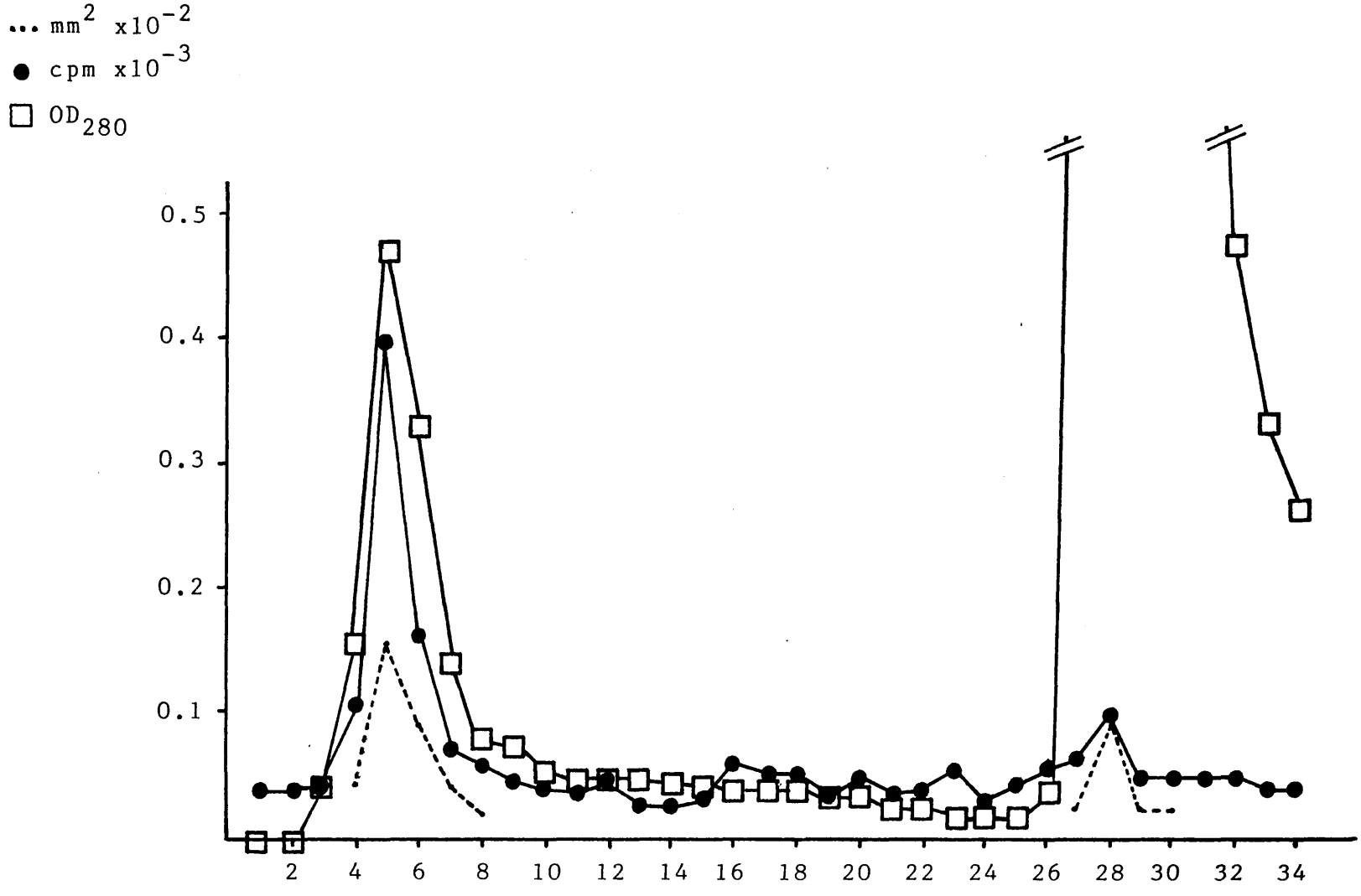
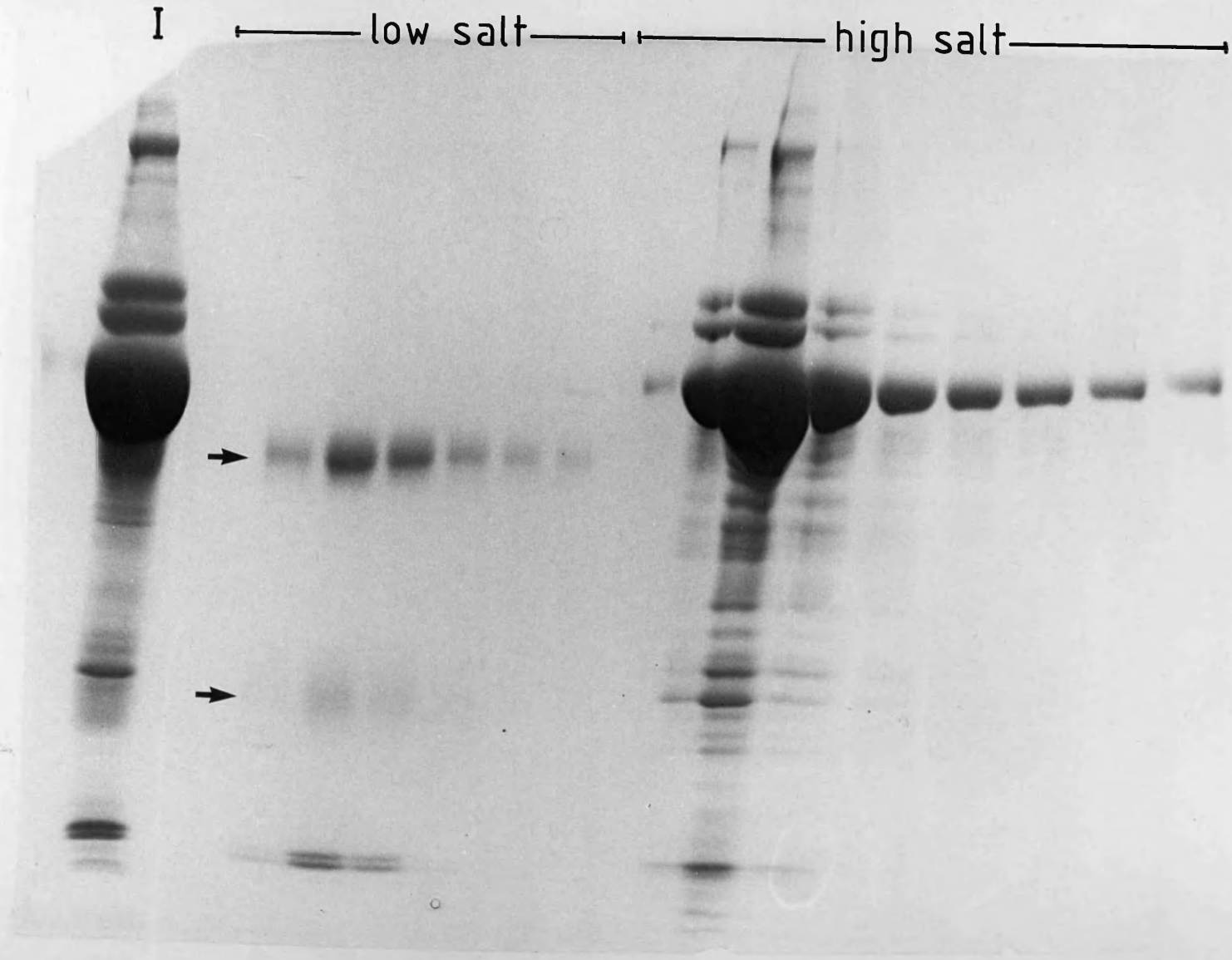

••• mm² x10⁻²
● cpm x10⁻³
□ OD280
0.5
0.4
0.3
0.2
0.1
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34

FIGURE 46

Purification of IgG. Fractions from the experiment shown in Figure 45 were separated by SDS-PAGE and stained with Coomassie blue. The position of the IgG heavy (approximately 50K) and light (approximately 25K) chains is indicated (➡).


I
low salt
high salt

of its size, and the fact that the level of this contaminant varies, depending on the degree of haemolysis in the serum sample.

Purified IgG was then coupled to Sepharose as described in method section V.2. The immunoaffinity columns produced are summarised in Table 7. These were then used in an attempt to purify 21K, running the columns under a number of different conditions : both whole cell extracts and partially-purified 21K were used as input material; the immunoaffinity beads were used either as a column or mixed end-over-end in eppendorf tubes with the extracts; incubation was carried out at different temperatures for varying lengths of time; both phosphate- and Tris-buffered solutions were used; NaCl concentrations were ranged from 50-500mM; ionic and non-ionic detergents were used at various concentrations - SDS from 0-1% and sodium deoxycholate and Nonidet P40 at 0, 0.5 and 1%; the antigens were heat-denatured, either by heating to 50°C and then incubating with the column at this temperature, or by heating to 90-100°C for 15-30 min and then incubating with the column at RT or 4°C; DNA was added to some samples before chromatography in an attempt to stabilise the protein; and the columns were run in the presence and absence of protease inhibitors.

None of these procedures led to good purification of 21K. Figure 47 shows the best purifications obtained by immunoaffinity chromatography and these produced only a low yield of 21K and very high levels of contaminating proteins. In addition, the proteins were eluted using 3M sodium thiocyanate - no protein was eluted using synthetic oligopeptide. The presence of the protease inhibitors PMSF and TLCK did not improve the purification of 21K very significantly, although

Table 7 : Immunoaffinity columns used to purify 21K.

Purified IgG was covalently coupled to Sepharose 4B (Seph), at the concentrations indicated. Rabbits 14190 and 14191 were immunised with purified 21K as described in result section I.6b.

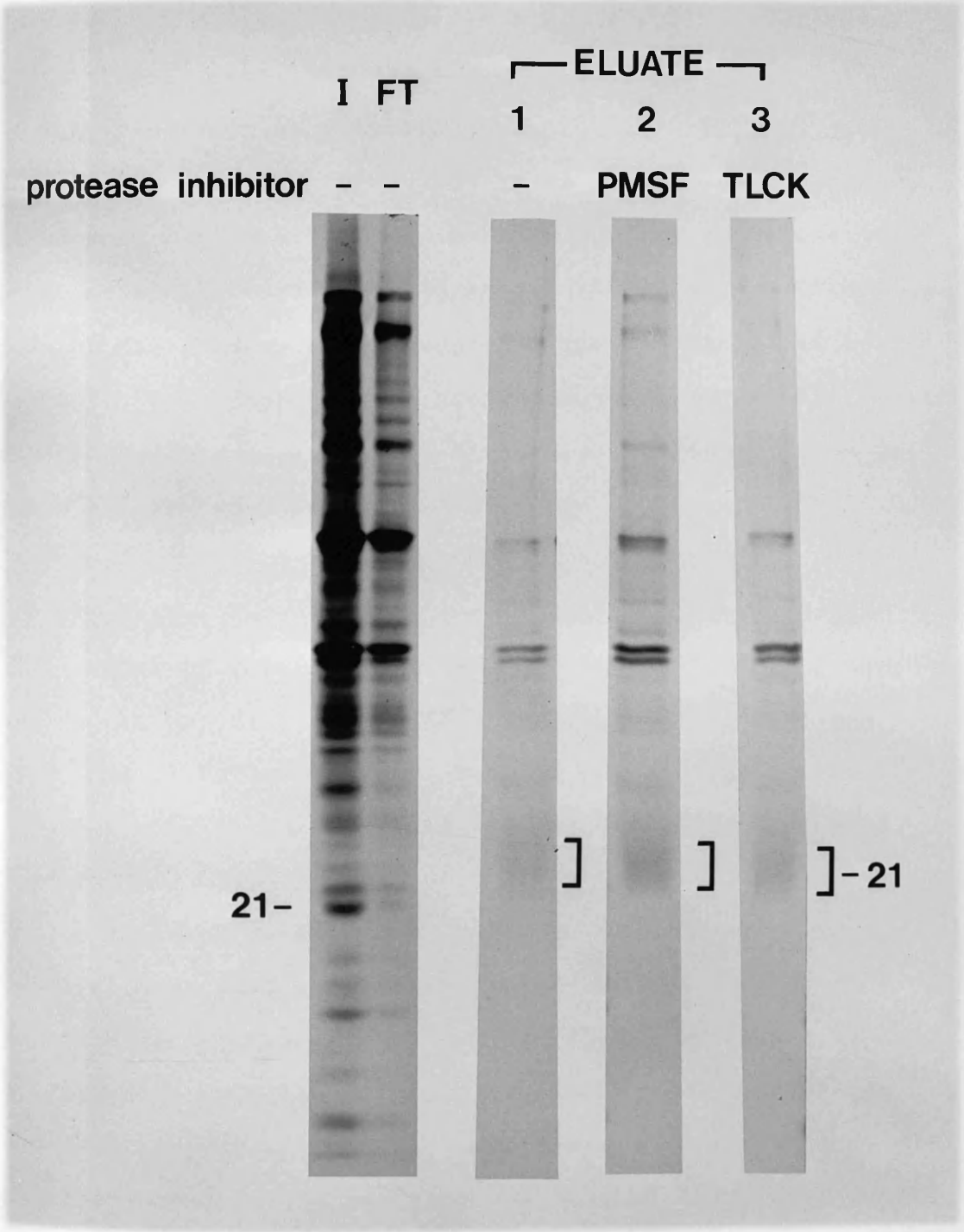
NRS normal rabbit serum

Table 7 : Immunoaffinity columns used to purify 21K

Column	IgG from Rabbit	Bleed	mg IgG / ml Seph
1. Seph:anti(1-11)	14473	3	4.8 mg/ml
2. Seph:anti(48-58)	14059	1	5.5 mg/ml
3. Seph:anti(151-161)[1]	14327	4	4.5 mg/ml
4. Seph:anti(151-161)[2]	14328	4	5.6 mg/ml
5. Seph:anti(156-161)	12848	11	2.6 mg/ml
6. Seph:anti 21K[1]	14190	4	3.4 mg/ml
7. Seph:anti 21K[2]	14191	4	3.8 mg/ml
8. Seph:NRS	NRS	-	5.6 mg/ml

FIGURE 47

Purification of 21K by immunoaffinity chromatography. Sepharose conjugated to IgG from anti-C_{11} serum 14328 (Seph:anti(151-161)[2]) was equilibrated in B2 buffer containing (1) no protease inhibitor, (2) 0.5 mM PMSF or (3) 0.1 mM TLCK, and then incubated end-over-end at RT for 90 min with 2 volumes of a ^{35}S-methionine labelled HSV-1-infected cell extract in the same buffer. Unbound material was collected (the flow-through) and the beads washed, before the more strongly bound proteins were eluted using 3M sodium thiocyanate, containing the appropriate protease inhibitor (the eluate). Fractions were analysed on 5-12.5% SDS-polyacrylamide gels. The input (I) and flow-through (FT) fractions in each case were identical, and thus only one example is shown. The region encompassing the 21K polypeptide, which appears as a rather diffuse band with an apparently increased mol. wt., is indicated (]).


I
FT
ELUATE
1
2
3
protease inhibitor
-
-
-
PMSF
TLCK
21-
-21

they do show slightly greater yields than the untreated control. On this gel 21K appears as a broad smear, rather higher in mol. wt. than in the whole cell extract. This appearance is frequently observed upon partial purification of the protein (see, for example, Figure 51).

Most purification attempts resulted in little, if any, 21K protein being recovered, and in no case could protein be eluted with peptide. That the protein was not remaining bound to the column following the 3M sodium thiocyanate elution was shown by analysing a small sample of the column matrix by SDS-PAGE. These samples were consistently devoid of 21K (results not shown). This approach was therefore considered to be of little use and was not pursued further.

III.2 Size exclusion, ion-exchange and affinity chromatography

A number of other procedures were tested for their usefulness in the purification of 21K. The results of these are summarised in Tables 8 and 9. Many of these techniques met with little or no success, and will be discussed here only briefly.

The starting material for these procedures was either whole infected cell extracts, prepared by the high salt extraction procedure (method section I.7b), or partially-purified 21K, prepared by DNA cellulose chromatography (see below). A consistent problem, particularly with the partially-purified material, was the poor recovery of 21K, irrespective of the presence of a protease inhibitor. The protease inhibitors PMSF, TLCK or α_2-macroglobulin were used. No attempt was made to systematically investigate the effect of different protease inhibitors, either alone or in combination, on the recovery of 21K, and it is possible that a combination of these, or

Table 8 : Purification of 21K by conventional chromatography

Column	Input	Recovery	Purification
Size exclusion			
Sephacryl S200	pp.21K	None	-
Sephacryl S300ø	pp.21K	Poor	Fair
Ion exchange			
DE-52	HSV-1 extract	Good	Poor
DEAE-Sepharose CL6B*	pp.21K	Fair	Poor
Affinity			
Blue Sepharose*	pp.21K	V.Poor	-
ATP Agarose	HSV-1 extract	None	-
	pp.21K	None	-
ds DNA cellulose	HSV-1 extract	Good	Good
ss DNA cellulose*	pp.21K	Good	Poor
Heparin Sepharose	HSV-1 extract	Good	Good
	pp.21K	None	-
Hydroxylapatite	HSV-1 extract	Good	Good
Phosphocellulose	HSV-1 extract	Good	Good
	pp.21K	Poor	Good

ø : Chromatography carried out by Dr Howard S Marsden

* : Chromatography carried out by Dr Graham Hope

Table 9 : Purification of 21K by HPLC and FPLC

Column	Input	Elution	Recovery	Purification
HPLC				
Anion Exchange (DEAE-5PW)	HSV-1 extract	1M $MgCl_2$	Poor	Fair
	pp.21K	0.5M NaCl	None	-
		1M NaCl	None	-
		1M $MgCl_2$	None	-
	pp.21K+DNA	1M $MgCl_2$	Good	None
Cation Exchange (SP-5PW)	HSV-1 extract	1M $MgCl_2$	Fair	Poor
	pp.21K	0.5M NaCl	None	-
		1M $MgCl_2$	None	-
Reverse Phase (Cu18 Bondapak)	pp.21K	Acetonitrile	Poor	Good
FPLC				
Anion Exchange (Mono Q)	pp.21K	400mM NaCl	None	-

other, protease inhibitors may have been required to stabilise this polypeptide.

Two points concerning the HPLC procedures are note-worthy. Firstly, dialysis of partially-purified 21K in the presence of salmon sperm DNA, prior to chromatography on the HPLC anion exchange column, prevented loss of the 21K polypeptide but, unfortunately, resulted in all the proteins eluting apparently equally over 2 fractions - ie. no increase in purification was obtained. Secondly, 21K was quite significantly purified by reverse-phase chromatography - however, this procedure involves highly denaturing elution conditions, and is therefore not a method of choice for the purification of a functional protein.

It has previously been shown that 21K binds to DNA with high affinity (Bayliss et al., 1975; Dalziel and Marsden, 1984). Therefore, a number of columns with an affinity for DNA-binding proteins were tested, including DNA cellulose, phosphocellulose and heparin-Sepharose (see below).

III.3 DNA cellulose

This procedure is described in method section V.3. Briefly, an HSV-1-infected cell extract was passed through a double-stranded calf thymus DNA cellulose column of approximately 100ml in volume. Immediately upon application of the extract to the column, fractions of approximately 10ml were collected and an aliquot of each assessed for radioactivity in a scintillation counter. The column was washed extensively with B2 buffer before proteins were eluted using a linear 50-900mM NaCl gradient, applied over 20 fractions (200ml), followed by a 2M NaCl wash. A peak of radioactivity eluted in fractions 66-76. The protein profiles of fractions 66-75 are shown in

Figure 48. The 21K and 22K proteins are present predominantly in two fractions, although low levels are present in several additional fractions. Coomassie blue staining of this gel (Figure 49) confirms that 21K is the predominant protein in these two fractions (69 and 70), representing probably 3-10% of the material present. The DNA cellulose purified 21K was often used as the starting material for other purification procedures.

III.4 Heparin-Sepharose and phosphocellulose

Other columns which work on a similar principle to DNA cellulose were also considered potentially useful in the purification of 21K. Heparin, being highly negatively charged, can mimic the ionic interactions between DNA and protein, while hydroxylapatite and phosphocellulose are both negatively charged and contain phosphate groups, and may therefore also have an affinity for phosphate-binding proteins. These three procedures were compared with DNA cellulose chromatography.

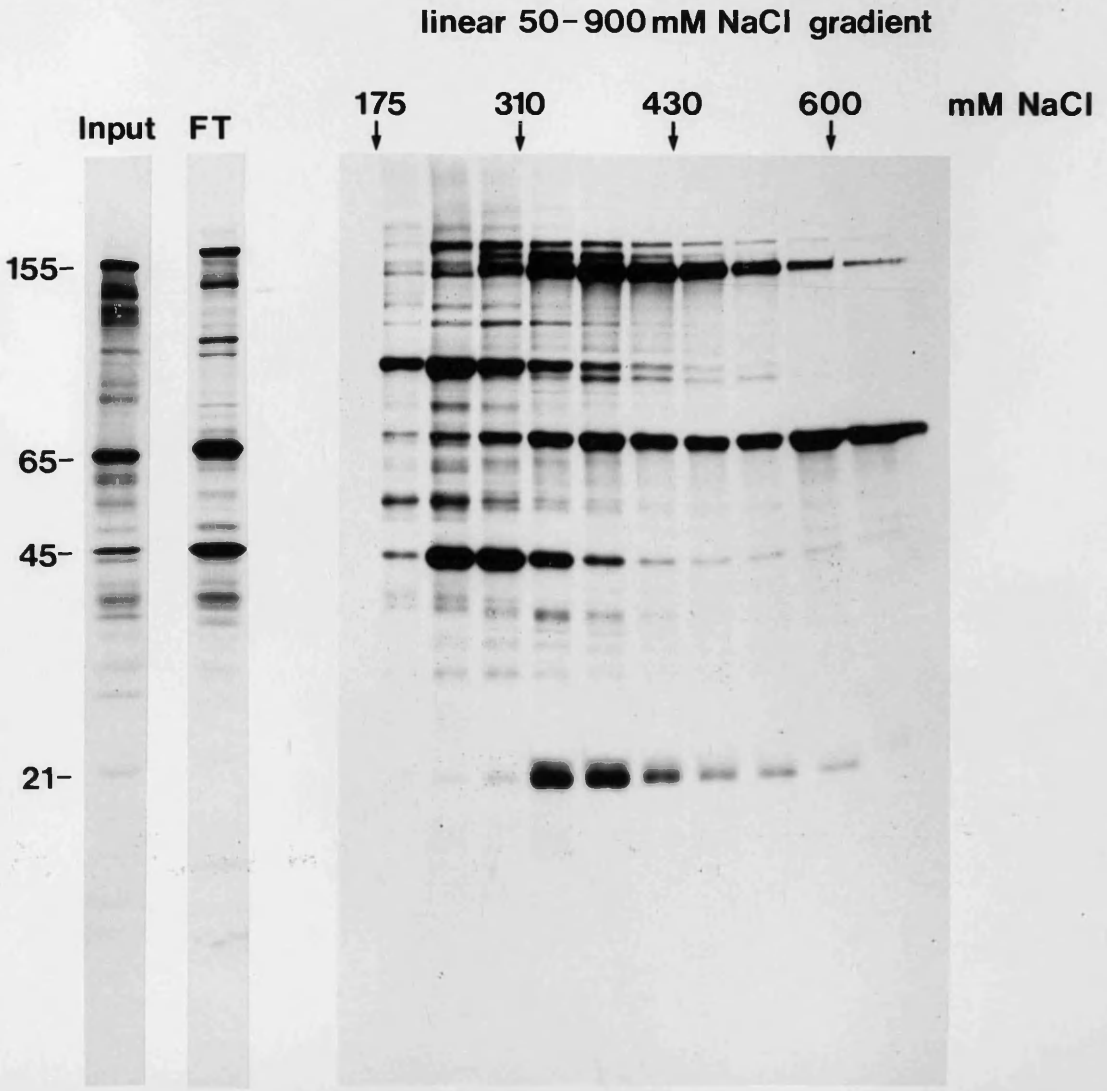
Columns of 0.5ml were prepared and 1ml of an HSV-1-infected cell extract applied. After extensive washing, proteins were eluted using a 5ml linear gradient from 50mM-2M NaCl. Fractions of 0.5ml were collected on ice and analysed by SDS-PAGE. The four methods produced a very similar purification of 21K. With both DNA cellulose and hydroxylapatite, 21K eluted predominantly with the bulk of the other proteins (results not shown), whereas with both heparin-Sepharose and, in particular, phosphocellulose 21K eluted slightly behind the majority of the proteins (Figure 50).

The use of partially-purified 21K as input material was tested only on these latter two columns. With phosphocellulose a further purification of 21K was obtained, although recovery

FIGURE 48

Purification of 21K by double-stranded DNA cellulose chromatography. A DNA cellulose column, approximately 100 ml in volume, was prepared as described in method section V.3. A ^{35}S-methionine labelled HSV-1-infected cell extract in B2 buffer (400 ml; prepared from 10 80oz roller bottles) was passed through the column and fractions of approximately 10 ml collected. Following extensive washing a linear 50-900 mM NaCl gradient was applied over 200 ml, followed by a 2M NaCl wash. Proteins eluted with the 50-900 mM NaCl gradient are shown. The approximate NaCl concentration at various points is indicated.

FT flow-through (ie. unbound material)


linear 50-900 mM NaCl gradient
Input
FT
175
310
430
600
mM NaCl
155-
65-
45-
21-

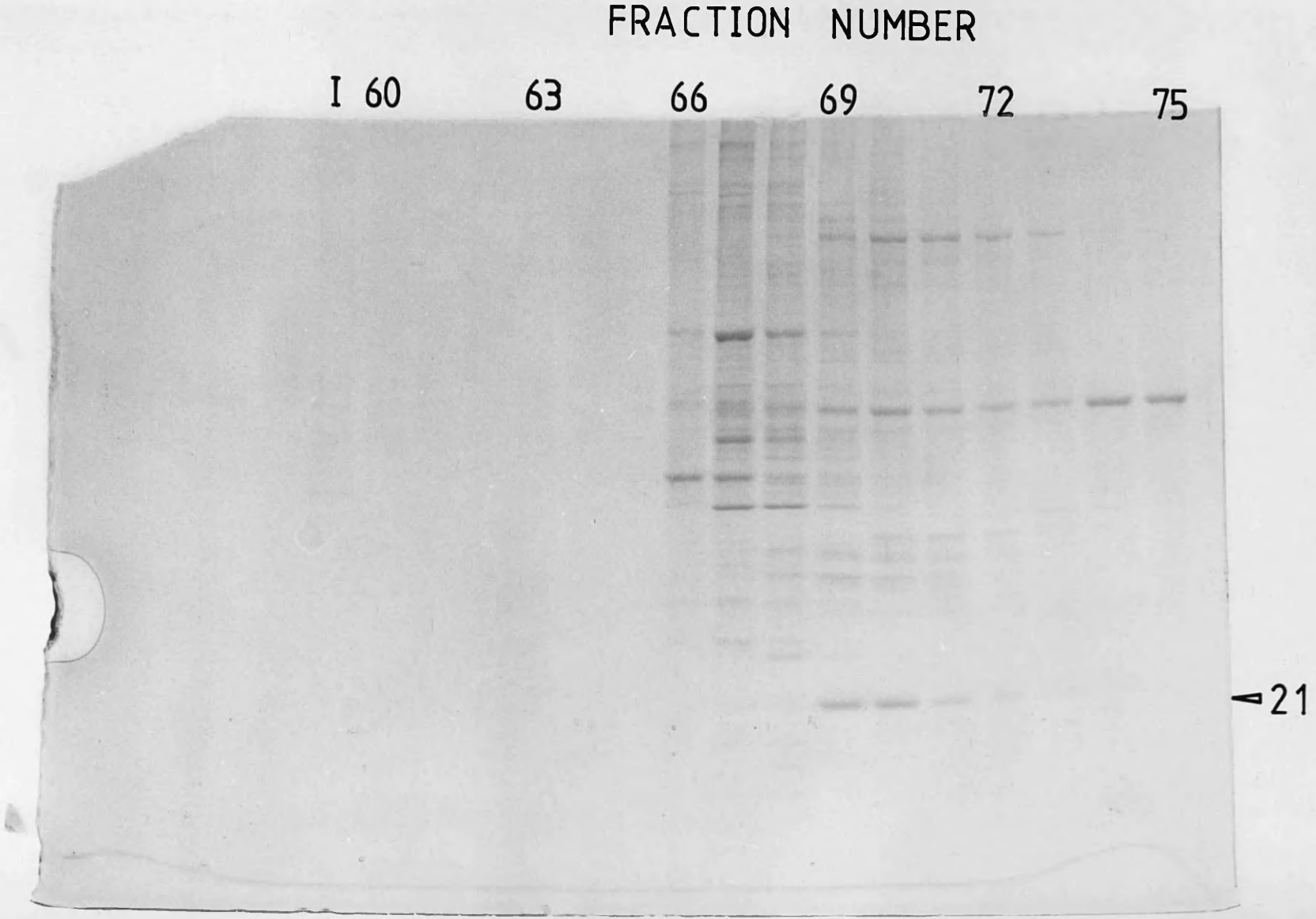
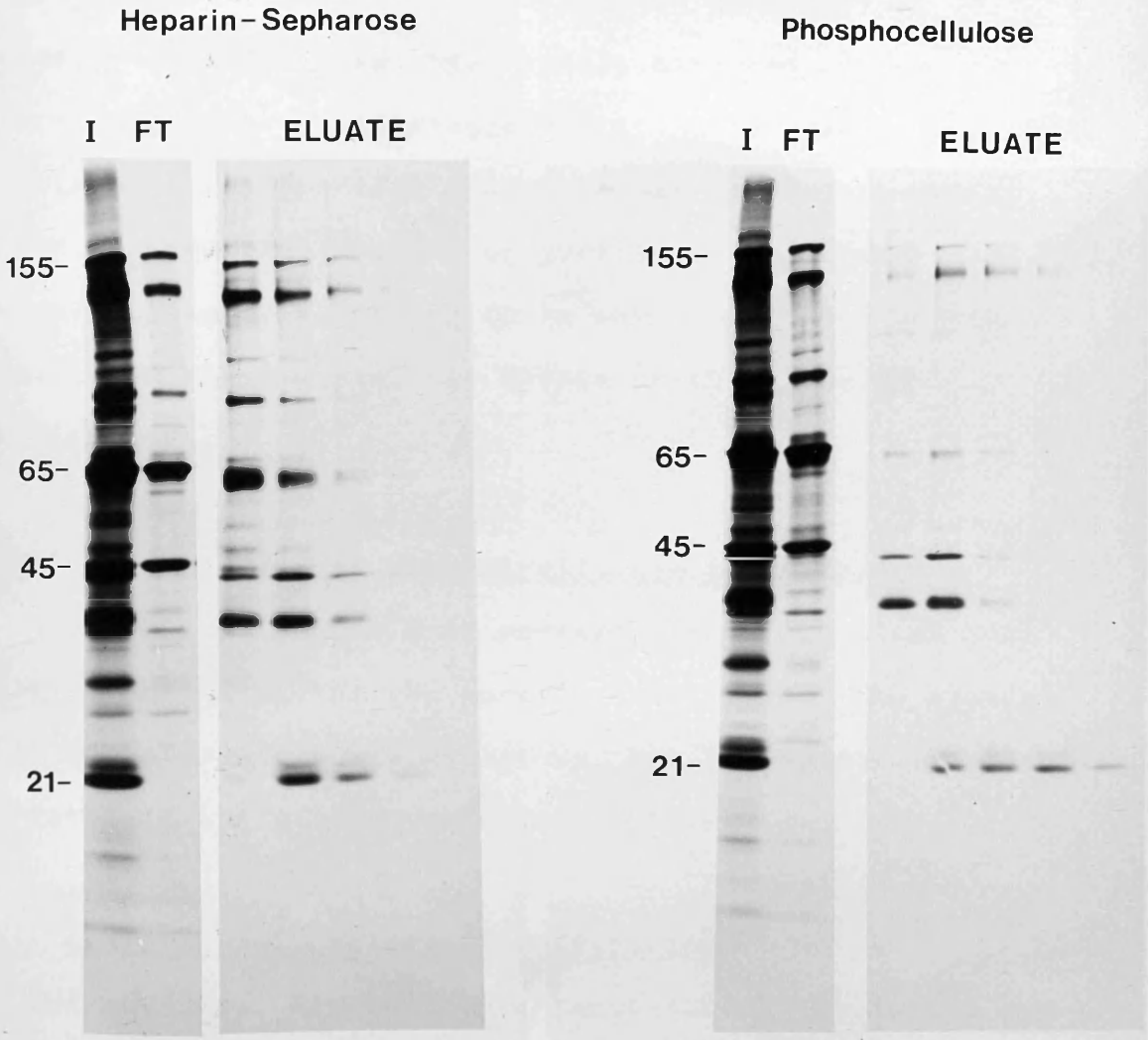

FRACTION NUMBER
I 60
63
66
69
72
75
21

FIGURE 49

Purification of 21K by double-stranded DNA cellulose chromatography. Coomassie blue staining of the 50-900 mM NaCl elution profile shown in Figure 48. Fraction numbers are indicated. The input sample (I) was diluted 1/20, to prevent overexposure on autoradiography, and is thus barely visible by Coomassie blue staining.

FIGURE 50

Purification of 21K by heparin-Sepharose and phospho-cellulose. Columns of 0.5 ml were prepared as described in method sections V.6 and V.4, respectively. One ml of ^{35}S-methionine labelled HSV-1-infected cell extract in B2 buffer (I) was run through the columns and the flow-through material collected (FT). After extensive washing, bound proteins were eluted with a linear 0.05-2M NaCl gradient. Only the major protein-containing fractions are shown (ELUATE). Samples were analysed on 5-12.5% SDS-polyacrylamide gels.


Heparin-Sepharose
I
FT
ELUATE
155-
65-
45-
21-
Phosphocellulose
I
FT
ELUATE
155-
65-
45-
21-

was difficult to judge since the polypeptide appeared rather diffusely spread on the gel (Figure 51). This is a fairly typical example of the frequently observed aberrant migration of partially-purified 21K. In contrast, with heparin-Sepharose, no 21K was recovered (results not shown).

The apparent instability of the 21K polypeptide, in partially-purified form, has greatly hindered the use of several successive chromatographic steps for the purification of this protein. For this reason it was decided to derive, as far as possible, a one-step purification procedure for 21K. Phosphocellulose, appearing to be the most useful of the methods described above, was chosen as the basis for purification.

III.5 Purification of 21K by phosphocellulose chromatography

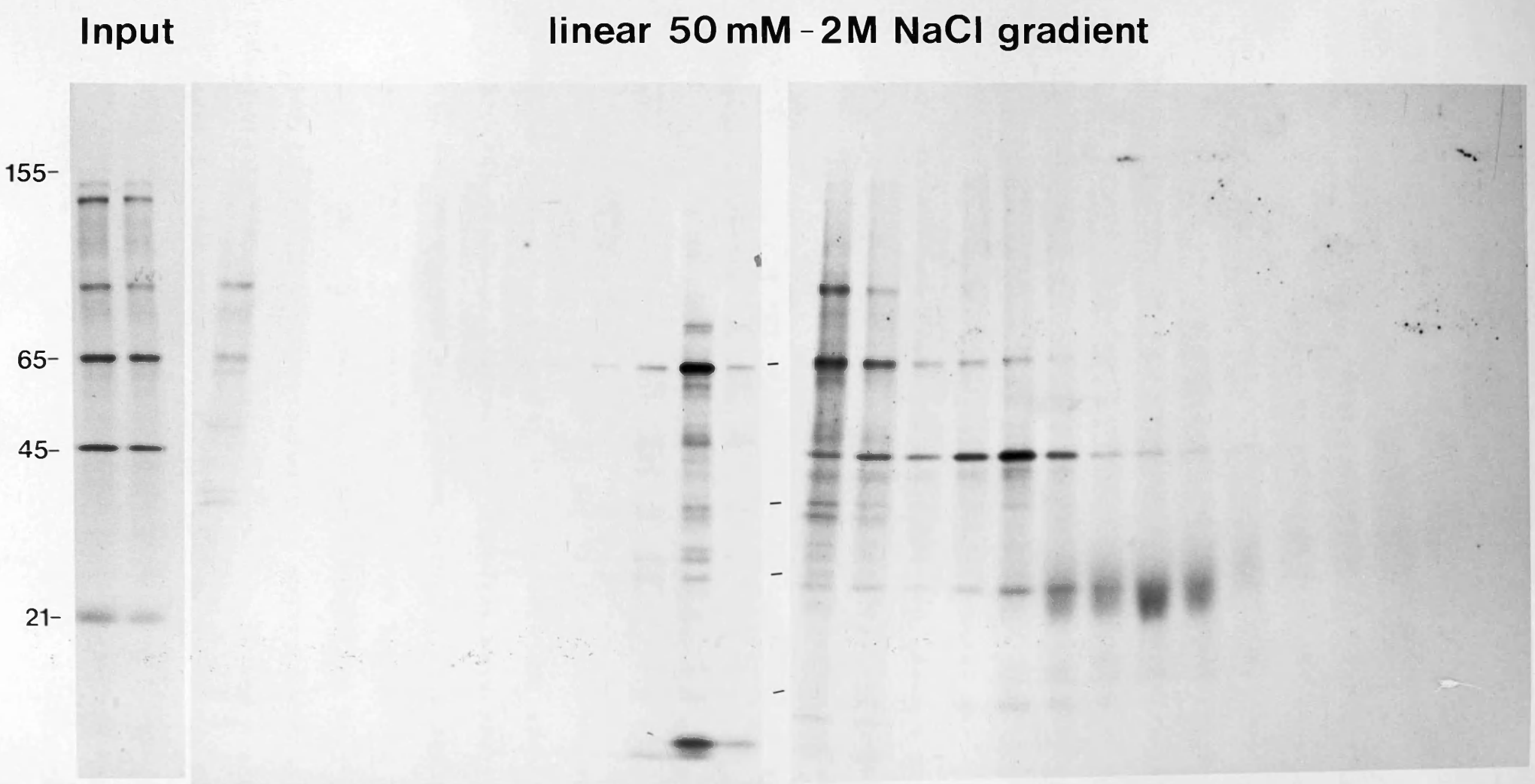
Initial experiments were carried out to establish more optimal conditions for the purification of 21K. The results of these experiments are not shown, but formed the basis for the purification procedures outlined below.

(a) Small scale phosphocellulose purification

Infected cell extracts were incubated on ice in 30% or 35% saturated ammonium sulphate and the precipitate removed by centrifugation at 7,000 rpm for 30 min in a Sorvall SM24 rotor. The supernatants were then dialysed extensively against B2 buffer containing 350mM NaCl. Phosphocellulose columns (2ml) were equilibrated in this buffer and the above samples, as well as an untreated infected cell extract (also in the presence of 350mM NaCl), were then applied to the columns. Bound proteins were eluted firstly with 450mM NaCl and subsequently with 2M NaCl. Fractions were analysed by SDS-PAGE

FIGURE 51

Purification of 21K by phosphocellulose chromatography. A 3.8 ml phosphocellulose column was prepared as described in method section V.4. One ml of 21K partially-purified by DNA-cellulose chromatography (fraction 69, Figure 49) was run through the column (I) and, after washing, bound proteins were eluted with a linear 0.05-2M NaCl gradient, applied over 40 ml. One ml fractions were collected and analysed on 5-12.5% SDS-polyacrylamide gels. The input material (undiluted) represents a 36 h exposure of the original gel. The eluted material represents a 151 day exposure. 21K is seen eluting in later fractions as a rather diffuse, smeared band apparently increasing in mol. wt.


Input
linear 50 mM - 2M NaCl gradient
155-
65-
45-
21-

(Figure 52).

The untreated infected cell extract under these conditions showed only a very faint elution of 21K with 450mM NaCl (not shown), while the 2M NaCl eluate contained predominantly 21K but also a number of contaminating bands (Figure 52). From extracts precipitated with ammonium sulphate no 21K was eluted from the column with 450mM NaCl. However, 2M NaCl eluted 21K almost devoid of contaminating bands. The yield of 21K was significantly better with the 30% rather than the 35% ammonium sulphate supernatant, although the 30% supernatant contained a faint lower mol. wt. band. Whether this band is related to 21K is unknown.

(b) Large scale phosphocellulose purification

Scaling up this procedure, however, was unsuccessful. The 21K polypeptide, although by far the most predominant band on autoradiography, was contaminated by a number of other proteins (Figure 53), while Coomassie blue staining revealed several polypeptides, of which 21K was not one (results not shown).

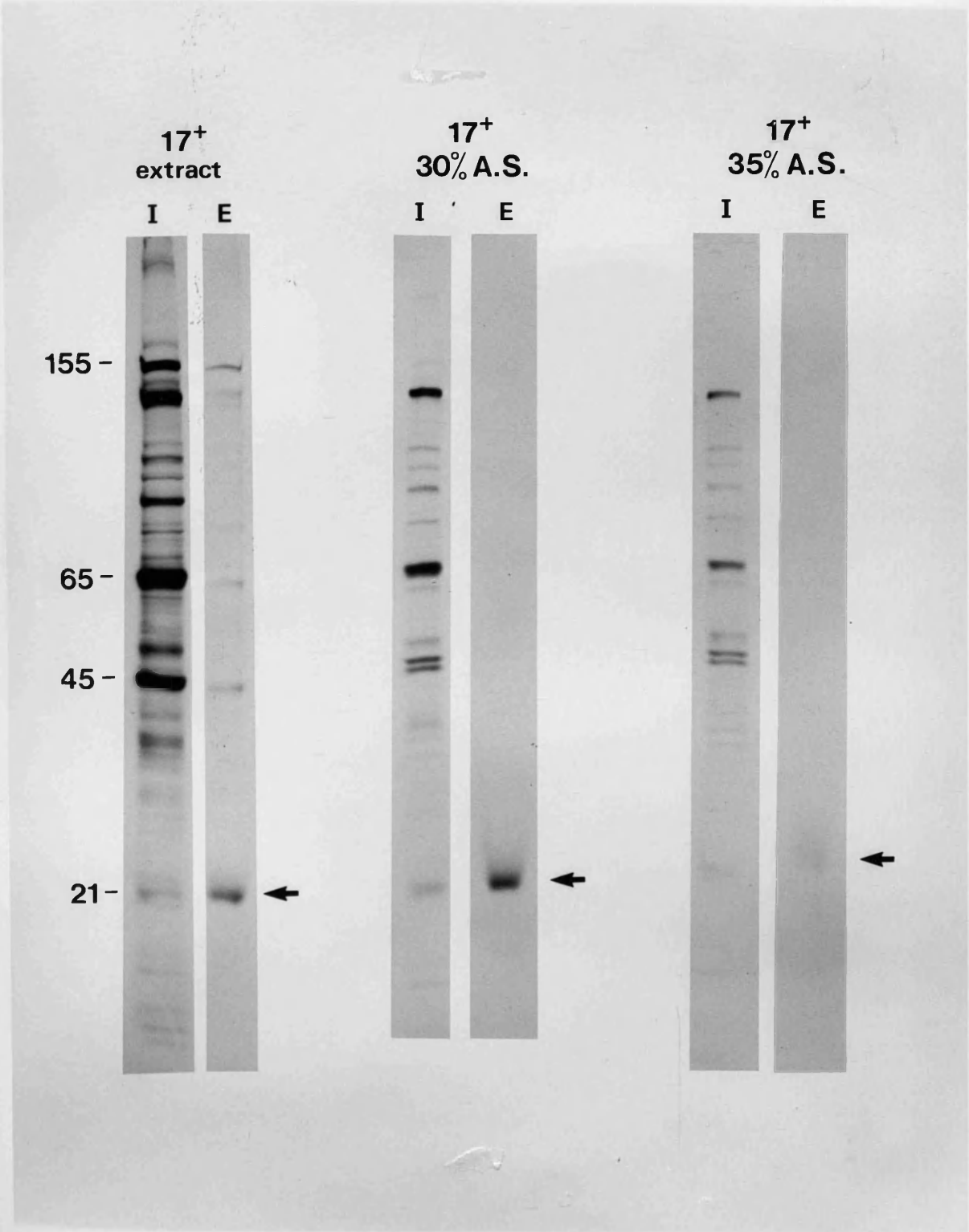
In conclusion, attempts to purify 21K have met with limited success. We had originally hoped to purify 21K to apparent homogeneity, in order to further investigate its DNA-binding properties. We therefore decided to approach this problem from another angle - namely, to investigate 'a' sequence-specific DNA-protein interactions, and the role of the 21K polypeptide in these interactions.

FIGURE 52

Phosphocellulose purification of 21K. ^{35}S-methionine labelled HSV-1-infected cell polypeptides were precipitated with 30% or 35% saturated ammonium sulphate (A.S.) and the supernatant collected and dialysed against B2 buffer containing 350 mM NaCl. Fifteen ml of each supernatant, or 10 ml of the original infected cell extract, adjusted to a concentration of 350 mM NaCl, were passed through 2 ml phosphocellulose columns equilibrated in B2 buffer containing 350 mM NaCl. After washing, bound proteins were eluted firstly with 450 mM and subsequently with 2 M NaCl. Samples were analysed on 5-12.5% SDS-polyacrylamide gels.

I input material

E 2 M NaCl eluate


17+
extract
I
E
17+
30% A.S.
I
E
17+
35% A.S.
I
E
155 -
65 -
45 -
21 -

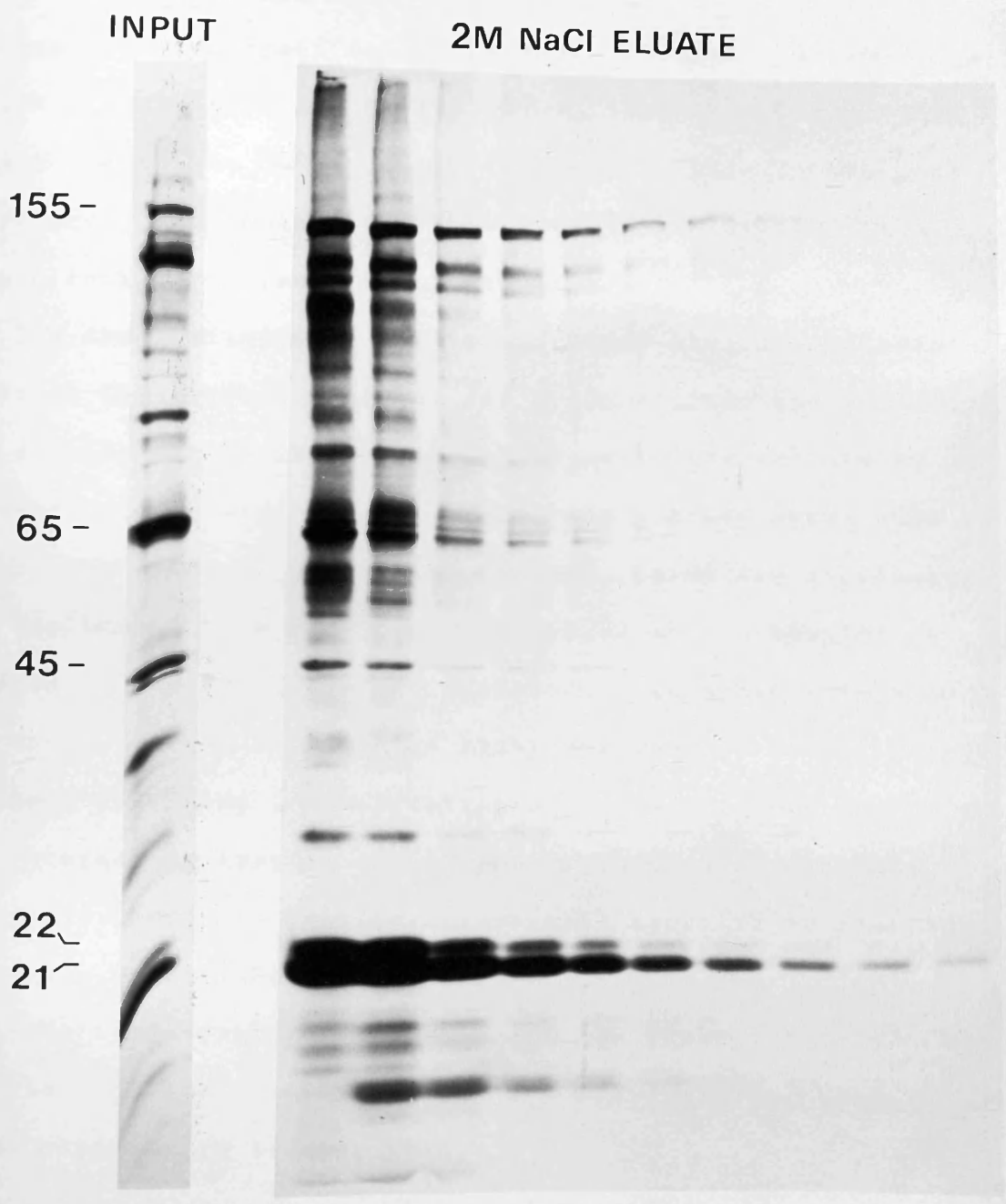

INPUT
2M NaCl ELUATE
155
65
45
22
21

FIGURE 53

Large scale phosphocellulose purification of 21K. ^{35}S-methionine labelled HSV-1-infected cell polypeptides in high salt extraction buffer (2M NaCl) were precipitated with 20% saturated ammonium sulphate, and the supernatant dialysed against B2 buffer containing 450 mM NaCl. Approximately 185 ml of supernatant (INPUT) was applied to a 50-60 ml phosphocellulose column, the column washed with B2 buffer containing 450 mM NaCl, and bound protein eluted with a 2M NaCl wash. Fractions were analysed on 5-12.5% SDS-polyacrylamide gels. A pool from the second, third and fourth eluate fractions shown was used in the tryptic peptide fingerprint experiments described in result section II.2.

SECTION IV : DNase I FOOTPRINTING ON THE HSV-1 'a' SEQUENCE

It has previously been shown that the 21K and 22K DNA-binding proteins specifically interact with the 'a' sequence of HSV-1 (Dalziel and Marsden, 1984). However, the precise region within the 'a' sequence involved in this interaction is unknown, and it has yet to be established whether this is a direct or indirect interaction.

The assay originally used to identify the 'a' sequence-specific DNA-binding proteins was a competition assay involving the elution of proteins bound to DNA cellulose columns by fragments containing HSV DNA. This rather crude assay uses large quantities of competitor DNA and, in my own experience, is complicated by a very high background of non-specific binding to the DNA fragment. Therefore, two other assays were attempted - a gel retardation assay and DNase I footprinting. Of these the DNase I footprinting produced the more immediate and interesting results and therefore this assay was subsequently chosen to further investigate specific interactions occurring within the 'a' sequence.

Before presenting the results of the DNase I footprinting experiments, the construction of a plasmid ($pASO_3$) used in later experiments is described.

IV.1 Construction of plasmid $pASO_3$

Our original intention was to obtain a plasmid containing tandem copies of a nucleotide sequence from within the U_b region of the HSV-1 'a' sequence which shares a high degree of homology with a similar region within the 'a' sequence of HSV-2. This corresponds to the pac-1 packaging signal described by Deiss *et al.* (1986).

Two 43 base synthetic oligonucleotides whose sequences were partially complementary but contained 5' end overhangs corresponding to Bgl II and Bam HI sites, respectively, were synthesised. Annealing of these two oligonucleotides should form the product shown in Figure 54 (ii). Since the 5' extensions at each end of the annealed product are both complementary and palindromic, this product could self-ligate in either orientation. Head-to-head or tail-to-tail ligation (inverted repeats) would result in the formation of either a Bam HI site, 5'-GGATCC-3', or a Bgl II site, 5'-AGATCT-3', respectively, at the junction between fragments. In contrast, head-to-tail or tail-to-head ligation (direct repeats) would not generate these sites, producing instead the sequence 5'-GGATCT-3', or 5'-AGATCC-3', at the junctions. Therefore, direct repeats could be selected by digestion of the ligation products with both Bam HI and Bgl II. We used this approach to generate the plasmid $pASO_3$, as follows.

The synthetic oligonucleotides (which lack 5' phosphate groups) were 5' end-labelled in the presence of T4 polynucleotide kinase and γ^{32}P-ATP as described (method section IX.2b). The reaction was continued for a further 30 min in the presence of excess (cold) ATP to ensure maximal phosphorylation of the 5' ends (a final concentration of 1mM ATP was used compared with approximately 44uM oligonucleotide). Approximately 10ug of each oligonucleotide were mixed together in a final volume of 25ul and allowed to anneal at 37°C for 1 h. The annealed strands were then ligated and following extraction and precipitation the DNA was digested with an excess (40 units) of Bam HI and Bgl II (method sections XII.3 and VIII.1). The digested DNA was again extracted and precipitated and then subjected to a second round of ligation/

FIGURE 54

Construction of plasmid $pASO_3$.

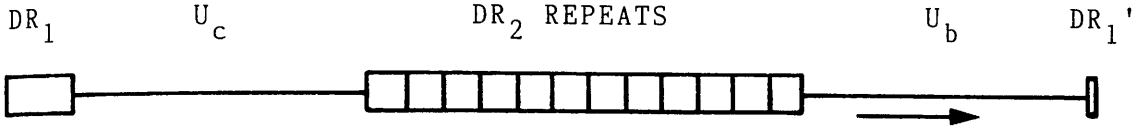
(i) Schematic representation of the HSV 'a' sequence in plasmid pR9. This 'a' sequence was obtained from the S terminus, and thus has an incomplete terminal DR_1 (DR_1'). In HSV-1 strain 17syn$^+$, the 'a' sequence contains only a single DR_4 homology, and therefore, for simplicity, this is included in the U_c region shown. The sequence to which the synthetic oligonucleotides correspond is indicated (⟶).

(ii) Product obtained after annealing of the synthetic oligonucleotides. The two oligonucleotides are complementary, with the exception of 4 bases at the 5' end of each. Annealing of these oligonucleotides would form a structure possessing 5' extensions which are both complementary and palindromic, and these could therefore anneal in either orientation. The sequence is shown in the orientation indicated in (i), and those nucleotides corresponding to the U_b region indicated are underlined.

(iii) Structure of $pASO_3$. The synthetic oligonucleotides were annealed and ligated as described in the text, and then cloned into the Bam HI site of the vector pUC8 (method section XII.3). A plasmid was obtained which contained three tandem repeats of the oligonucleotide sequence, in the orientation shown. This plasmid was termed $pASO_3$.

H Hind III; P Pst I; B Bam HI; S Sma I;
R Eco RI

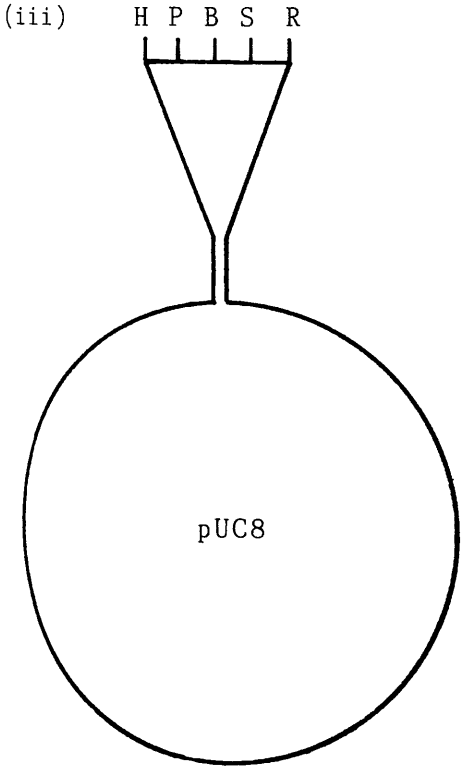
(i)

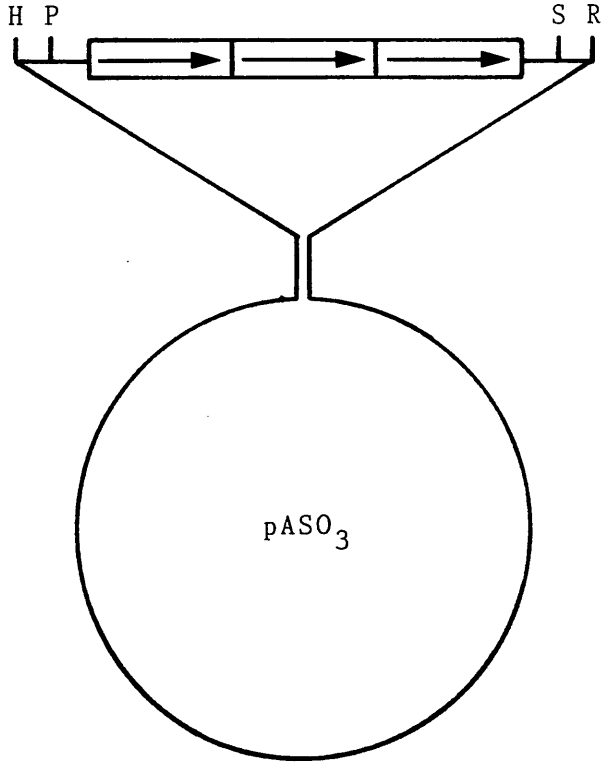


(ii) GATCTAACGGGCCCCCCCCAAAACACACCCCCCGGGGGTCGCG

ATTGCCCGGGGGGGGGTTTTGTGTGGGGGGCCCCCAGCGCCTAG

(iii)





digestion. Since small fragments of DNA ligate more efficiently than large fragments, the digested products were separated on an acrylamide gel. No attempt was made to isolate individual bands since in previous experiments this had met with no success. Instead two groups of bands were isolated - (a) those fragments estimated to contain 4-6 copies of the oligonucleotide, and (b) those fragments estimated to contain more than 6 copies of the oligonucleotide. Fragments containing less than 4 copies of the oligonucleotide were discarded.

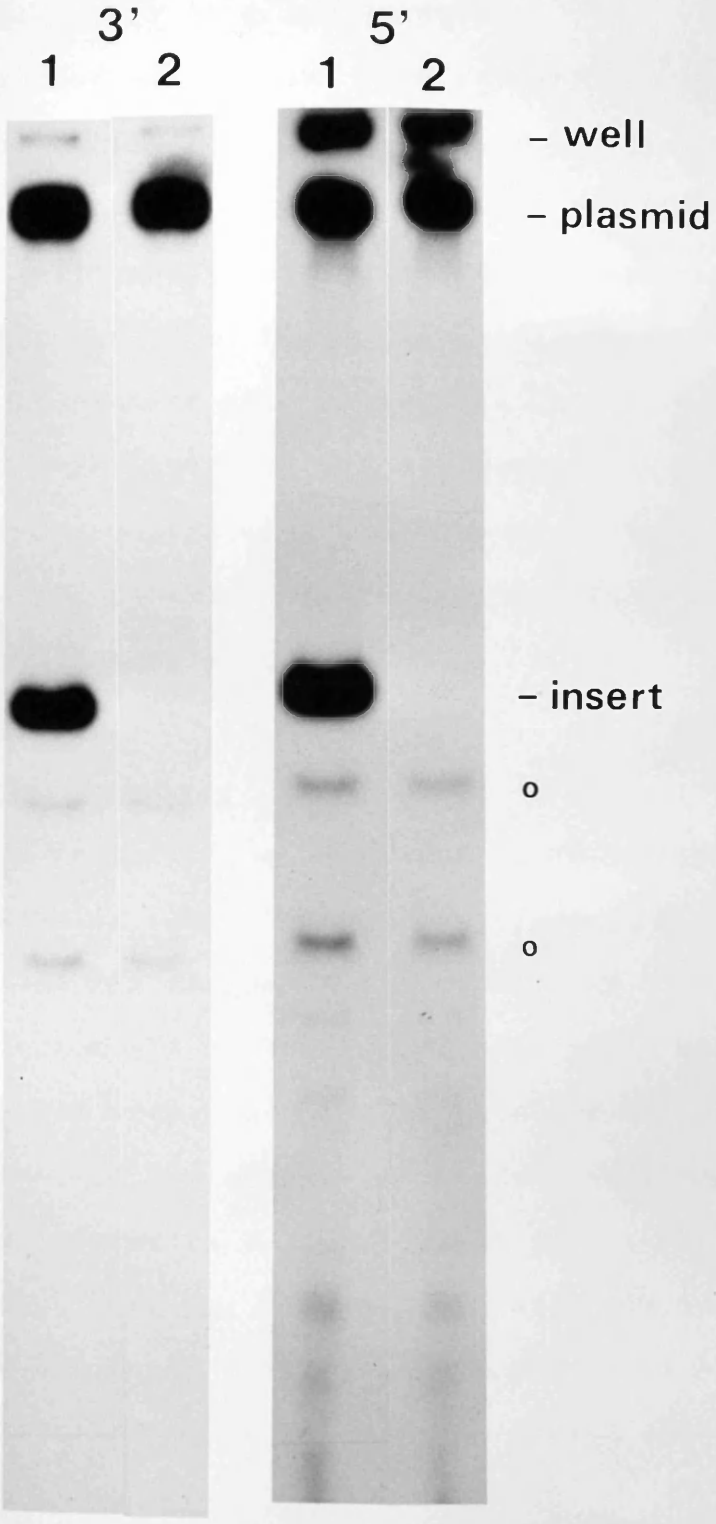
The purified DNA was cloned into the Bam HI site of pUC8 as described (method section XII.3) and used to transform DH1 cells to ampicillin resistance. Plaques were analysed by restriction enzyme digestion. Of 72 plaques analysed, one plasmid clone was isolated which appeared to contain 3 repeats of the oligonucleotide (ie. an insert of approximately 120 bp). This was therefore grown in larger quantity and the DNA isolated and purified by CsCl gradient centrifugation (method sections VII.1, VII.2 and VII.3).

To determine the nature of the inserted DNA, the Hind III/ Eco RI fragment containing the insert (see Figure 54 (iii)) was purified and sequenced as follows. The plasmid was linearised by digestion with Hind III and either 3' or 5' end-labelled at this site (method sections IX.2a,b). The labelled DNA was then digested with Eco RI and the products separated on a 10% polyacrylamide gel (Figure 55). The large fragment represents vector sequences, while the smaller, strongly labelled, fragment represents the insert contained within the polylinker site. Two smaller fragments (indicated by the open circles) were also consistently observed, although only in low quantities. The size of these latter fragments would be compatible with the presence within the polylinker site of

FIGURE 55

Purification of end-labelled insert DNA. Plasmid $pASO_3$ was digested with Hind III and either 3' or 5' end-labelled as described (method section IX.2a,b). The labelled DNA was digested with Eco RI (see Figure 54), separated on 10% polyacrylamide gels (method section II.2), and exposed for autoradiography for approximately 1 min (lanes 1). Insert DNA was cut out from the gel, and the gel re-exposed for autoradiography (lanes 2).

o represents two lower mol. wt. bands observed in both 3' and 5' end-labelled preparations.


3'
1
2
5'
1
2
– well
– plasmid
– insert
o
o

only one or two copies, respectively, of the oligonucleotide, suggesting that deletion of one or more copies of this sequence was occurring at a low frequency.

The insert band was cut out from the gel and the DNA eluted (method section IX.3). Re-exposure of the gel confirmed that the correct band had been efficiently removed (Figure 55, lanes 2). The purified end-labelled fragments were then sequenced by the method of Maxam and Gilbert (1980), (method section X.2). An example gel is shown in Figure 56. From this and other experiments it was concluded that the plasmid contained 3 tandem copies of the oligonucleotide, with the expected sequence, in the orientation shown in Figure 55 (iii). This plasmid was termed $pASO_3$.

IV.2 DNase I footprinting assay

The DNase I footprinting assay was based on the method of Galas and Schmitz (1978) and is described in method section X.3. End-labelled DNA fragments were incubated with various amounts of infected and mock-infected cell extracts and then partially digested with DNase I. The patterns of cleavage seen in the presence and absence of protein were then compared by analysis on denaturing polyacrylamide gels alongside sequence reaction markers. Proteins specifically bound to regions of the DNA would interfere with the DNase I digestion, resulting in alterations or gaps in the normal cleavage pattern.

In these experiments, two plasmids, pR9 and pR10, were used (see Figure 57). Plasmid pR10 contains an 'a' sequence derived from the L terminus plus about 200 bp of flanking 'b' sequence, orientated such that the U_c end of the 'a' sequence lies close to the Hind III site and the 'b' sequence lies

FIGURE 56

Sequence of the insert within $pASO_3$. Plasmid $pASO_3$ was 3' or 5' end-labelled at the Hind III site, and the insert DNA isolated following Eco RI digestion. Purified end-labelled fragments were sequenced as described in method section X.2, and analysed on 6% sequencing gels.

| indicates the restriction enzyme linker sequence within the oligonucleotide insert.

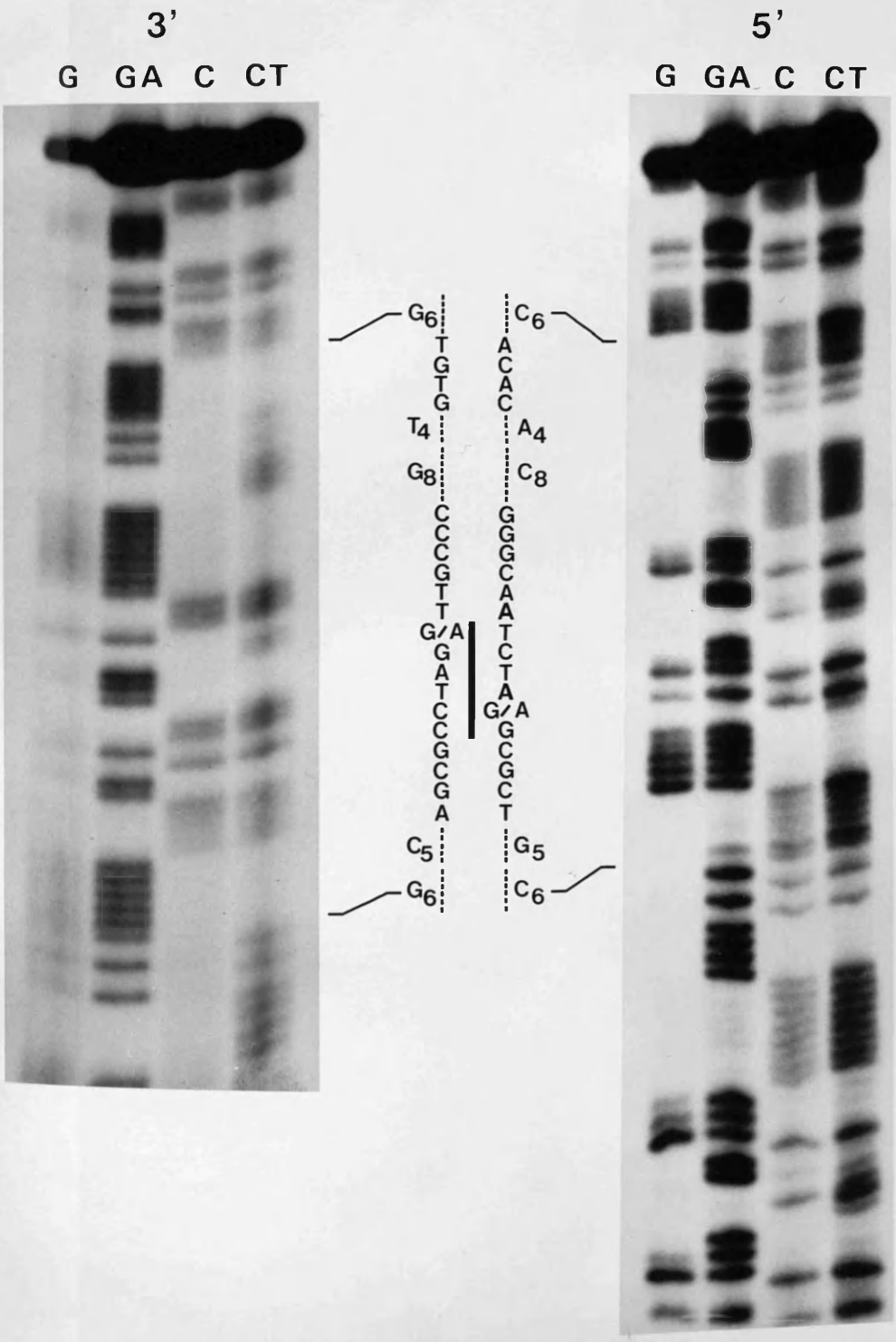
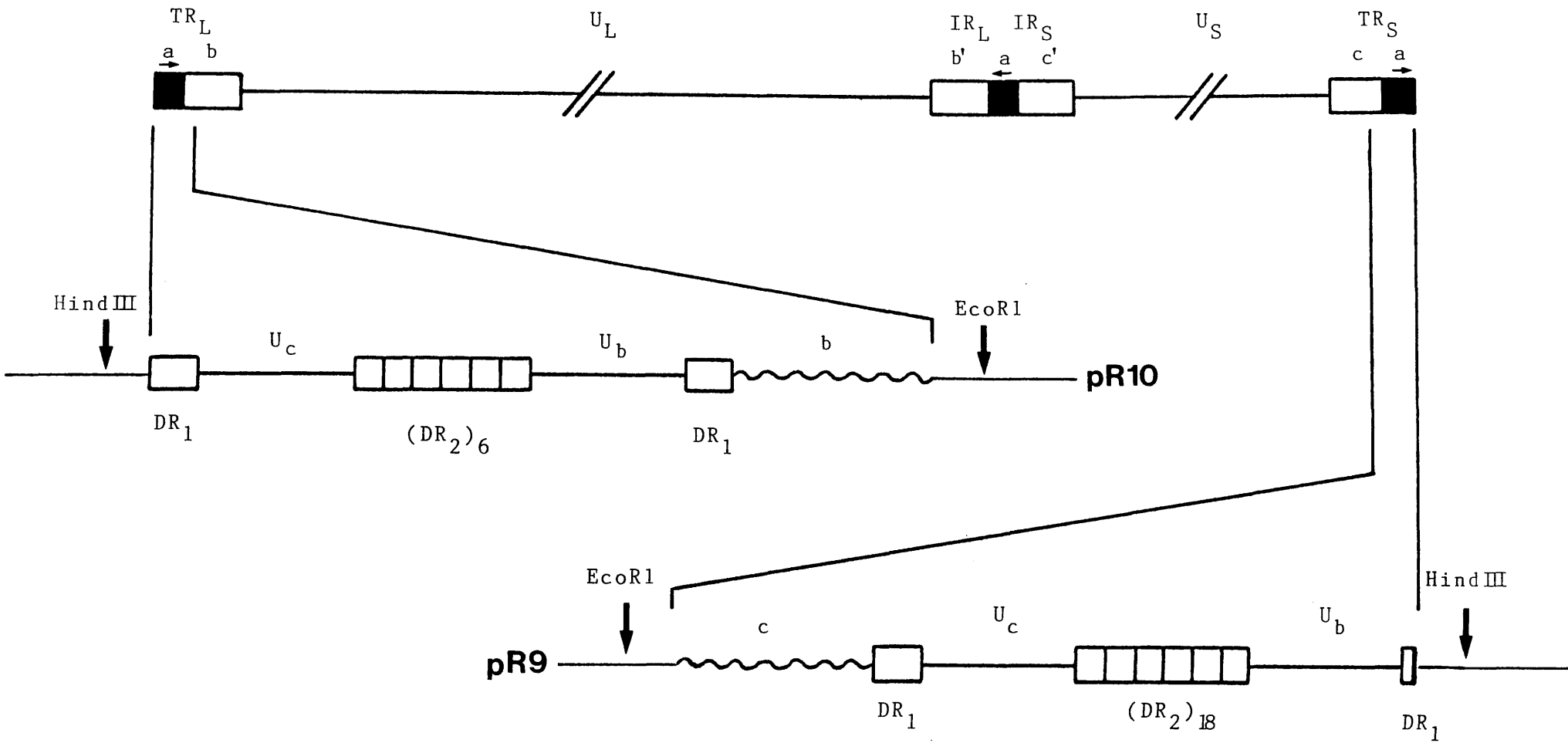

3'
G GA C CT
5'
G GA C CT
G6
T
G
T
G
T4
G8
C
C
C
G
T
T
G/A
G
A
T
C
C
G
C
G
A
C5
G6
C6
A
C
A
C
A4
C8
G
G
G
C
A
A
T
C
T
A
G/A
G
C
G
C
T
G5
C6

FIGURE 57

Schematic representation of the insert DNA contained within plasmids pR9 and pR10. Plasmid pR10 contains an 'a' sequence derived from the L terminus of HSV-1 plus about 200 bp of flanking 'b' sequence, orientated such that the U_c region lies toward the Hind III site, and the 'b' sequence lies toward the Eco RI site. Plasmid pR9 contains an 'a' sequence derived from the S terminus of HSV-1 plus about 150 bp of flanking 'c' sequence, orientated such that the U_b region lies toward the Hind III site, and the 'c' sequence lies toward the Eco RI site. Since this 'a' sequence is from the S terminus, it contains only 1 bp of the terminal DR_1 (DR_1'). Again, since the HSV-1 strain $17syn^+$ 'a' sequence contains only one copy of the DR_4 homology, it is included in the region designated U_c.


TR_L
a
b
U_L
IR_L
IR_S
b'
a
c'
U_S
TR_S
c
a
HindIII
EcoR1
U_c
U_b
b
pR10
DR_1
(DR_2)_6
DR_1
EcoR1
HindIII
c
U_c
U_b
pR9
DR_1
(DR_2)_18
DR_1

close to the Eco RI site. Thus end-labelling at the Hind III site allows protein interactions within the U_c and DR_2 repeat regions to be analysed. Plasmid pR9 contains an 'a' sequence derived from the S terminus and about 150 bp of flanking 'c' sequence, orientated such that the U_b end lies close to the Hind III site and the 'c' sequence lies close to the Eco RI site. Thus, end-labelling at the Hind III site in this plasmid allows analysis of protein interactions within the U_b and DR_2 repeat regions. Since this 'a' sequence is from the S terminus it contains only 2 bp of the terminal DR_1.

IV.3 Interpretation of the DNase I footprinting assay

Figure 58 illustrates the products generated by either a Maxam and Gilbert sequence reaction or by DNase I digestion. In the sequence reaction, the base being chemically attacked is eliminated to leave both a 5' and a 3' monophosphate group on the adjacent bases. Thus, for example, bands designated 'G' in a G sequence track will lack that terminal G residue. DNase I cleavage, in contrast, generates a 5' monophosphate and a 3' hydroxyl group, without loss of a base.

Thus, for example, a 5' end-labelled fragment containing the internal sequence 5'-pXpGpY-3', subjected to a G sequence reaction, would generate a labelled fragment of length 5'-Xp. DNase I digestion at the 5' side of the G residue would produce a similar fragment, differing only in possessing a 3' hydroxyl group, in contrast to a 3' monophosphate. These fragments were found to run almost identically, the 3' hydroxyl fragment showing a very slightly faster migration in the better resolved (lower) portion of the gel (see Figure 64). Similarly, a 3' end-labelled fragment containing the internal sequence 5'-pXpGpY-3', subjected to a G sequence reaction,

FIGURE 58

Interpretation of the DNase I footprinting assay.

(i) A hypothetical DNA fragment, containing the internal sequence 5'-pXpGpY-3' is shown (a). A Maxam and Gilbert 'G' sequence reaction would eliminate the G residue, leaving a monophosphate group on each of the adjacent bases, as shown in (b). If this DNA strand was 5' end-labelled, then DNase I digestion to the 5' side of the G residue, shown in (c), would produce a labelled fragment of similar apparent mol. wt. to that shown in (b). If the DNA strand was 3' end-labelled, DNase I digestion to the 3' side of the G residue (d) would produce an identical end-labelled fragment to that shown in (b).

(ii) A second hypothetical sequence is shown, displaying enhancement of cleavage at the dinucleotide bonds indicated (➡) in the presence of DNase I. (For simplicity, enhancement rather than protection of cleavage is shown). As mentioned above, Maxam and Gilbert sequence reactions generate fragments of DNA which lack the nucleotide being chemically attacked. Thus, DNase I digestion at a given dinucleotide bond will generate a labelled DNA fragment of the same length as the sequence marker of the nucleotide distal to the label - ie. on a 3' end-labelled strand it will be equivalent to the 5' partner, and on a 5' end-labelled strand, to the 3' partner. This is illustrated diagramatically in the sequence gels shown.

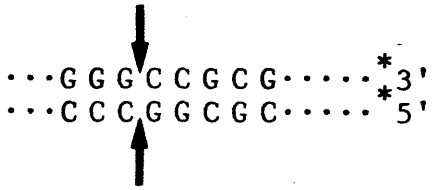
(i) a. 5'——————pXpGpY——————3'

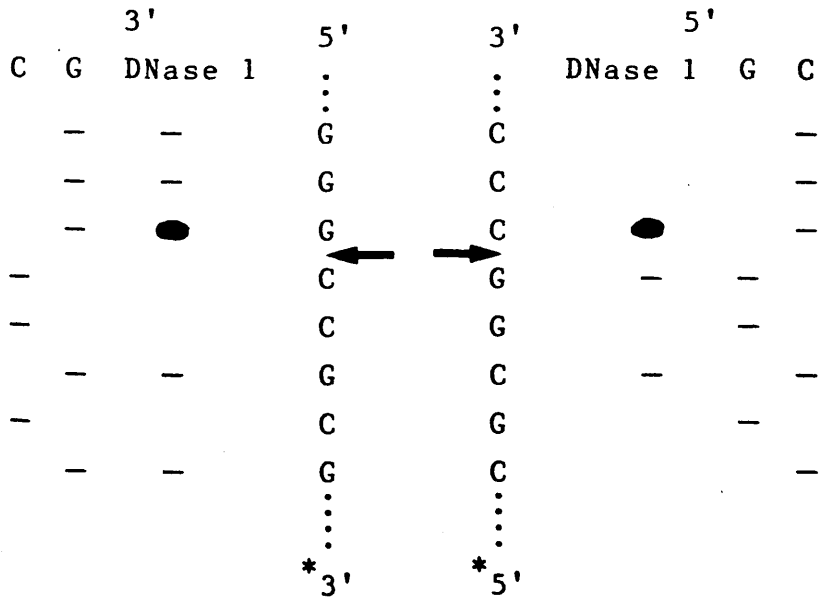
b. 5'——————pXp pY——————3'

c. 5'——————pX pGpY——————3'

d. 5'——————pXpG pY——————3'

(ii)





would generate a labelled fragment of length pY-3'. DNase I digestion at the 3' side of the G residue would produce an identical fragment. Thus, by aligning the DNase I digestion pattern with a sequence marker the dinucleotide bond being broken can be identified.

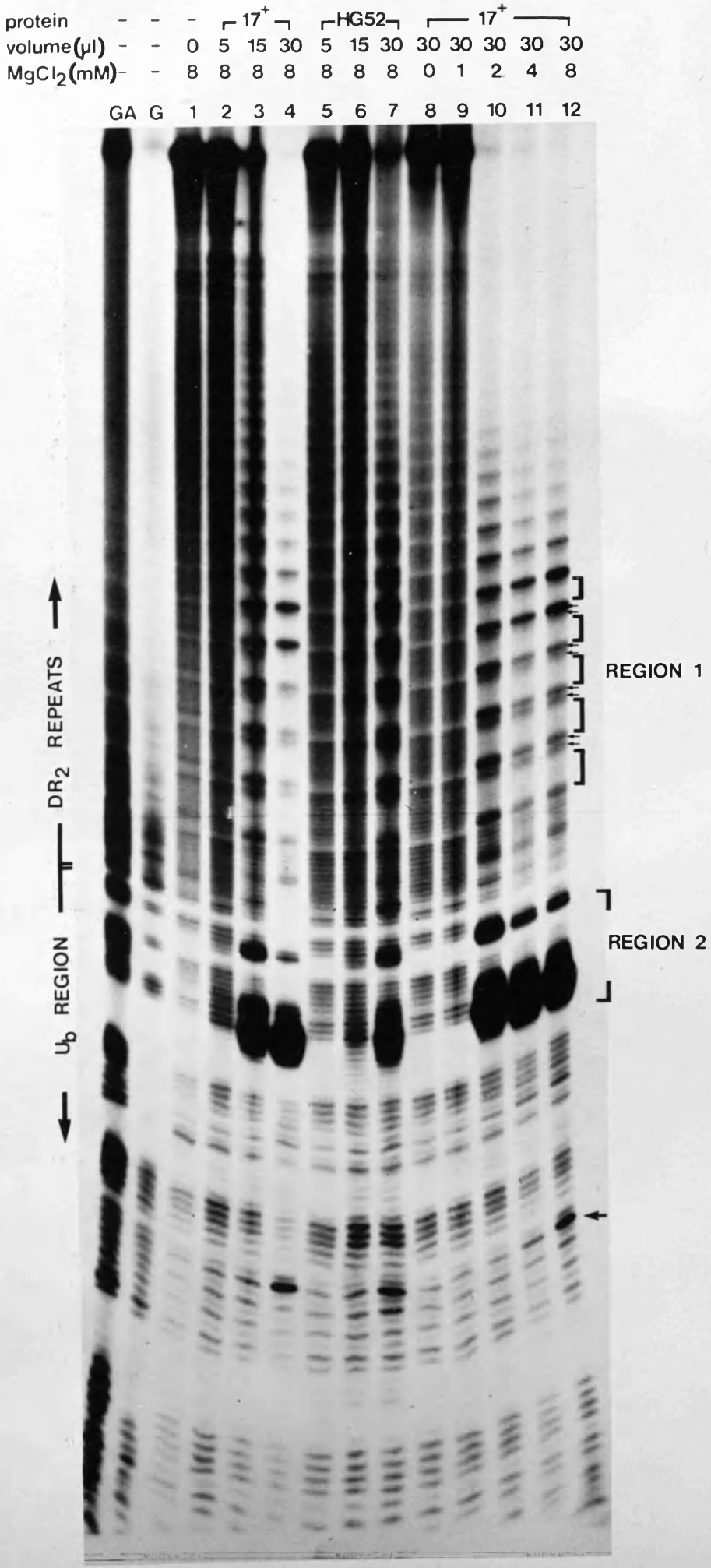
IV.4 Infected cell extracts alter the DNase I digestion pattern of the HSV-1 'a' sequence

The DNase I digestion pattern within U_b and the DR_2 repeats is altered in the presence of infected cell extracts (Figure 59). Within the DR_2 repeat region a repetitive pattern is seen, consisting of apparently protected regions spanning approximately 7 nucleotides (indicated by brackets) flanked on either side by two nucleotides showing enhanced cleavage (indicated by small arrows). Between each of these repeated elements is one apparently unaffected nucleotide. This area has been termed Region 1. A number of bands within U_b show a very marked increase in sensitivity to DNase I. In some experiments, but not all, some protection can be seen around this region (compare, for example, lanes 1,4 and 7). This area has been termed Region 2. Region 1 and Region 2 are shown more clearly in Figure 60. There was no evidence to suggest that any sequence-specific DNA-protein interactions were occurring within U_c, under our assay conditions (results not shown).

In the experiments of Dalziel and Marsden (1984), 21K bound to the 'a' sequence in the presence of B2 buffer. Therefore, our original DNase I footprinting assays were carried out in B2 buffer containing additionally 8mM $MgCl_2$, 8ug/ml calf thymus DNA and 0.1mg/ml BSA. However, in subsequent experiments we altered the conditions in an attempt

FIGURE 59

DNase I footprinting on the HSV-1 'a' sequence. The Eco RI/Hind III fragment from plasmid pR9, 3' end-labelled at the Hind III site, was subjected to DNase I footprinting as described in method section X.3, except that the $MgCl_2$ concentration used was either 8 mM (lanes 1-7) or varied (lanes 8-12). End-labelled DNA was incubated in the absence (lane 1) or presence of HSV-1-infected (lanes 2-4, 8-12) and HSV-2-infected (lanes 5-7) cell extracts. The volume of extract added, and the final concentration of $MgCl_2$ in the reaction buffer, are indicated. Maxam and Gilbert sequence reactions (GA, G) are shown for alignment, and the relative positions of the DR_2 repeat and U_b regions are shown. Changes observed in the presence of extract within the DR_2 repeat region are designated Region 1. Areas of protection are indicated by brackets (]), while apparently enhanced bands are indicated by the small arrows (→). Changes observed within the U_b region are designated Region 2. The large arrow (➡) indicates a band enhanced only in the presence of 8 mM $MgCl_2$. Samples were analysed on a 6% sequencing gel.


protein - - - 17+ HG52 17+
volume(µl) - - 0 5 15 30 5 15 30 30 30 30 30 30
MgCl2(mM) - - 8 8 8 8 8 8 8 0 1 2 4 8
GA G 1 2 3 4 5 6 7 8 9 10 11 12
REGION 1
REGION 2
DR2 REPEATS
Ub REGION

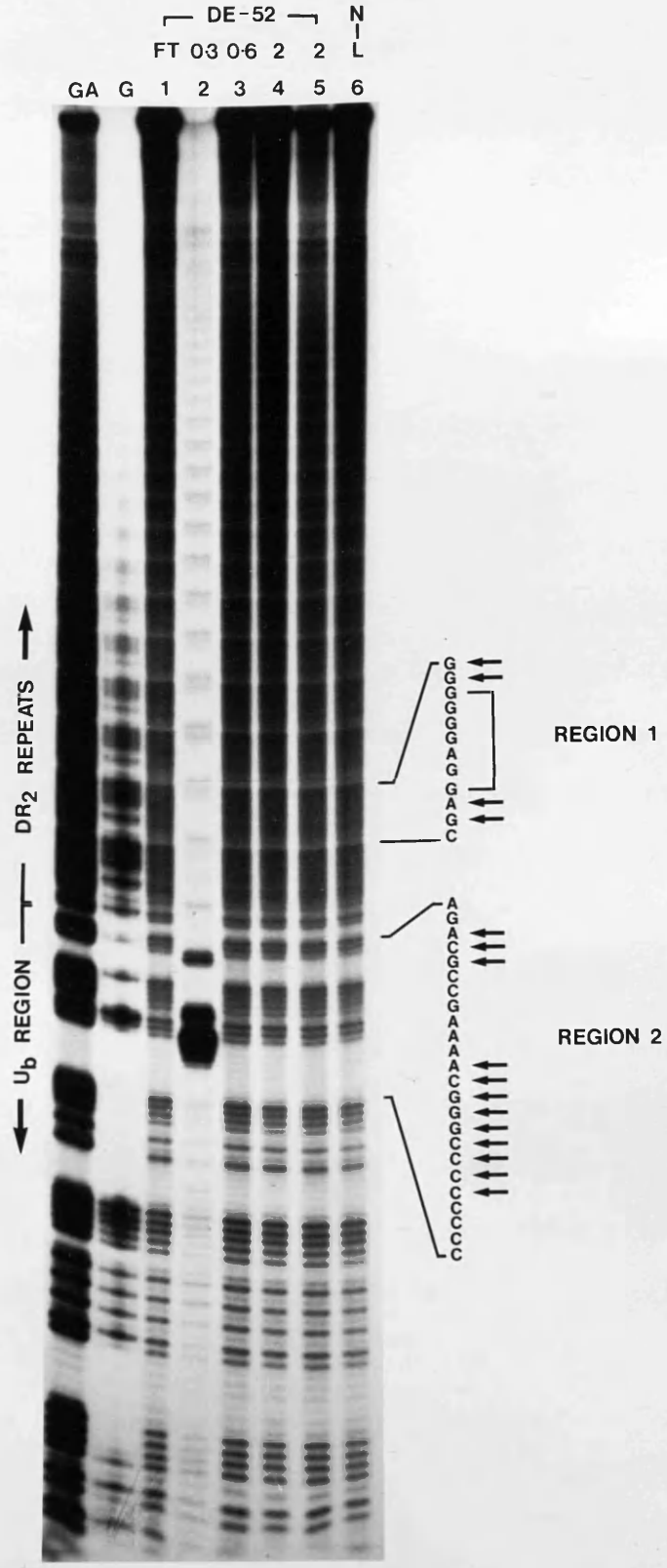

DE-52
N
L
FT 0.3 0.6 2 2
GA G 1 2 3 4 5 6
DR_2 REPEATS
U_b REGION
REGION 1
REGION 2

FIGURE 60

Altered DNase I digestion pattern in the presence of HSV-1-infected cell polypeptides. An HSV-1-infected cell extract was applied to a DE-52 cellulose column in B2 buffer, and fractions collected into 0.5 inhibitory units of α_2-macroglobulin. After collecting the flow-through (FT), the column was washed and bound protein eluted stepwise with 0.3M, 0.6M and 2M NaCl. Fractions were dialysed against B2 buffer before use.

The Eco RI/Hind III fragment from plasmid pR9, 3' end-labelled at the Hind III site, was incubated with either no protein (lane 6) or 30 ul of the DE-52 purified protein fraction indicated. Samples were analysed on a 6% sequencing gel. GA and G sequence reactions serve as marker tracks, and the position of the DR_2 repeat and U_b regions are indicated. The DNA sequences around Region 1 and Region 2 are shown. Arrows represent nucleotides apparently enhanced in cleavage at their 3' side, while the bracket represents nucleotides protected from DNase I digestion.

to optimise for the effects we had observed (Figure 59, and results not shown). These latter experiments showed that the reaction is absolutely dependent on the presence of Mg^{2+} ions, being optimal at 2mM $MgCl_2$. At concentrations much higher than 10mM $MgCl_2$ there was a significant loss of recoverable counts. Neither Mn^{2+} nor Ca^{2+} could replace the Mg^{2+} ions. Raising the salt concentration to 100mM, using either NaCl or KCl, significantly inhibited the reaction. The reaction also appeared to be dependent on the presence of a reducing agent, the level of 2-mercaptoethanol being optimal at 1-3mM. The level of competitor calf thymus DNA could be increased to a final concentration of 20ug/ml. This resulted in a significant increase in the recovery of counts (ie. precipitable end-labelled DNA) without affecting the DNase I digestion pattern seen. However, at 60ug/ml the reaction was inhibited. Incubation for 5 min at 37°C was sufficient for the reaction to occur, with longer incubations resulting in significant degradation of the DNA. The reaction would not occur at 4°C, even after 30 min incubation. However, following incubation at 37°C samples could be left at 4°C without significantly affecting the results, for at least 15 min. Thus, 4°C could be used as a 'stop' for the DNase I footprinting reactions. Where possible, extracts were prepared and used while fresh. However, samples could be kept at -70°C for up to 6 weeks and freeze-thawed at least twice with about only a 50% reduction in the level of activity. Incubation at 37°C for 30 min resulted in a significant loss of activity.

Therefore, in all experiments shown, with the exception of Figure 59, the assays were carried out as described in method section X.3. The alterations to the reaction conditions did not result in a change in the DNase I digestion pattern

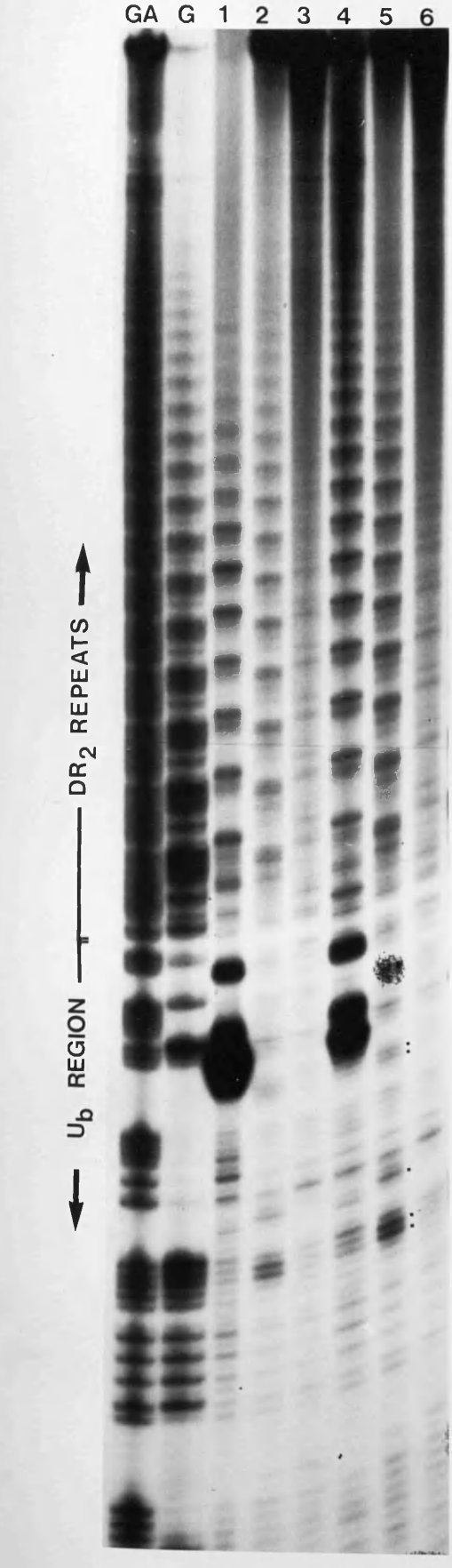
seen, with one minor exception. An additional band within U_b (indicated by the heavy arrow in Figure 59) was consistently found to be enhanced in the presence of 8mM $MgCl_2$. However, in the presence of 2mM $MgCl_2$ this band was no longer enhanced (compare lanes 10 and 12). In contrast, with 2mM $MgCl_2$ there appeared to be an even greater level of enhancement in Region 2, within U_b. The reason for this difference in $MgCl_2$ sensitivity is unknown.

IV.5 Extracts from HSV-2-infected cells, but not mock-infected cells, produce the same DNase I footprint as the HSV-1-infected cell extracts

Extracts from cells infected with HSV-2 generated an identical DNase I digestion pattern to HSV-1-infected cell extracts (Figure 59, lanes 5-7), both with respect to the protection in the DR_2 repeats and the enhanced cleavage seen within U_b. In contrast, mock-infected cell extracts did not induce this change, but instead resulted in a different pattern of cleavage in the DR_2 repeats (Figure 61). There was no enhancement in Region 2, although some bands within U_b appeared to be slightly enhanced in cleavage. This result is shown more clearly in Figures 62 and 63. Thus, the reaction observed with the infected cell extracts is specific for virus-infected cells.

IV.6 Alterations in the DNase I digestion pattern of the lower strand

So far, only results for the pR9 insert 3' end-labelled at the Hind III site have been shown. This represents the upper strand with respect to the 5'-3' sequence shown in Figure 63. Therefore, to investigate interactions on the


protein – – 17+ MI – 17+ MI –
volume (µl) – – 30 30 – 15 30 –
GA G 1 2 3 4 5 6
Ub REGION
DR2 REPEATS

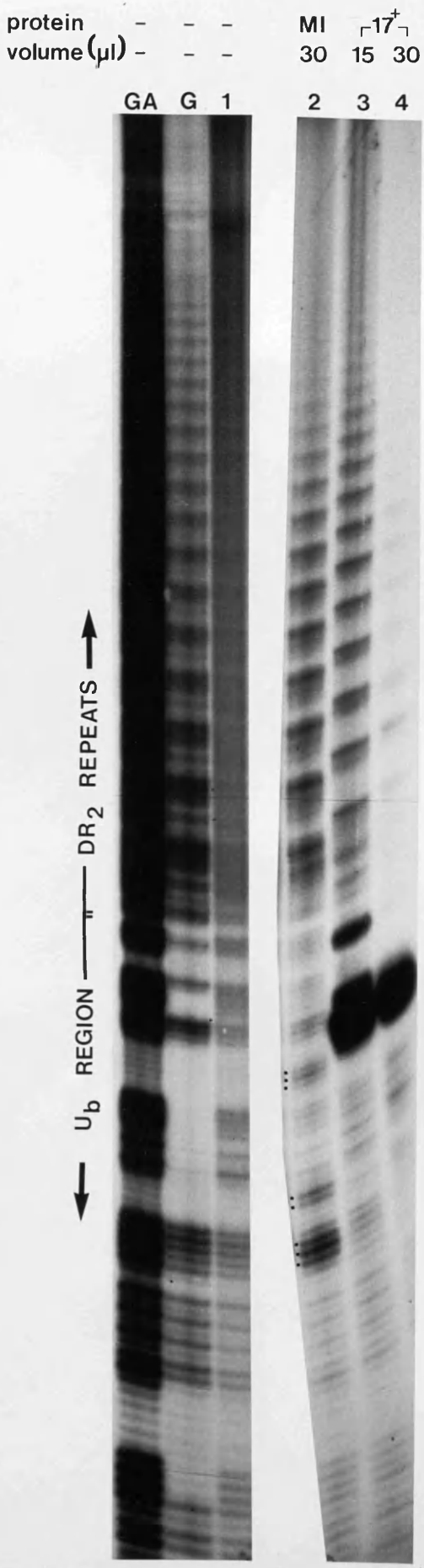

protein – – – MI 17+
volume (µl) – – – 30 15 30
GA G 1 2 3 4
Ub REGION
DR2 REPEATS

FIGURE 61

DNase I digestion pattern in the presence of mock-infected cell extracts. The Eco RI/Hind III fragment from plasmid pR9, end-labelled at the Hind III site, was incubated with 15 ul or 30 ul of either mock-infected or HSV-1-infected cell extract, as indicated. Samples were analysed on a 6% sequencing gel. GA and G sequence reactions serve as markers, and the position of the DR_2 repeat and U_b regions are shown. Bands within U_b apparently enhanced in cleavage in the presence of mock-infected cell extracts are indicated (▪).

FIGURE 62

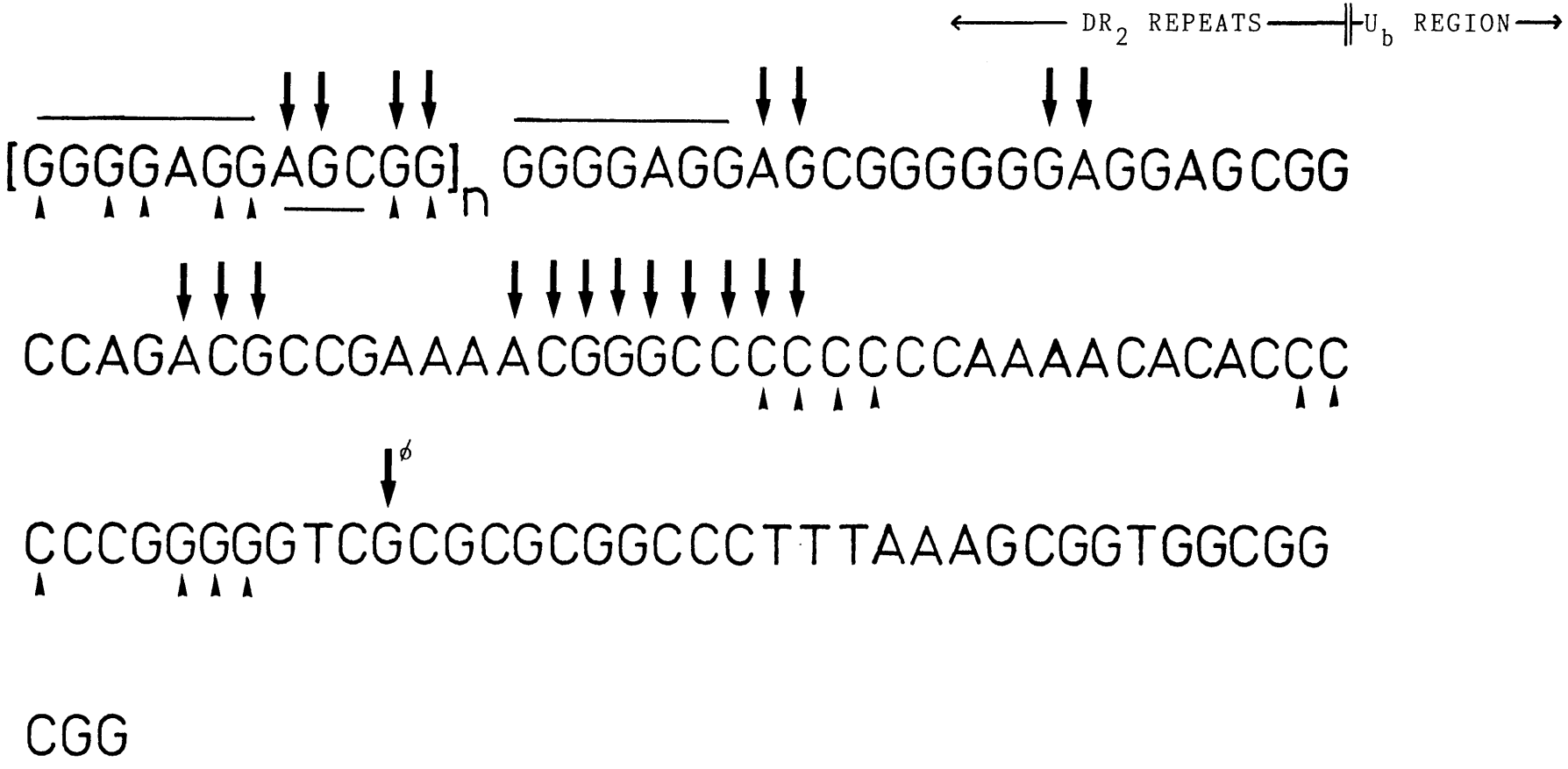
Comparison of the DNase I footprint generated by infected and mock-infected cell extracts. The Eco RI/Hind III fragment from pR9, 3' end-labelled at the Hind III site, was incubated with either no protein (-), 30 ul of mock-infected cell extract (MI), or 15 ul or 30 ul of HSV-1-infected cell extract (17^+). Samples were analysed on a 6% sequencing gel. GA and G sequence reactions serve as markers, and the position of the DR_2 repeat and U_b regions are indicated. Part of the DNA sequence within U_b and DR_2 is presented - alterations in the DNase I digestion pattern generated by mock-infected cell extracts are indicated to the left (MI), while those generated by infected cell extracts are indicated to the right (17^+). Nucleotides apparently enhanced in cleavage to their 3' side in the presence of mock-infected (▪ , ⟶) or infected (➡) cell extracts, are indicated. Nucleotides apparently protected from DNase I digestion are indicated by brackets (]).

GA G - MI
17+
30 15 MI - G GA
MI 17+
Ub REGION DR2 REPEATS
Ub REGION DR2 REPEATS
GGGGGGAGGAGC
AGACGCCGAAAACGGGCCCCCCCCCCAAAACACACCCCCCCCGGGGGTC

FIGURE 63

DNase I digestion patterns of mock-infected and infected cell extracts. The nucleotide sequence of the DR_2/U_b region of the 'a' sequence is shown. This is adapted from, and in the opposite orientation to, that shown in Davison and Wilkie (1981), and represents the top strand, in a 5'-3' direction, of the 'a' sequence present in plasmid pR9. Those nucleotides on this strand enhanced in cleavage at their 3' side in the presence of infected (➡) or mock-infected (➡) cell extracts are indicated. The overline represents those nucleotides apparently protected from DNase I digestion in the presence of infected cell extracts; the underline represents those nucleotides apparently protected from DNase I digestion in the presence of mock-infected cell extracts.

G^{ϕ} nucleotide enhanced in cleavage at its 3' side only in the presence of 8 mM $MgCl_2$.


← DR2 REPEATS ⫴ Ub REGION →
[GGGGAGGAGCGG]n GGGGAGGAGCGGGGGGGAGGAGCGG
CCAGACGCCGAAAACGGGCCCCCCCCAAAACACACCC
ϕ
CCCGGGGGTCGCGCGCGGCCCTTTAAAGCGGTGGCGG
CGG

lower strand, the plasmid was 5' end-labelled at the Hind III site (Figure 64). DNase I footprinting on 5' end-labelled fragments has proved relatively unsuccessful, due apparently to the presence of an endogenous 5'-3' exonuclease activity within the extracts. However, long exposures of such footprints (lanes 7 and 8) suggest that there is little additional activity on the lower strand. Only one band appears to be significantly enhanced (indicated by the heavy arrow) although two additional bands appear to be weakly enhanced (indicated by the open circles).

IV.7 DNase activity within the whole cell extract

The high level of nuclease activity within the infected cell extracts has been a consistent problem. The question of whether the enhanced cleavage seen in Region 2 is due to a specific cleavage event generated by a protein, or proteins, within the infected cell extract, or whether it is simply enhancement of cleavage by DNase I, cannot be answered using crude whole cell extracts. Incubation of 3' end-labelled DNA with infected cell extracts produces an identical footprint in the presence or absence of DNase I (Figure 65, compare lanes 2 and 4, and 5 and 7), suggesting that a DNase I-like activity resides within the extract.

IV.8 Partial purification of the DNase I footprinting activity

To identify the protein(s) involved in this interaction and to separate them from the endogenous nuclease activity, a number of purification procedures were attempted. Infected cell extracts were applied in B2 buffer to DEAE-cellulose (DE-52), phosphocellulose and heparin-Sepharose and then eluted stepwise with 0.3M, 0.6M and 2M NaCl. The activity

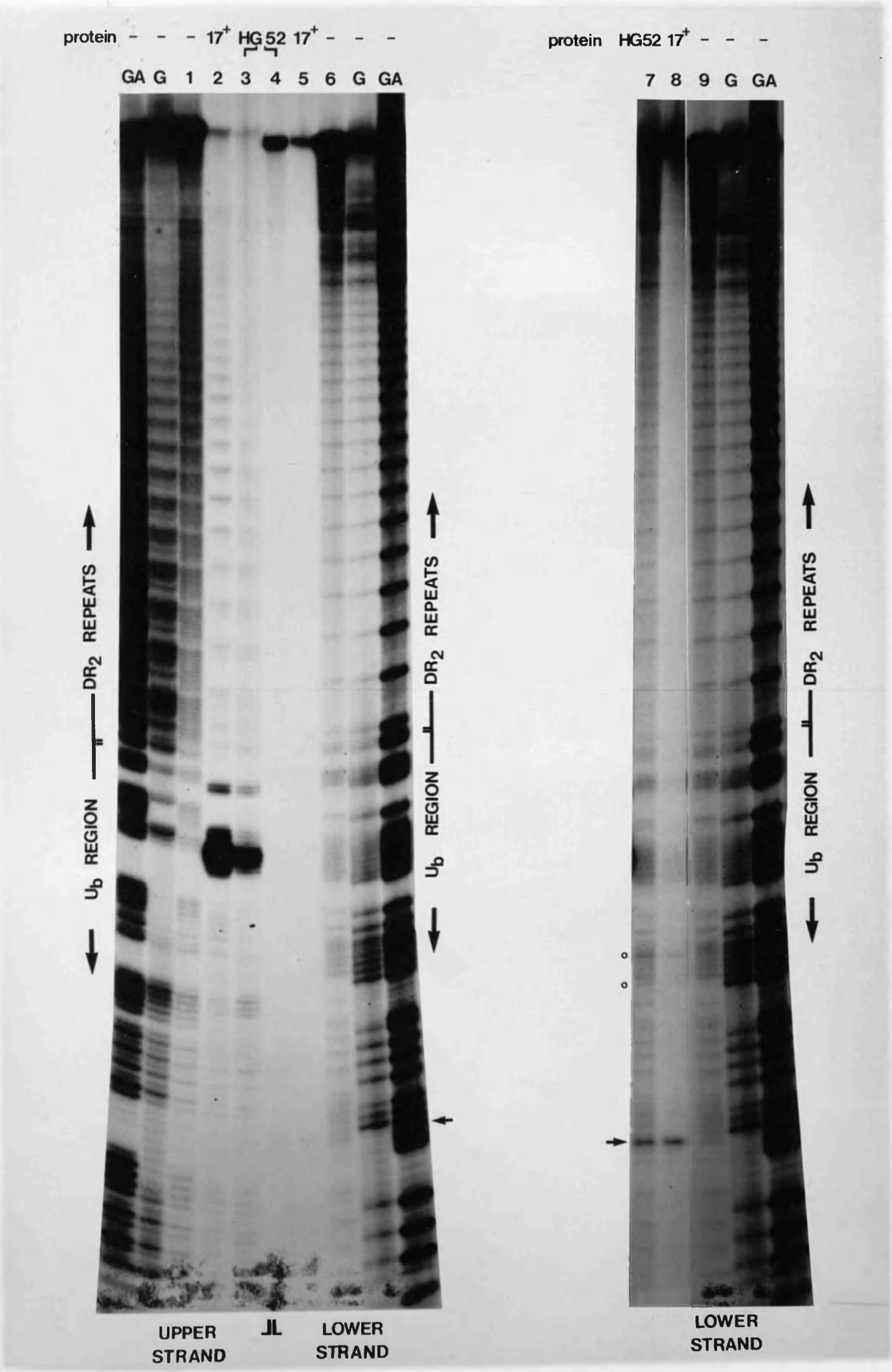
FIGURE 64

Alterations in the DNase I digestion pattern of the lower strand in the presence of infected cell extracts. Eco RI/ Hind III fragments from plasmid pR9, 3' (upper strand) or 5' (lower strand) end-labelled at the Hind III site, were incubated with either no protein (-), or with 30 ul of HSV-1 (17^+) or HSV-2 (HG52) -infected cell extracts. Samples were analysed on 6% sequencing gels. GA and G sequence reactions serve as markers, and the positions of the DR_2 and U_b regions are indicated.

➔ a nucleotide on the lower strand apparently enhanced in cleavage at its 5' side.

o nucleotides on the lower strand possibly enhanced in cleavage at their 5' side.

Lanes 7 and 8 represent a longer exposure of lanes 4 and 5; lane 9 is the same as lane 6.


protein - - - 17+ HG 52 17+ - - -
GA G 1 2 3 4 5 6 G GA
Ub REGION
DR2 REPEATS
UPPER STRAND
LOWER STRAND
protein HG52 17+ - - -
7 8 9 G GA
LOWER STRAND

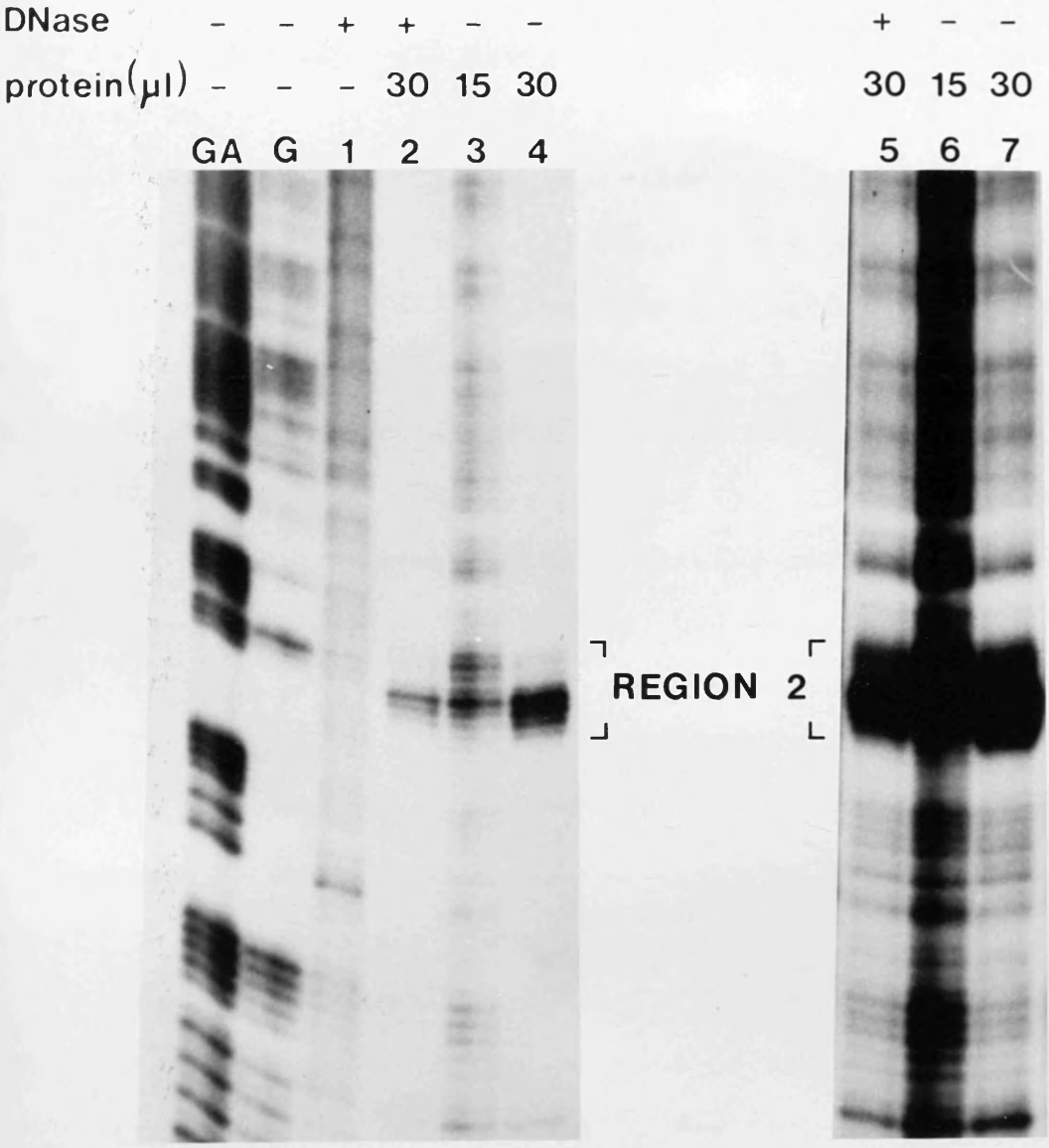

DNase - - + + - - + - -
protein(μl) - - - 30 15 30 30 15 30
GA G 1 2 3 4 5 6 7
REGION 2

FIGURE 65

Effect of endogenous DNase I on the pattern of digestion. Eco RI/Hind III fragments, 3' end-labelled at the Hind III site, were incubated with an HSV-1-infected cell extract and then either treated (+) or not treated (-) with DNase I. Samples were analysed on a 6% sequence gel. GA and G sequence reactions serve as markers. Only a portion of the gel is shown - the DR_2 repeats occupy approximately the top third of the gel, and Region 2 within U_b is indicated. Lanes 5-7 represent a longer exposure of lanes 2-4.

was recovered entirely within the 0.3M eluate from DE-52 (Figure 60), appeared to be lost by passage through phosphocellulose, and was recovered predominantly in the 0.6M eluate from heparin-Sepharose (Figure 66A). However, the majority of proteins eluted in these fractions (results not shown).

An HSV-1-infected cell extract was fractionated on DNA cellulose, as described in result section III.3, by Dr Graham Hope, and the eluted fractions were then assessed for activity in the DNase I footprinting assay (Figure 66B). The activity was found to spread broadly across the gradient, being most predominant in fractions 1 and 9. Fraction 9 was the purest fraction with respect to protein content, and had a low level of endogenous nuclease activity (Figure 66 and results not shown). This fraction was therefore further purified using an FPLC mono Q anion exchange column. Despite reasonable recovery of most proteins, the DNase I footprinting activity was lost (results not shown).

Loss of the DNase I footprinting activity could be due to a number of factors. Firstly, the assay is relatively insensitive and therefore significant dilution of the activity would render it undetectable. Secondly, the activity is unstable, and may have become denatured or degraded during chromatography. Thirdly, the activity may involve more than one protein, and these proteins may have become separated by the chromatographic procedures.

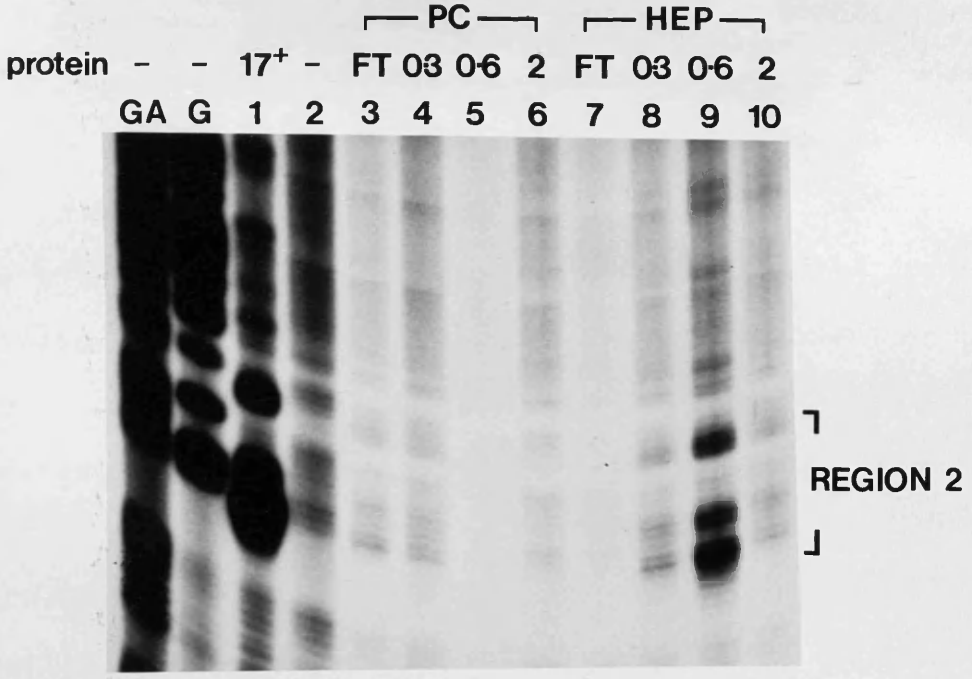
IV.9 Role of the 21K polypeptide in the DNase I footprinting activity

The 21K polypeptide is known to bind to the 'a' sequence and is therefore a potential candidate for the DNase I footprinting activity. To further investigate which protein

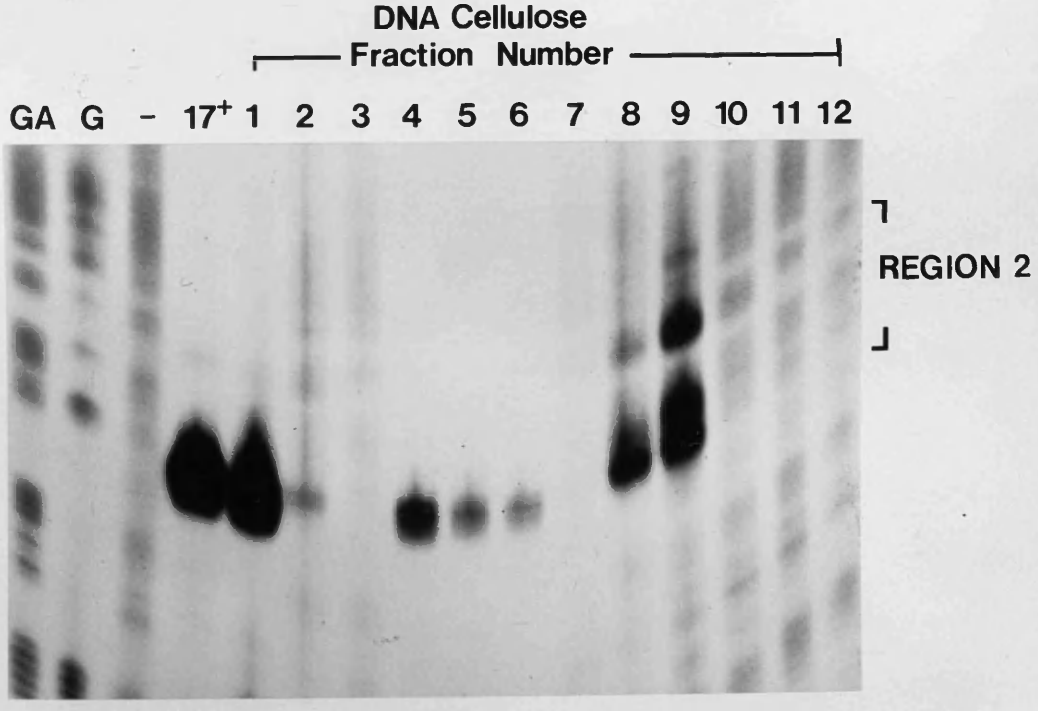
FIGURE 66

Partial purification of the DNase I footprinting activity. HSV-1-infected cell extracts were applied to either phosphocellulose (PC), heparin-Sepharose (HEP) or DNA cellulose columns. Flow-through (FT) material was collected and bound proteins were eluted either stepwise with 0.3M, 0.6M and 2M NaCl (1.5 ml fractions being collected into 0.5 inhibitory units of α_2-macroglobulin), or, for the DNA cellulose, with a linear 50-900 mM NaCl gradient. Following dialysis against B2 buffer, 30 ul of each fraction was incubated with the Eco RI/Hind III fragment of plasmid pR9, 3' end-labelled at the Hind III site. Only the relevant portion of the gel is shown, and GA and G sequence reactions serve as mol. wt. markers. Samples were analysed on 6% sequencing gels.

A


PC
HEP
protein - - 17+ - FT 0·3 0·6 2 FT 0·3 0·6 2
GA G 1 2 3 4 5 6 7 8 9 10
REGION 2

B


DNA Cellulose
Fraction Number
GA G - 17+ 1 2 3 4 5 6 7 8 9 10 11 12
REGION 2

or proteins were involved in this activity, and specifically to determine whether the 21K DNA-binding protein played a role, we first attempted to inhibit the interaction using various antisera. The IgG was purified from a general anti-HSV-1 antiserum and also from the antisera raised against the amino- and carboxy-terminus of 21K. None of these antibodies inhibited the reaction (results not shown).

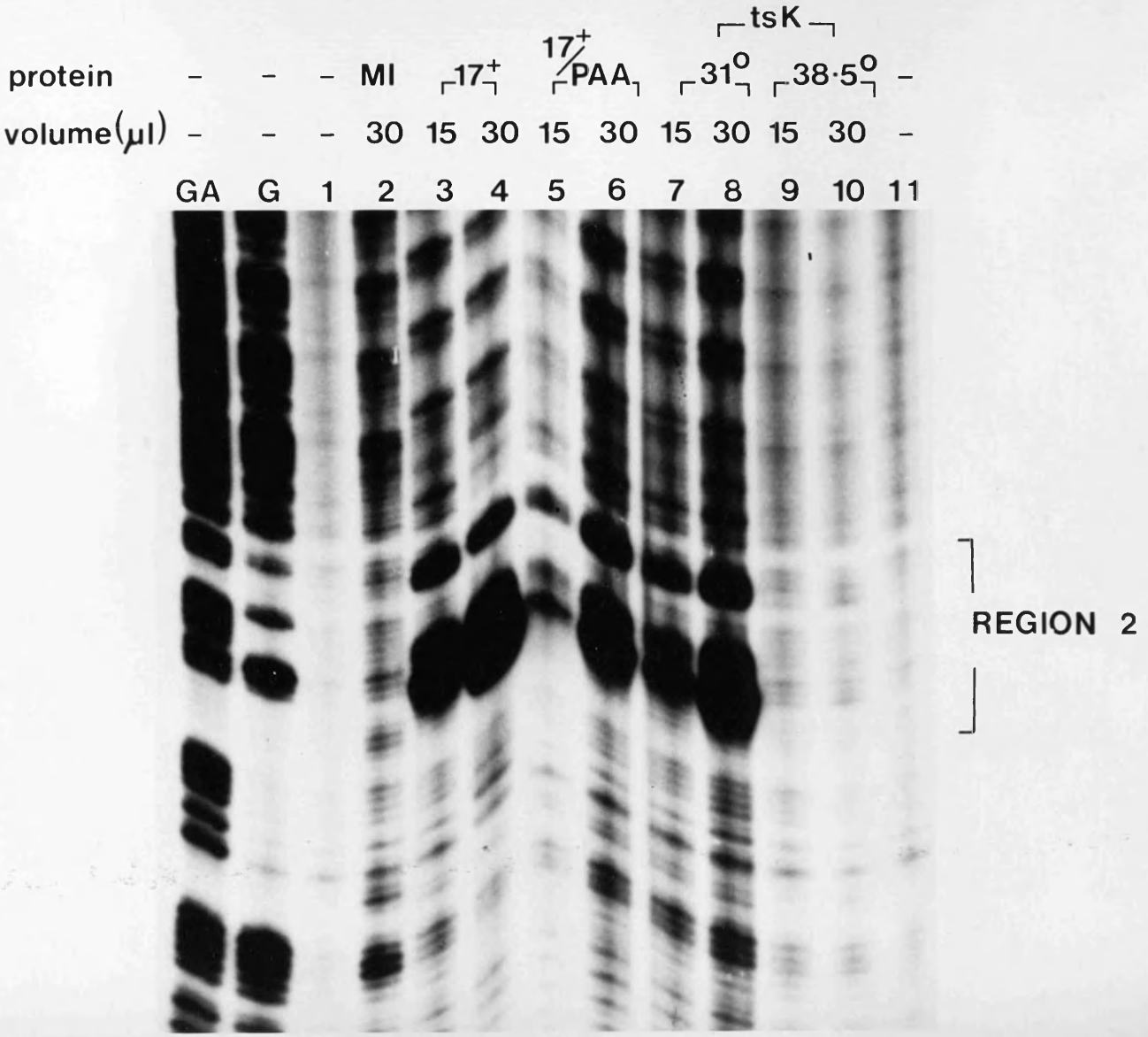
We next studied the timecourse of appearance of the DNase I footprinting activity. The temperature-sensitive mutant, tsK, defective in the IE protein Vmw175, overproduces IE polypeptides but fails to synthesise early and late polypeptides, at the non-permissive temperature. This virus did not induce the DNase I footprinting activity at the non-permissive temperature, although it clearly did so at the permissive temperature (Figure 67), suggesting that, with the possible exception of Vmw175, this activity does not involve an IE polypeptide alone.

Treatment of infected cells with phosphonoacetic acid (PAA) inhibits viral DNA replication and thus significantly reduces the synthesis of true late proteins. Figures 67 and 68 show that treatment with PAA does not significantly inhibit the appearance of the DNase I footprinting activity, reducing this activity by only approximately 2-fold (compare lanes 3 and 5 in Figure 68), suggesting that this does not require a true late function and arguing against the involvement of 21K. This conclusion was supported by the observation that, as expected, PAA inhibited the synthesis of, among others, the 21K polypeptide in this experiment (results not shown).

An HSV-2 mutant has recently been isolated which has a large deletion from the right hand end of the short region and lacks the U_S11 gene equivalent (Brown and Harland, 1987).

FIGURE 67

Investigation of the temporal class of viral polypeptides involved in the DNase I footprinting activity. Eco RI/ Hind III fragments from plasmid pR9, 3' end-labelled at the Hind III site, were incubated with either no protein (lanes 1 and 11), or with extracts from mock-infected cells (lane 2), cells infected with HSV-1 alone (lanes 3 and 4) or in the presence of PAA (lanes 5 and 6), or cells infected with the temperature sensitive mutant tsK at either the permissive (lanes 7 and 8) or non-permissive (lanes 9 and 10) temperature. Samples were analysed on a 6% sequencing gel. Only the relevant portion of the gel is shown. GA and G sequence reactions serve as markers, and Region 2 is indicated.


protein
volume(µl)
MI
17+
17+/PAA
tsK
31°
38·5°
GA
G
1
2
3
4
5
6
7
8
9
10
11
REGION 2

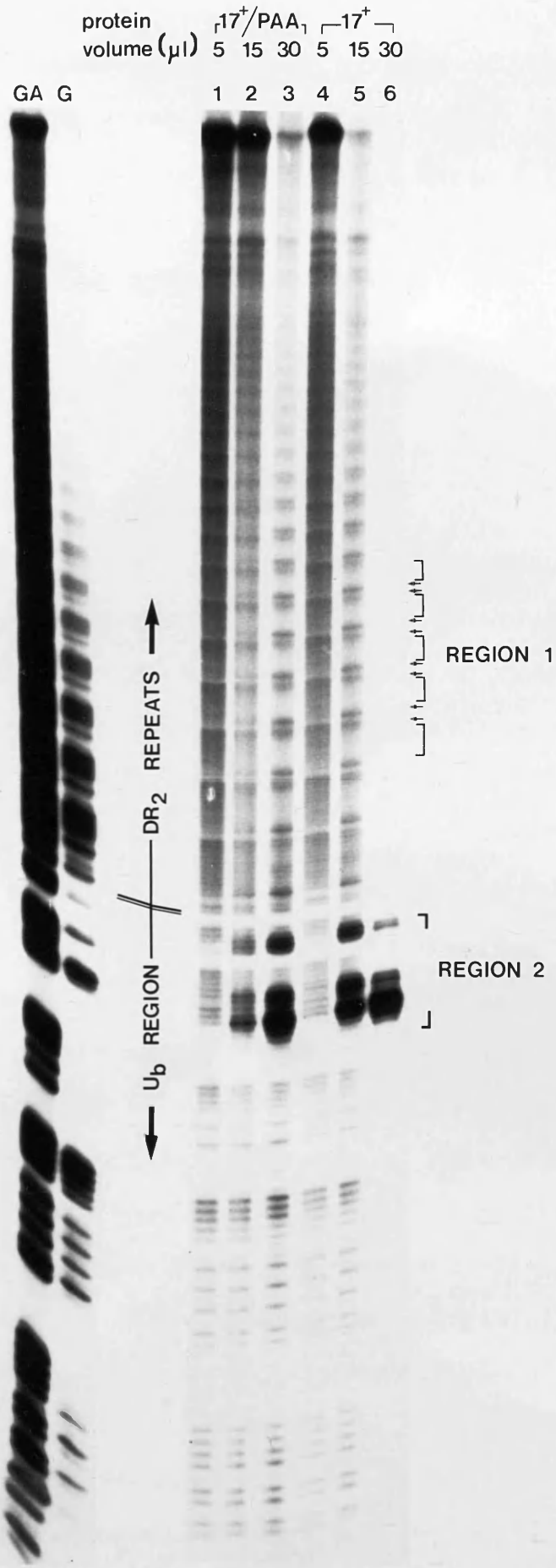

protein
volume (µl)
17+/PAA
17+
5 15 30 5 15 30
GA G
1 2 3 4 5 6
REGION 1
REGION 2
DR2 REPEATS
Ub REGION

FIGURE 68

DNase I digestion pattern with early and late infected cell extracts. Eco RI/Hind III fragments from plasmid pR9, 3' end-labelled at the Hind III site, were incubated with 5, 15 or 30 ul of extracts from cells infected with HSV-1 in the presence (lanes 1-3) or absence (lanes 4-6) of PAA. Samples were analysed on a 6% sequencing gel. GA and G sequence reactions serve as markers, and the positions of the DR_2 and U_b regions are indicated.

Extracts were prepared from cells infected with this mutant (X12). These produced an identical pattern of cleavage to the HSV-2 wild-type virus, HG52 (Figure 69).

In conclusion, the presence of this DNase I footprinting activity would not appear to involve the product of gene U_S11, or any other true late gene product.

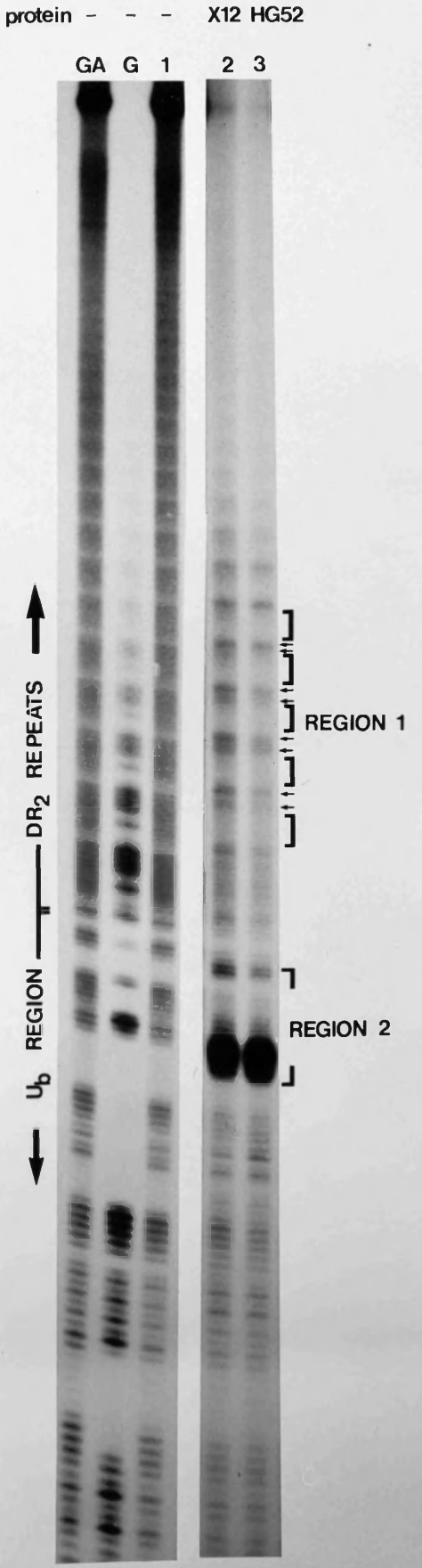
IV.10 Involvement of the U_b homology region

(a) DNase I footprinting with plasmid $pASO_3$

The HSV-1 and HSV-2 'a' sequences are heterogeneous and contain only short stretches of homology (Davison and Wilkie, 1981). However, the most extensive region of homology lies within U_b and overlaps the region (Region 2) in which enhancement of cleavage is evident. Therefore, since both HSV-1 and HSV-2-infected cell extracts generate enhancement of cleavage, it is possible that the U_b homology region may be involved in the interaction. A synthetic oligonucleotide representing the homology region was synthesised (see Figure 54). The original intention (not subsequently attempted) was to use this oligonucleotide to prepare an affinity column to purify sequence-specific binding proteins. Therefore, we attempted to clone multimers of the oligonucleotide. One plasmid, termed $pASO_3$, contained 3 tandem repeats of this sequence, linked head-to-tail (result section IV.1, Figure 54). Inserts either 3' or 5' end-labelled at the Hind III site were isolated and tested in DNase I footprinting assays (Figure 70). Incubation with HSV-1-infected cell extracts resulted in the enhancement of cleavage of certain bands, with no obvious signs of protection. Mock-infected cell extracts did not produce this pattern. Again, enhancement was only visible on one strand. The

FIGURE 69

DNase I digestion pattern with X12-infected cell extracts. Eco RI/Hind III fragments from plasmid pR9, 3' end-labelled at the Hind III site, were incubated with either no protein (lane 1), or with 30 ul of extract from cells infected with HSV-2 strain HG52 (lane 3), or the HSV-2 mutant X12 (lane 2), which lacks (among others) gene U_S11. Samples were analysed on 6% sequencing gels. GA and G sequence reactions were used as markers, and the relative positions of the DR_2 repeat and U_b regions are indicated.


protein - - - X12 HG52
GA G 1 2 3
REGION 1
REGION 2
U_b REGION
DR_2 REPEATS

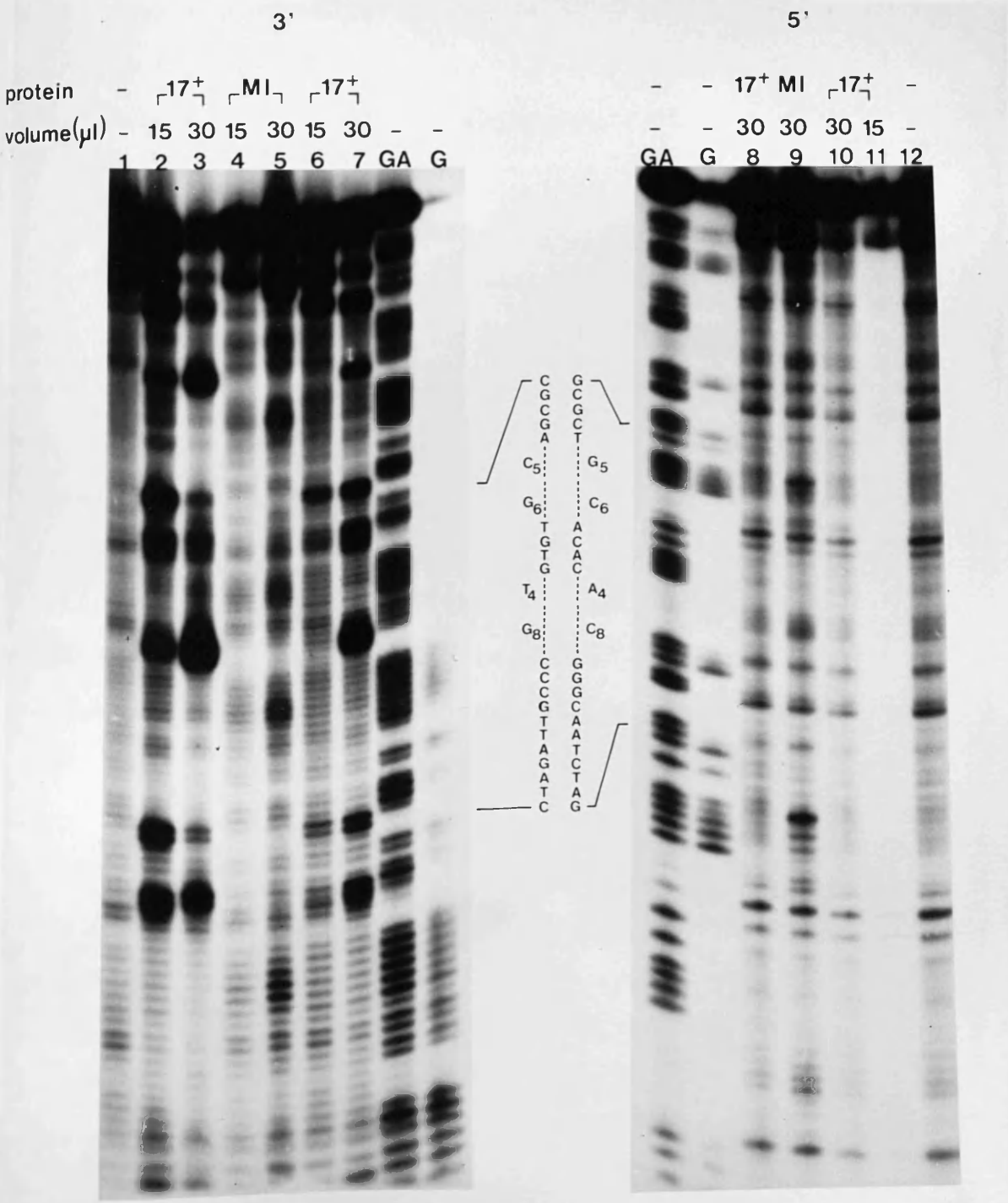

3'
5'
protein
volume(µl)
17+
MI
17+
1 2 3 4 5 6 7 GA G
GA G 8 9 10 11 12
C G C G A
C5
G6
T G T G
T4
G8
C C C G T T A G A T C
G C G C T
G5
C6
A C A C
A4
C8
G G G C A A T C T A G

FIGURE 70

DNase I footprinting on the synthetic oligonucleotide trimer. Eco RI/Hind III fragments from $pASO_3$, 3' or 5' end-labelled at the Hind III site (as indicated), were incubated with either no protein (lanes 1, 12) or with 15 or 30 ul of either mock-infected (MI) or HSV-1-infected (17^+) cell extracts, for either 5 min, as usual, (lanes 1-5, 9-12), or for only 2.5 min (lanes 6-8). Samples were analysed on 6% sequencing gels. GA and G sequence reactions were used as markers. One copy of the oligonucleotide sequence is shown.

sequence tracks for the 3' end-labelled fragment are not very clear, and therefore, from this figure, the enhanced bands can only be located to within one base. However, by comparison with other experiments, the enhanced cleavages were located as shown in Figure 71. The pattern did not correlate with that seen in the intact 'a' sequence (Figure 71).

(b) DNase I footprinting with the HSV-2 'a' sequence

In addition, very preliminary results have been obtained with the HSV-2 'a' sequence. Plasmid pAH_2, obtained from Mr Martin Murray, contains the HSV-2 'a' sequence derived from the L-S junction, and about 200 bp of flanking 'b'' sequence (materials, Figure 17). The HSV-2 'a' sequence contains an Eag I restriction enzyme site within the DR_4 region which could be labelled to study interactions occurring in the region equivalent to U_b. Although the DNase I footprint reaction shown in Figure 72 is poor, it can clearly be seen that certain bands are markedly enhanced in cleavage in the presence of HSV-1-infected cell extracts. The sequence tracks are unclear, partly due to the fact that the Eag I cleavage site is 5'-C↓GGCCG-3', and therefore 3' end-labelling using [α^{32}P]-dGTP resulted in two populations of molecule differing in size by 1 bp. This was a consistent problem with 3' end-labelling at restriction enzyme sites of this nature. However, from the general pattern of the sequence tracks and by comparison with the sequence tracks for the pR9 insert end-labelled at the Hind III site, it would appear that the region of enhancement lies within the U_b homology region - ie. the DR_2 repeat elements of the pR9 insert start at approximately 90 bp from the labelled end, while the U_b homology region within the pAH_2 insert lies between approximately 70-100 bp

FIGURE 71

DNA sequence of a monomer of the synthetic oligonucleotide trimer within $pASO_3$. The sequence is shown in the same orientation as Figure 63. The U_b homology sequence is indicated (⟼) as are those nucleotides forming the linker sequence (----), and therefore not present in the intact 'a' sequence.

► dinucleotide bonds enhanced in cleavage when the $pASO_3$ insert is used as substrate.

➞ dinucleotide bonds enhanced in cleavage when the intact 'a' sequence is used as substrate.

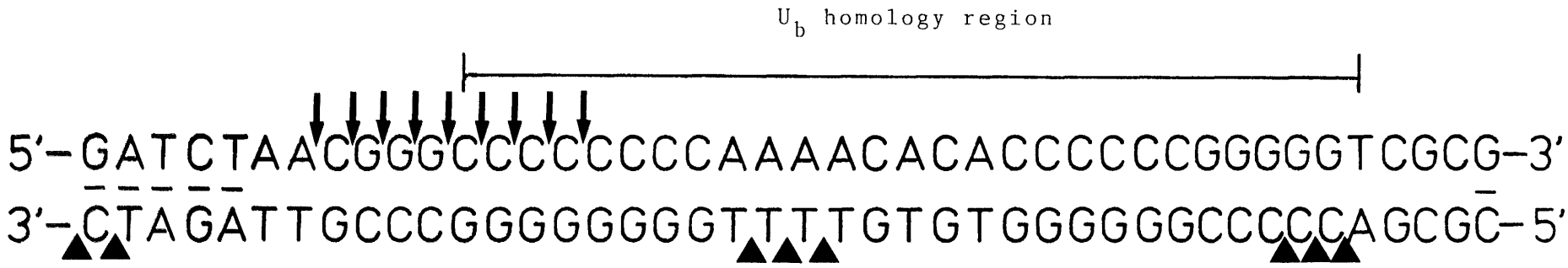
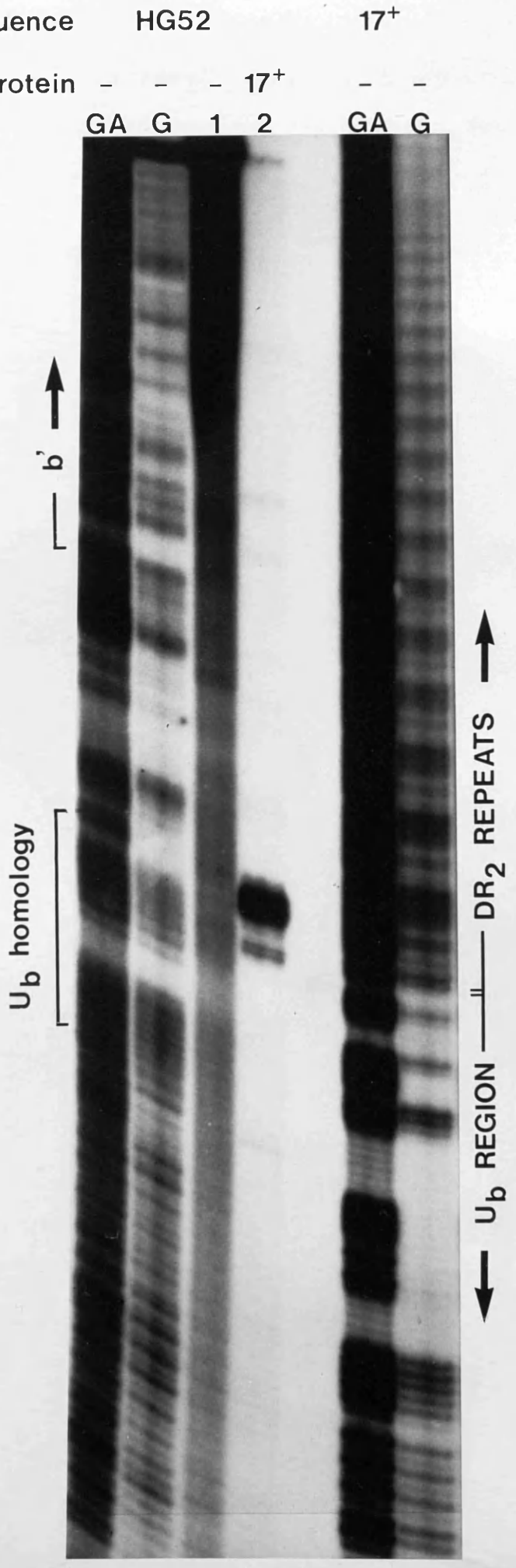

U_b homology region
5'-GATCTAACGGGCCCCCCCCCAAAACACACCCCCCGGGGGTCGCG-3'
3'-CTAGATTGCCCGGGGGGGGGTTTTGTGTGGGGGGCCCCCAGCGC-5'

FIGURE 72

DNase I footprinting on the HSV-2 'a' sequence. Plasmid pAH_2 contains the HSV-2 'a' sequence. Eco RI/Eag I fragments from pAH_2, 3' end-labelled at the Eag I site (located within the DR_4 region), were incubated with either no protein (lane 1) or 30 ul of an HSV-1-infected cell extract (lane 2). Samples were analysed on a 6% sequence gel. GA and G sequence reactions on both the Eco RI/Eag I fragment from pAH_2, and on an Eco RI/Hind III fragment from pR9, 3' end-labelled at the Hind III site, were used as markers. Between the HSV-2 and HSV-1 'a' sequence reactions is an empty lane.


'a' sequence
HG52
17+
protein
–
–
–
17+
–
–
GA
G
1
2
GA
G
b'
Ub homology
Ub REGION
DR2 REPEATS

from the labelled end. This result is preliminary and obviously requires further study, but it does suggest the possible involvement of the U_b homology region in the interactions observed.

DISCUSSION

DISCUSSION

SECTION I : ANTI-PEPTIDE SERA

The aim of this project was to further characterise the 21K/22K polypeptides known to specifically interact with the 'a' sequence of HSV-1, and to investigate in more detail their DNA-binding properties. As discussed in the introduction (section XI), it was thought highly likely that these polypeptides were encoded by gene U_S11. Therefore, use was made of the available DNA sequence information (Rixon and McGeoch, 1984) to obtain synthetic oligopeptides corresponding to several regions of the predicted gene product. The original intention was to use antisera raised against these oligopeptides to purify the 21K/22K proteins. Immunoaffinity chromatography against anti-peptide antibodies has the advantage that protein specifically bound to the column can then, in theory, be eluted using the oligopeptide, thus avoiding harsh and denaturing conditions. Therefore this procedure is potentially useful for purifying functionally active polypeptides.

Five synthetic oligopeptides were obtained over the course of this project. These represented amino acids 1-11, 48-58, 66-76, 132-138 (138-144) and 151-161, respectively (see result section I.1, Figure 18). All five peptides were immunogenic - ie. they induced antisera which reacted against the synthetic oligopeptide in a solid phase radioimmunoassay. However, only four of the five peptide antisera reacted convincingly with HSV-induced polypeptides. One antiserum, anti-(132-138), failed to react strongly in either immunoblotting or immunoprecipitation reactions, although a very

weak interaction was detected. This was the smallest peptide, an 8-mer (7 amino acids from the U_S11 gene product plus an amino-terminal tyrosine residue), and internal peptides of this size have reportedly a low success rate in inducing protein-reactive antisera (Palfreyman et al., 1984b). In addition, this peptide represents part of the region within the predicted U_S11 gene product which contains 24 tandem repeats of the sequence X-Pro-Arg. It is possible that this repetitive sequence may confer a distinct structure to this region of the polypeptide, which the small 8-mer oligopeptide may not be able to mimic.

We routinely coupled synthetic peptides to the carrier protein BSA for immunisation. However, it has been reported that free, uncoupled peptides can induce strong anti-peptide and anti-protein responses (Young et al., 1983), and therefore two peptides were tested as immunogens in an uncoupled form. For both these peptides the anti-peptide and anti-protein responses were considerably weaker than those generated by the coupled peptides (see result section I, Table 6 and Figure 23). However, it must be mentioned that no attempts have been made to optimise the immunisation procedure.

A number of rabbits showed a decline in the RIA and immunoblotting reactions, despite continued immunisation (see result section I, Table 6 and Figure 27), suggesting the development of tolerance to the immunogen. This illustrates the importance of assessing the immune response to each immunisation, to reduce the risk of missing the optimal anti-protein response. No attempt was made to boost these rabbits using peptide coupled to an alternative carrier protein.

There was no apparent correlation between the anti-peptide titre measured by RIA and the anti-protein response

detected by immunoblotting (result section I.5, Figure 27). However, the peak immunoblotting reaction was often found to coincide with the initial decline in RIA titre. The RIA is an easy and relatively quick assay to perform (one can readily do 500 tests in a day), and could be used to assess each consecutive bleed with a view to deciding at what point to stop immunisation and bleed out the animal. However, this is obviously a very limited study, and how an anti-protein response assessed by immunoblotting correlates with a response to native protein is unclear, since in this study no anti-serum showed a good reaction with native protein by either immunoprecipitation or immunoaffinity chromatography.

The original intention of this project was to use the anti-peptide sera to purify the 21K polypeptide. However, immunoaffinity chromatography proved unsuccessful, the synthetic oligopeptides failing to specifically elute any bound protein from the columns (result section III.1). A low level of 21K was eluted using 3M sodium thiocyanate, but this was contaminated by a number of other polypeptides. In addition, sodium thiocyanate is a highly denaturing eluting agent, and would be unlikely to be suitable for the purification of a functionally active protein.

It has been demonstrated that a high proportion of monoclonal antibodies raised against synthetic oligopeptides cross-react with the native protein (Niman et al., 1983). Therefore, in the hope of selecting an anti-peptide antibody with a higher affinity for 21K, we attempted to raise monoclonal antibodies against the available oligopeptides. In addition, we attempted to raise monoclonal antibodies against the intact 21K polypeptide, by immunising mice with DNA cellulose purified material. Both these approaches failed,

due, at least in part, to the lack of a reliable assay system (result section I.6).

Therefore, our original intention of purifying the 21K/22K polypeptides by immunoaffinity chromatography against anti-peptide sera was not achieved. However, this approach resulted in the generation of some useful antisera with which to further characterise the U_S11 gene products.

SECTION II : CHARACTERISATION OF THE U_S11 GENE PRODUCTS

The anti-peptide sera specifically recognised not only proteins of apparent mol. wt. 21K and 22K, but also at least four lower mol. wt. polypeptides (17.5K, 15K, 14K and 11K). The tryptic peptide fingerprints shown in result section II.2 (Figure 36) clearly confirm that these polypeptides are related to 21K and 22K. However, the precise relationship between these products is unclear. Although the amino acid sequence of the predicted U_S11 gene product contains 24 tandem repeats of the tripeptide X-Pro-Arg, there is no extensive reiteration within the DNA sequence, as all the potential codons for proline and arginine are used (Rixon and McGeoch, 1984). However, there is a short 18 bp sequence which is present as three tandem repeats in HSV-1 strain $17syn^+$ (Rixon and McGeoch, 1984), but only as two tandem repeats in HSV-1 strain Patton (Watson and Vande Woude, 1982). It has been suggested (Rixon and McGeoch, 1984) that variation in the number of repeats of this 18 bp sequence may account for the interstrain variability in the apparent mol. wt. of Vmw21 reported by Lonsdale et al. (1979). It is also possible that this could account for the difference between the 21K and 22K polypeptides. However, the fact that the apparent

mol. wt. of Vmw21, Vmw22 was unaltered in 72 separate plaque isolates of HSV-1 strain 17syn$^+$ (Alasdair R MacLean, personal communication), might argue against this possibility.

Carboxy-terminal antisera recognised products of apparent mol. wt. 17.5K, 14K and 11K which were not recognised by amino-terminal antisera. Antisera against internal oligopeptide 48-58 recognised the 17.5K and possibly the 14K polypeptides, but not the 11K polypeptide. This might suggest that the 17.5K, 14K and 11K products, while retaining the carboxy-terminus, lack progressively larger regions from the amino-terminus of 21K. However, this interpretation is not readily compatible with the distribution of the three methionine residues in the predicted U_S11 gene product : these are all situated in the amino-terminal region (residues 1, 27 and 41, respectively). An alternative interpretation is that the amino-terminus is present in the 17.5K, 14K and 11K polypeptides, but inaccessible to the amino-terminal antisera in immunoblotting reactions. It is unlikely that the 11K polypeptide is generated without the loss of some amino acids, since the predicted mol. wt. of the U_S11 gene product is 17,756 (McGeoch *et al.*, 1985) and the *in vitro* translation product has an apparent mol. wt. of 21K (Rixon and McGeoch, 1984). Another hypothesis for the observed reactivity of the antisera is that the lower mol. wt. polypeptides arise as a consequence of splicing within the U_S11 gene - however, there is no evidence for this (Rixon and McGeoch, 1984; Frazer J Rixon, personal communication).

The 15K polypeptide is recognised by the amino-terminal antiserum, anti-(48-58) and possibly by anti-(66-76) sera, but not by the carboxy-terminal antisera. The simplest interpretation of this result is that this polypeptide lacks

part of the carboxy-terminus of the U_S11 gene product.

Polypeptides of apparent mol. wt. 21K and 22K, designated Vmw21 and Vmw22, were previously shown to be produced in abundance late in infection (Marsden et al., 1976) and to map to the right hand end of the short region of the virus genome (Marsden et al., 1978). Timecourse experiments (result section II.1) confirmed that the 21K and 22K U_S11 gene products are synthesised late in infection, and PAA, an inhibitor of viral DNA replication, severely impaired their synthesis (approximately 100-fold), in contrast to an approximately 5-fold reduction in the synthesis of gD, an early ($\beta\gamma$) polypeptide. Thus, it was concluded that the 21K/22K polypeptides encoded by gene U_S11 could be defined as true late gene products.

One reason for studying the U_S11 gene products was to determine their relationship with the previously described 21K and 22K DNA-binding proteins shown to specifically interact with the 'a' sequence of HSV-1 (Dalziel and Marsden, 1984). Three attempts were made to repeat the competition experiments of Dalziel and Marsden, and these resulted in a very high level of non-specific elution of DNA-binding proteins. Therefore, we could not obtain the 'a' sequence-specific DNA-binding proteins for immunoblotting or immunoprecipitation experiments, and so it was not possible to demonstrate unambiguously that the U_S11 gene products interacted with the 'a' sequence of HSV-1. However, by immunoblotting proteins eluted from DNA cellulose columns we could confirm that the 21K and 22K U_S11 gene products were DNA-binding proteins (result section II.3, Figure 37). The existence of a second, completely unrelated, pair of late 21K/22K DNA-binding proteins would seem sufficiently unlikely that

it can be concluded with reasonable confidence that the 21K/22K proteins which specifically interact with the HSV-1 'a' sequence are the products of gene U_S11.

The subcellular localisation of the U_S11 gene products was determined by immune electron microscopy. The U_S11 gene products localised within two areas of the infected cell, the cytoplasm and the nucleolus. The cytoplasmic labelling varied in intensity between individual cells, but the reason for this was not clear. Nor was the functional significance of this behaviour apparent. Attempts to compare the distribution of 21K within a population of infected cells by immunofluorescence proved unsuccessful, due to high levels of non-specific staining of infected cells. In contrast, the immune electron microscopy was highly specific (result section II.4).

The predominant site of localisation of the U_S11 gene products was the nucleolus (result section II.4). The structure and function of nucleoli has recently been reviewed (Sommerville, 1986). The primary, and only described, function of the nucleolus appears to be the generation and maturation of ribosomal particles. Nucleoli are therefore generated around ribosomal chromatin and are often surrounded and penetrated by so-called 'nucleolus-associated chromatin' (Jordan, 1984). This was clearly seen, for example, in Figures 40 and 42.

Studies on the effects of HSV infection on cellular metabolism have led to the conclusion that the nucleolus disaggregates to produce spherical densely staining components (dense bodies) and a less densely staining, irregularly shaped, granular component (Sirtori and Bosisio-Bestetti, 1967; Schwartz and Roizman, 1969; Dupuy-Coin et al., 1978). In contrast to the granular component, the dense bodies

contain no DNA or RNA, although they have been shown to contain at least one nucleolus-specific protein (Puvion-Dutilleul *et al.*, 1985). No association was found between the U_S11 gene products and dense bodies.

The predominant association of the U_S11 gene products with the granular component of the nucleolus, the site of production, processing and maturation of pre-ribosomal particles (Sommerville, 1986) resembles that previously shown for ribocharin, a protein believed to be involved in nucleocytoplasmic transport of ribosomes (Hügle *et al.*, 1985a) and S1, a structural component of ribosomal particles (Hügle *et al.*, 1985b). This might suggest some preferential association of the U_S11 gene products with ribosomal RNA or ribonucleoproteins in infected cell nuclei, although an association with ribosomal DNA cannot be excluded. Disaggregation of the nucleolus and inhibition of ribosomal RNA synthesis occur early following infection (Schwartz and Roizman, 1969; Wagner and Roizman, 1969) and therefore it would seem unlikely that the U_S11 gene products play a major role in these events.

Nii *et al.* (1968) have previously shown localisation of virus-induced antigens to similar structures using ferritin-conjugated antibodies. However, since only general anti-HSV antisera were used, the identity of the antigens recognised remains unknown. Therefore, whether other viral antigens accumulate within these residual nucleoli is unclear.

Although the 21K/22K polypeptides appear to interact with the HSV-1 'a' sequence *in vitro*, their nucleolar localisation suggests that they may not bind to viral DNA *in vivo*. Viral DNA is located in the nucleoplasm but is specifically excluded from nucleoli (Rixon *et al.*, 1983; Puvion-Dutilleul *et al.*, 1985; Randall and Dinwoodie, 1986). Similar nucleolar

localisation has not been observed for other HSV-specified DNA-binding proteins, for example IE Vmw175, the major DNA-binding protein and the DNA polymerase (Randall and Dinwoodie, 1986). In these cases the distribution reflects that of viral DNA, ie. localisation to the nucleoplasm and exclusion from the nucleolus.

The different patterns of localisation of the virus DNA and 21K might suggest that 21K plays no direct role in virus DNA replication or maturation. This suggestion is supported by the recent isolation of a number of HSV deletion mutants which lack gene U_S11 and are viable in tissue culture (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987). In vivo studies with these mutants have not yet been reported. Thus, whether the U_S11 gene products influence the host-range dependence of the virus, or whether they play a role in latency or pathogenesis in vivo, remains to be determined.

SECTION III : ATTEMPTS TO PURIFY THE 21K POLYPEPTIDE

Attempts to purify the 21K polypeptide met with limited success (result section III). A major problem was that the use of partially-purified 21K for subsequent chromatographic procedures generally resulted in loss of the protein - it was neither recovered significantly in the flow-through or eluate, nor did it remain bound to the column. The presence of various protease inhibitors (PMSF, TLCK and α_2-macro-globulin) did not significantly influence recovery. However, no systematic study of the effect of protease inhibitors was carried out, and it is possible that a combination of these and/or other protease inhibitors may have helped.

A two step purification procedure was developed which involved ammonium sulphate precipitation followed by chromatography of the dialysed supernatant against phosphocellulose (result section III.5). Under certain conditions, this yielded an apparently homogeneous preparation of 21K, as assessed by autoradiography. However, Coomassie blue staining revealed several major contaminants and no detectable 21K polypeptide.

One interesting feature that emerged from these studies was the aberrant migration of partially-purified 21K in SDS-PAGE. It often ran as a very diffuse, smeared band, frequently with an increased apparent mol. wt. This aberrant migration was particularly apparent as the protein became more pure : the cause is not clear, but may represent a structural instability of the polypeptide in a purified form.

From the purification attempts described it became clear that we were unlikely to achieve a homogeneous purification of significant quantities of 21K for either the generation of monospecific antisera or for functional studies. It was also possible that purification of the 21K polypeptide might result in loss of its DNA-binding activity. Therefore, since the original intention was to study the interaction between the 21K polypeptide and the HSV-1 'a' sequence, we decided at this point to look instead for evidence of DNA-protein interactions occurring within the 'a' sequence, and then to investigate whether or not these interactions involved the 21K polypeptide.

SECTION IV : DNA-PROTEIN INTERACTIONS WITHIN THE HSV-1 'a' SEQUENCE

To investigate sequence-specific DNA-protein interactions within the 'a' sequence we used a DNase I footprinting assay (result section IV). Incubation of end-labelled 'a' sequence-containing DNA fragments with crude whole cell extracts resulted in alterations in the DNase I digestion pattern which were specific for infected cell extracts (Figure 62). For ease of discussion these alterations have been divided into two areas, termed Region 1 and Region 2 (see Figure 60, result section IV.4).

Region 1 occurs within the DR_2 repeat elements, and comprises a protected region spanning 7 nucleotides, flanked on either side by 2 nucleotides at which cleavage is enhanced. Between each element is one nucleotide, cleavage of which is apparently unaffected. Region 2 occurs within U_b and encompasses a number of sites showing a markedly enhanced sensitivity to DNase I digestion. Occasionally, protection was seen within this region.

A major disadvantage to the use of whole cell extracts was the high level of nuclease activity present. This severely limited the amount of protein which could be added to the reaction, and significantly impaired the analysis of 5' end-labelled fragments. The selective loss of counts seen at the top of the gels with 3' end-labelled fragments, and the fact that 5' end-labelling of a blunt ended fragment did not improve recovery of counts (results not shown), would suggest that a 5'-3' exonuclease activity, and not a single-strand nuclease activity or a phosphatase activity, was responsible. It is well established that HSV infection induces high levels

of nuclease activity in infected cells, and one candidate for this would be the virus-encoded alkaline exonuclease (Keir and Gold, 1963; Morrison and Keir, 1968). However, for reasons discussed later, this does not seem likely to be the source of the putative 5'-3' exonuclease activity.

Mock-infected cell extracts also induce changes in DNase sensitivity within the DR_2 repeat element. The relationship between these changes and those induced by HSV-infected cell extracts is unclear (see Figure 62, result section IV.5, for a comparison). It is possible that the DNA-binding properties of a cellular protein could be modified following infection, possibly via interaction with viral protein(s). Alternatively, however, a virus-encoded or virus-induced protein may be responsible for the effect seen with infected cell extracts. HSV-1 and HSV-2-infected cell extracts induce identical DNase I digestion patterns in this region. Interestingly, the HSV-2 'a' sequence contains only a single DR_2 homology (Davison and Wilkie, 1981).

The enhancement of cleavage seen in Region 2 is also virus-specific, and HSV-1 and HSV-2-infected cell extracts again generate identical DNase I digestion patterns in this region. Enhancement has often been reported near or within protected regions in DNase footprinting studies (for example, Dynan and Tjian, 1983; Wildeman et al., 1986), but here the enhancement is occurring in the absence of any significant protection. Whether enhanced sensitivity to DNase I is indicative of a DNA-protein interaction is debatable. However, the enhancement seen in Region 2 is very marked and must at least suggest the possibility of some form of DNA-protein interaction within or near this region.

The HSV-1 and HSV-2 'a' sequences share limited homology (Davison and Wilkie, 1981; Mocarski and Roizman, 1981; Mocarski et al., 1985; Varmuza and Smiley, 1985). However, within U_b there is a 28 bp region which is highly conserved between these 'a' sequences (Davison and Wilkie, 1981). Bands enhanced in the presence of HSV-infected cell extracts overlap and flank this region (see later, Figure 74), possibly reflecting some undetected binding within the U_b homology region. Preliminary experiments suggest that a similar very marked enhancement of cleavage occurs in the HSV-2 'a' sequence, within the U_b homology region. However, the precise nucleotides affected were not determined. Nevertheless, this observation strengthens the possibility that the U_b homology region may be involved in the effects seen in Region 2.

A number of mechanisms could lead to increased cleavage between particular dinucleotides. Firstly, a specific cleavage event could be generated by protein(s) within the infected cell extract. To test this possibility the putative U_b-binding protein will have to be purified away from the nuclease activity present within the extract. Secondly, protein binding may induce a conformational change in the DNA, leading to exposure of DNase hypersensitive sites. Thirdly, protein-protein interactions could occur between the DNase I enzyme and protein bound to DNA, leading to enhanced cleavage nearby. Although the latter two mechanisms may explain the enhanced cleavage often described near or within protected regions in DNase I footprinting, the lack of any obvious protection within Region 2 may suggest that, alone, they do not satisfactorily account for the marked level of enhancement observed here.

Given the endogenous 5'-3' exonuclease activity, an alternative explanation is that these enhanced cleavages mark the 5' boundaries of protein binding sites. Exonuclease digestion has previously been used to map sites of protein binding within labelled DNA fragments (Wu, 1984). This principle is illustrated in Figure 73. Such a mechanism could explain why the markedly enhanced cleavages are only observed on the 3' end-labelled strands of either the oligonucleotide representing the homology region, or the intact 'a' sequence, and could also explain the differences observed in the pattern of enhancement on these two substrates. A composite picture of these enhanced cleavages is shown in Figure 74.

The presence of two regions of enhanced cleavage on each strand might suggest that two separate binding sites are involved. One potential mechanism by which the enhanced cleavages within U_b could have arisen is shown in Figure 74b. A sequence of 17 bp is present as an imperfect, overlapping direct repeat within this region. This is shown in Figure 74c. In the presence of a 5'-3' exonuclease, protein bound to the leftmost sequence (i) could generate the leftmost blocks of enhancement on the upper and lower strands, while protein bound to the rightmost sequence (ii) could generate the rightmost blocks of enhancement on the upper and lower strands (Figure 74b). This appears to be the best candidate for a conserved sequence to account for the four regions of enhancement. However, the possibility that additional unrelated binding sites are involved cannot be excluded.

Although the region protected from exonuclease digestion might be expected to extend further than the nucleotides directly involved in the DNA-protein interaction, this mechanism does not explain the very extensive degree of enhancement spread over a large number of nucleotides seen in the right-

FIGURE 73

Exonuclease footprinting. This diagram illustrates a hypothetical interaction between a sequence-specific DNA-binding protein and a fragment of DNA. Protein, binding to a specific region of the DNA (indicated by the hatched area) will protect it from nuclease attack. The number of bases in the protected and unprotected regions are indicated. Incubation of a 5'-3' exonuclease with the protein-bound DNA, 3' end-labelled on either the upper (U) or lower (L) strand (a), will lead to the generation of labelled fragments terminating at the 5' boundary of the protein binding site on that strand (b). Analysis of the products alongside sequence markers (c) then allows these boundaries to be mapped. In a similar manner, a 3'-5' exonuclease can be used to map the 3' boundaries of the protein binding site, using 5' end-labelled DNA.

* radioactive label

➡ direction of cleavage by the exonuclease

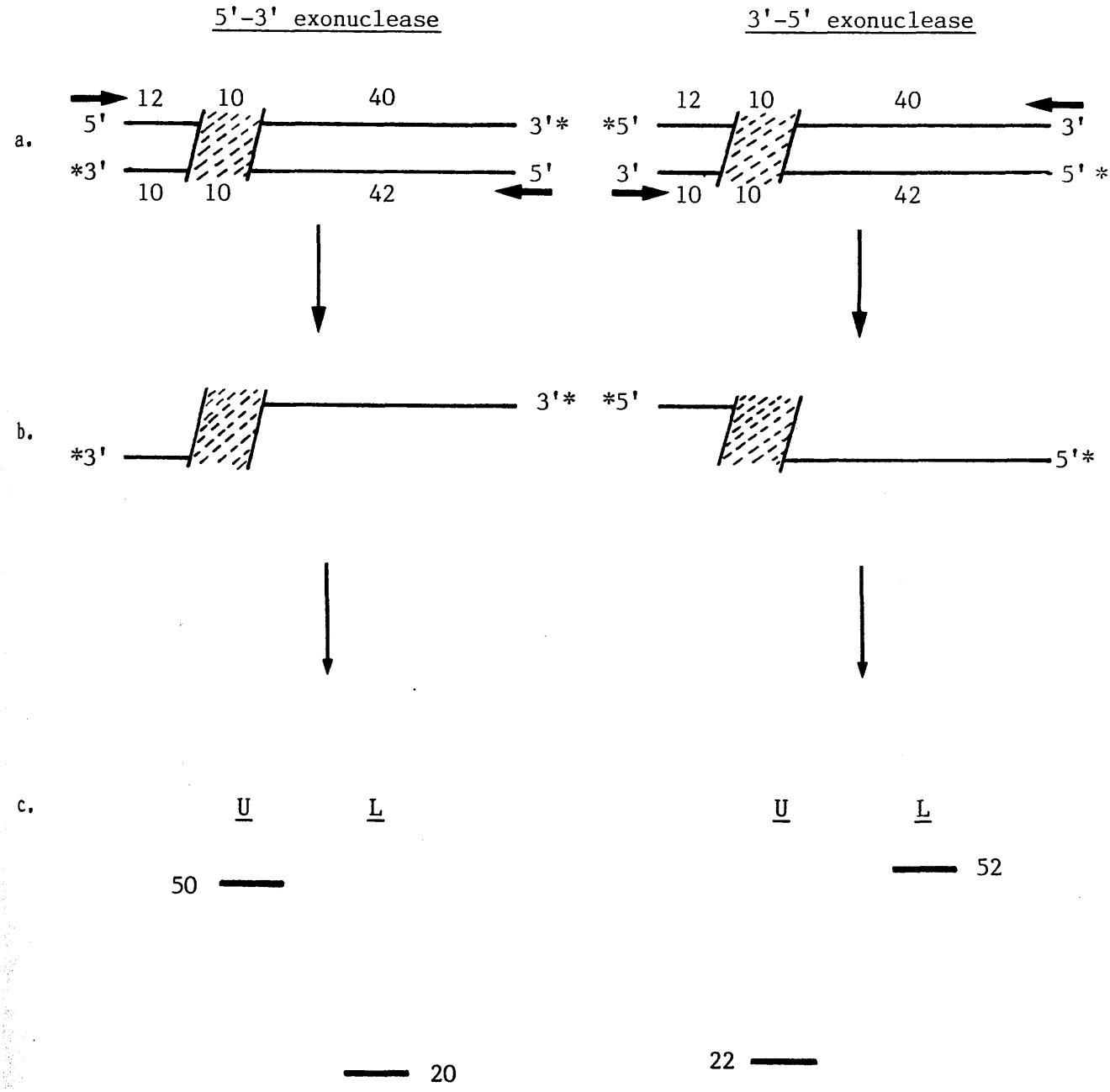

5'-3' exonuclease
3'-5' exonuclease
a.
12
10
40
5'
3'*
*3'
5'
10
10
42
*5'
3'
3'
5' *
b.
c.
U
L
50
52
20
22

FIGURE 74

A possible mechanism for the enhanced cleavage within U_b.

(a) A composite picture of those dinucleotide bonds at which cleavage is enhanced when either the intact 'a' sequence (upper strand, ⟶) or the oligonucleotide sequence from $pASO_3$ (lower strand, ▶) is used as substrate. These groups have been termed E1-E4. ◇ indicates the relative position of the nucleotides within the linker sequence at which cleavage is enhanced in the latter case. The U_b homology region is indicated (⟷).

(b) One potential mechanism by which cleavage may appear to be enhanced at these sites. Enhanced cleavage has only been observed on 3' end-labelled strands. A 5'-3' exonuclease, processively digesting the labelled DNA until blocked by bound protein, would result in the over-representation of 3' end-labelled fragments terminating at the 5' boundary of the protein binding site. Protein, bound to the right of E1 and E2, and to the left of E3 and E4, in (a), could therefore generate these apparently enhanced cleavages. The presence of two regions of enhancement on each strand would suggest two separate binding sites. One possible candidate, a sequence present as an imperfect direct repeat, is indicated.

(c) Putative U_b-binding site. (|) indicates those nucleotides conserved between the two copies of the imperfect direct repeat shown in (b).

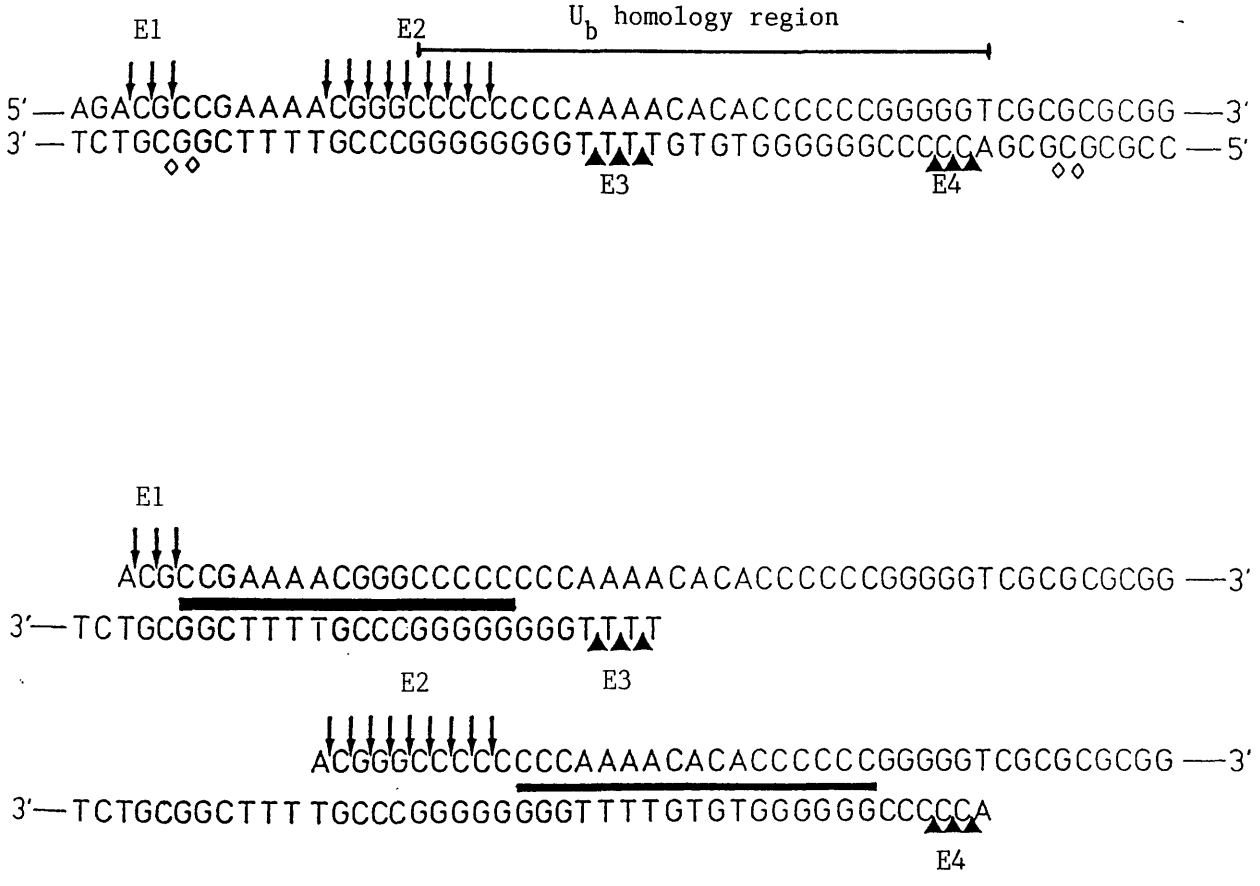
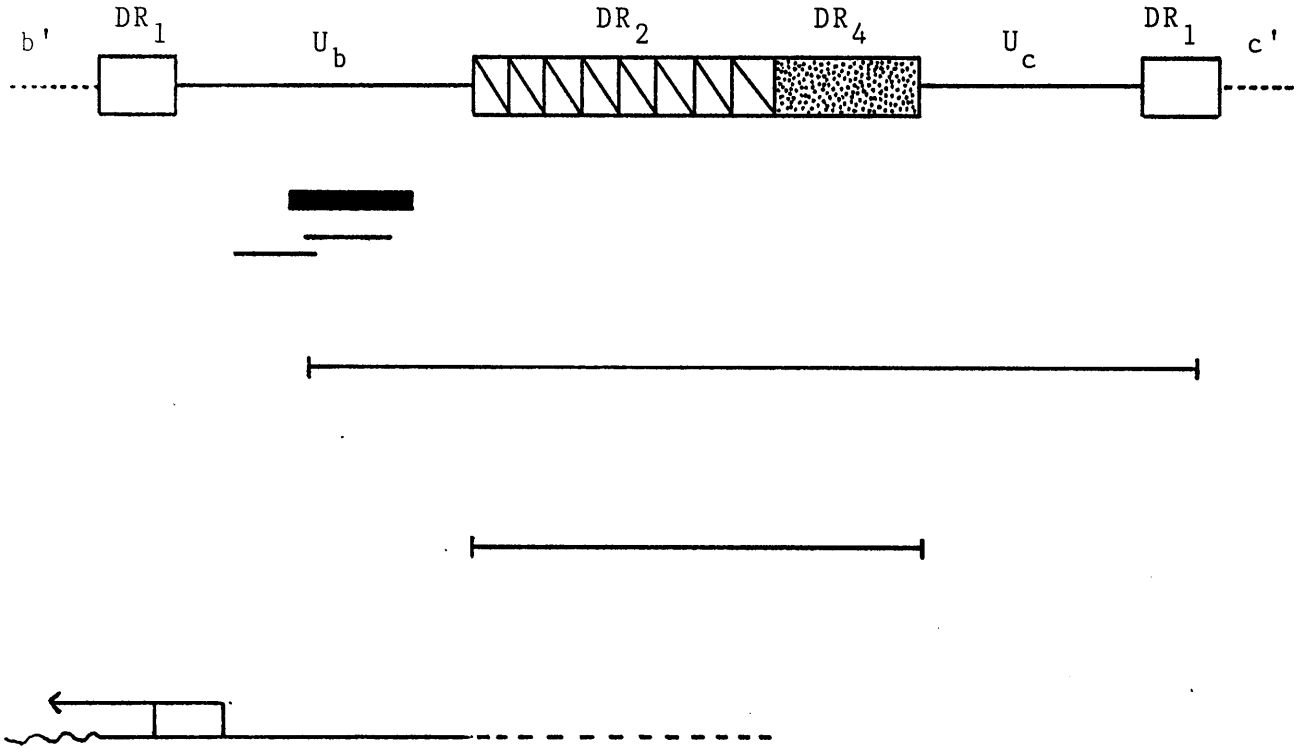

E1
E2
U_b homology region
a.
5′—AGACGCCGAAAACGGGCCCCCCCCAAAACACACCCCCCGGGGGTCGCGCGCGG—3′
3′—TCTGCGGCTTTTGCCCGGGGGGGGGTTTTGTGTGGGGGGCCCCCAGCGCGCGCC—5′
E3
E4
b.
E1
ACGCCGAAAACGGGCCCCCCCCCAAAACACACCCCCCGGGGGTCGCGCGCGG—3′
3′—TCTGCGGCTTTTGCCCGGGGGGGGGTTTT
E2
E3
ACGGGCCCCCCCCCAAAACACACCCCCCGGGGGTCGCGCGCGG—3′
3′—TCTGCGGCTTTTGCCCGGGGGGGGGTTTTGTGTGGGGGGCCCCCA
E4
c.
(i) CCGAAAACGGGCCCCCC
(ii) CCCAAAACACACCCCCC

FIGURE 75

Schematic representation of functional domains within the 'a' sequence. The HSV-1 'a' sequence is shown in the orientation found at the L-S junction. The location of the U_b homology region (pac-1) is indicated (▬), as are the putative U_b-binding sites proposed in Figure 74 (—). Below are shown the regions within which different functional signals have been mapped (see text). The region involved in cleavage/packaging corresponds to the Sma I F fragment of Varmuza and Smiley (1985).


b'
DR1
Ub
DR2
DR4
Uc
DR1
c'
Ub homology region
putative Ub-binding sites
cleavage/packaging
inversion
promoter/transcription start sites

most block of enhancement on the upper strand, nor the additional enhancement seen within the restriction enzyme linker in the oligonucleotide trimer. It is possible that the effects observed reflect several different, possibly unrelated, DNA-protein interactions. In addition, it should be stressed that the mechanism postulated above is purely speculative, and the precise binding site, or sites, have yet to be elucidated.

Making the assumption that the enhanced cleavages represent DNA-protein interactions within the 'a' sequence, then two main questions arise : (i) what function(s) are served by such interactions, and (ii) what viral protein(s), if any, are involved.

A number of functional signals reside within the HSV 'a' sequence (see introduction section X). These are summarised in Figure 75. Chou and Roizman (1985) mapped the sequences required for site-specific inversion to the repeat elements of the HSV-1 (F) 'a' sequence (DR_2 and DR_4), and demonstrated that the U_b and U_c regions were not required. Varmuza and Smiley (1985) mapped sequences sufficient for cleavage and packaging of viral DNA to the Sma I F fragment of HSV-1 (KOS). It was concluded that there are two cleavage/packaging signals, one within U_b and one within U_c, and it has been suggested that the U_b-derived signal involves, at least in part, the 28 bp U_b homology region (Varmuza and Smiley, 1985; Deiss et al., 1986). In addition, the 'a' sequence contains the promoter-regulatory domain and transcription initiation site of a gene within the 'b' sequence of the long repeat region (Chou and Roizman, 1986). Two transcription initiation sites were mapped in the HSV-1 (F) 'a' sequence, one within DR_1 and the other 15 bp upstream at the edge of a TTTAAA run within U_b, suggested to be the

nearest equivalent to a 'TATA' box. The authors claimed to have preliminary evidence that the promoter resides within the U_b/DR_2 repeat region of the 'a' sequence.

Thus, sequences within U_b are involved in the cleavage and packaging of the viral genome, contain promoter elements for a gene within the adjacent 'b' sequence, and serve as transcription initiation sites. Sequences within DR_2 may also have a promoter/regulatory function, and are involved in site-specific inversion events. Whether the DR_2 repeat region is involved in packaging is unknown.

The change in the DNase I digestion patterns within U_b and DR_2 occurred simultaneously - neither was observed in the absence of the other. These activities copurified through the limited chromatographic procedures used. Therefore, it would be interesting to use the synthetic oligonucleotide representing the U_b homology region to compete with the binding of proteins to the intact 'a' sequence, to determine whether binding to the U_b region influences the binding in the DR_2 repeats, and indeed whether the same protein(s) are involved. The sequence within DR_2, however, shares limited homology with the putative binding sites - two possible alignments are shown in Figure 76. The intensity of the cleavages seen within U_b would suggest that, if the same protein(s) are involved, then the affinity for the U_b region is much stronger than the affinity for the DR_2 repeat elements.

The proteins involved in these interactions are unknown. The 21K/22K DNA-binding proteins previously shown to interact specifically with the 'a' sequence of HSV-1 (Dalziel and Marsden, 1984) are late polypeptides and the products of gene U_S11. Extracts from cells infected in the presence of PAA

FIGURE 76

Possible homology between DR_2 and the putative U_b-binding sites.

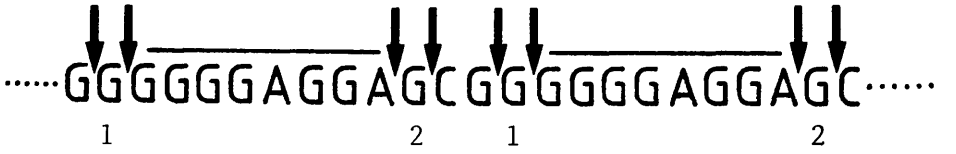
(a) Nucleotides showing enhanced cleavages in DR_2. The sequence is drawn in the 5'-3' direction for the upper strand, and shows a repetitive pattern of enhanced cleavages (arrows). The groups of enhancement are labelled 1 and 2 for ease of reference. Protection is indicated by the solid bar.

(b) Sequence of the two putative U_b-binding sites, and a consensus sequence derived from these. This is shown in the 5'-3' direction for the lower strand (compare Figure 74).

(c) Two possible alignments of the DR_2 repeat sequence with the putative U_b-binding site consensus. One possible mechanism by which apparently enhanced cleavages at sites 1 and 2 might be generated, in the presence of a 5'-3' exonuclease, is if protein bound to alignments (i) and (ii), respectively (cleavage would take place on the 5' side, close to the binding site). An alternative possibility is that the enhancement within DR_2 represents true enhancement of DNase I cleavage, possible via protein binding within the sequence GGGGAGG, the region protected in the DNase I footprints (see (a)).

* represents those bases shared with the consensus sequence in (b).

a.



b.

site 1 GGGGGGTGTGTTTTGGG

site 2 GGGGGGCCCGTTTTCGG

consensus GGGGGGY[G/C]YGTTTT[G/C]GG

c.

(i) GGGAGGAGCGGGGGAG

(ii) GGGGGGAGGAGCGGGGG

produced an identical DNase I digestion pattern to untreated infected cell extracts, as did extracts from cells infected with the HSV-2 deletion mutant, X12, which lacks gene U_S11 (result section IV.9). This would rather strongly suggest that the 21K/22K 'a' sequence-specific DNA-binding proteins are not involved in these interactions.

The presence of this activity in the PAA-treated extracts suggests that the interactions involve an IE or early polypeptide. The temperature-sensitive mutant, tsK, failed to induce this activity when grown at the non-permissive temperature, suggesting that IE polypeptides, with the possible exception of Vmw175, are not involved alone.

The polypeptides involved in cleavage and packaging of viral DNA and in the inversion of the viral genome are unknown. The presence of a promoter-regulatory domain and transcription initiation site within the DR_2/U_b region of the 'a' sequence could result in the presence of a number of potential binding sites for either cellular or viral transcription factors. Viral transcription factors include Vmw175, Vmw65 and the major DNA-binding protein (introduction, section IV.3). Chou and Roizman (1986) classified the 'a' sequence promoter as a γ_1 promoter, due to its slight sensitivity to PAA. Vmw65 plays a role in IE transcription, and is therefore an unlikely candidate. In contrast, the major DNA-binding protein is involved in IE, early and late gene expression. The use of ts mutants should help to elucidate the role, if any, of this polypeptide. Vmw175 appears to bind, either directly or indirectly, to a number of promoter-regulatory sequences, and a consensus sequence 5'-ATCGTCNNNNYCGRC-3' was proposed (Faber and Wilcox, 1986). There is no obvious homology with this sequence within either the U_b or DR_2 repeat

region. However, Vmw175 may interact with sites other than this consensus sequence (introduction, section VI.6a). The use of extracts prepared from cells infected with wild-type virus under conditions which allow the synthesis of only the IE gene products, should help to clarify this point.

Although the enhanced cleavages within U_b are generated only by infected cell extracts, this does not exclude the possibility that cellular transcription factors are involved. The U_b homology region, and flanking sequences, were compared with known binding sites for a number of cellular transcription factors, including nuclear factor I, nuclear factor III, 'TATA'-box-binding protein, and the transcription factor Sp1 (Table 10). Only one apparent homology was found (Figure 77). The sequence 5'-TGGGGGGGGC-3', present on the lower strand shown in Figure 74, correlates at 9/10 nucleotides with the Sp1 consensus sequence 5'-$^{G}_{T}$GGGCGG$^{GGC}_{AAT}$-3' (Kadonaga et al., 1986). Within the Sp1 consensus, the hexameric sequence 'GGGCGG' is very highly conserved and has been found, with only two exceptions, in all known Sp1-binding sites to date (Jones et al., 1986; Kadonaga et al., 1986). The two exceptions are Sp1-binding sites within the HIV promoter, containing the sequences 5'-GGGAGT-3', and 5'-AGGCGT-3', respectively. The affinity of Sp1 for different binding sites appears to correlate with the degree of homology with the consensus sequence (Kadonaga et al., 1986), and it remains to be determined whether the sequence 5'-GGGGGG-3' is an Sp1-binding site.

The DR_2 repeat element also shows homology to the Sp1 consensus. The sequence 5'-GGGGAGGAGC-3' differs only at the A at position 5 (GGGAGG) and in this regard resembles one of the Sp1-binding sites within the HIV promoter (GGGAGT).

Table 10 : DNA sequence elements believed to interact with transcription factors.

N any nucleotide

References

1. Pruijn et al., 1986
2. Bohmann et al., 1987
3. Jones et al., 1987
4. Kadonaga et al., 1986
5. Schlokat et al., 1986
6. Singh et al., 1986
7. Karin et al., 1984
8. Topol et al., 1985
9. Sawadogo and Roeder, 1985
10. Sivaraman et al., 1986
11. Zenke et al., 1986

Table 10 : Transcription factor binding sites

		Reference
A. General transcription factor binding sites		
NF III	TATGATAATGAG	1
	(ATGCAAATAG)	2
NF I / CTF	TGG[A/C]NNNNNGCCAA	3
Sp1	[G/T]GGGCGG[GGC/AAT]	4
B. Specific regulatory elements		
Ig enhancer elements	CAGGTGGC	5
	TGTGGCAA	5
Ig NF-A	ATTTGCAT	5,6
Heavy metal ion response element (MRE)	[TG/CT]CG·CCCGG·C[T/C]C	7
Glucocorticoid receptor element (GRE)	[T/C]GGT·[A/T]CA[AA/CT]·TGT[T/C]CT	7
Heat shock element	CNNGAANNTTCNNG	8
AdV major late promoter USF	GGCCACGTGACC	9
AdV E2A promoter	TGGAGATGACGTAGTTT	10
SV40 enhancer motifs		11
Sph motif	AAG[T/C]ATGCA	
TC motif	TCCCCAG	
GT motif	G[C/G]TGTGGAA[A/T]GT	
P motif	TCAATTAGTCA	

FIGURE 77

Sequences within the U_b and DR_2 repeat regions which share homology with the Sp1 consensus sequence.

* indicates conserved bases.


Sp1
GGGGCGGGGC
TAAT
* * * * * * * * * *
U_b
TGGGGGGGGC
DR_2
GGGGAGGAGC
* * * * * * * * * *
Sp1
GGGGCGGGGC
TAAT

The significance of the Sp1-like elements within these two regions is unclear. Only a single Sp1-like sequence is present within the U_b region, a fact difficult to reconcile with the pattern of enhancement seen. The Sp1-like sequence also overlaps the predominant region of enhancement on the top strand. These features, together with the fact that the enhanced cleavages are specific for virus-infected cell extracts, would argue against a role for Sp1, at least with respect to the enhanced cleavages within U_b. It is possible that Sp1 could be involved in the effects seen with mock-infected cell extracts within the DR_2 repeat region. Competition experiments, using DNA containing a known Sp1-binding site (or alternatively, the use of purified Sp1) would help to elucidate the role of the Sp1 transcription factor.

Another question which arises is the nature of the apparent exonuclease activity. If a 5'-3' exonuclease is indeed responsible for the apparent increase in sensitivity to DNase I, by the production of an exonuclease footprint, then it must copurify with the 'a' sequence-binding protein(s) through the limited chromatographic procedures used. Two procedures however, phosphocellulose chromatography and FPLC, resulted in loss of the footprinting activity, and this could have arisen had the exonuclease and 'a' sequence-binding proteins become separated. Equally, the footprinting activity could simply have become inactivated by these procedures. It would be interesting to carry out exonuclease footprint experiments, using the extracts and, especially, the partially-purified material, to determine whether a known 5'-3' exonuclease could generate a similar pattern of cleavage, and also whether a 3'-5' exonuclease could produce a corresponding pattern on the opposite strand. Ideally, this would require

the partial purification of the 'a' sequence-binding proteins away from the endogenous exonuclease activity.

The possibility that the exonuclease and 'a' sequence-binding proteins are more directly related must also be considered. It has not been excluded that a specific cleavage event is responsible for the enhanced cleavages within U_b. Purification of the 'a' sequence-binding protein(s) will be required to directly test this possibility. The HSV-encoded alkaline exonuclease possesses both exonuclease and endonuclease activities (Hoffmann and Cheng, 1979; Hoffmann, 1981). The exonuclease activity appears to digest both strands of DNA at an equal rate, to produce flush ends of digested DNA (Hoffmann, 1981), suggesting that the alkaline exonuclease is not responsible for the 5'-3' exonuclease activity observed here. However, this does not exclude a possible role for the endonuclease activity in generating these cleavages. The use of a ts mutant within the alkaline exonuclease gene (Francke et al., 1978) would help to more directly address this point.

Exonucleases have been reported to be involved in the process of recombination (Sadowski, 1982). However, the U_b region does not appear to be essential for the site-specific recombination events which generate segment inversion. Therefore, it might seem unlikely that an exonuclease activity would specifically interact with the U_b region of the 'a' sequence. In addition, if the enhancement observed does represent a specific cleavage event it would seem unlikely that this is directly related to the cleavage events which generate the normal genomic termini, since the termini are located within DR_1, some distance downstream from the enhanced cleavages within U_b. The presence of multiple enhanced bands would suggest considerable flexibility in the 'specific'

* The anti-peptide antisera used in this study appeared to have a low affinity for 21K. Fusion proteins, expressing either sub-regions of, or the intact, U_S11 coding sequences, could be used as immunogens to generate higher affinity antisera against the products of gene U_S11.

cleavage reaction.

In conclusion, we have demonstrated a DNA-protein interaction occurring within the 'a' sequence which does not involve the 'a' sequence-specific 21K/22K DNA-binding proteins. This interaction appears to involve, at least in part, a region within U_b which is highly conserved between different herpes viruses. However, much remains to be done to clarify the nature of this interaction and its possible significance with respect to the many functions associated with the HSV 'a' sequence.

Future work

The intense localisation of the 21K polypeptide to the nucleolus of infected cells raises a number of questions concerning its function. This localisation would not be predicted for an 'a' sequence-specific DNA-binding protein, since viral DNA is not present within the nucleolus. The fact that the 21K polypeptide binds to the 'a' sequence *in vitro* need not necessarily imply that it does so *in vivo*. Nevertheless, it would be important to investigate in more detail the specific interaction between the 'a' sequence and 21K. It would also be of interest to determine whether 21K binds to RNA. Since 21K is non-essential in tissue culture, it should be possible to construct deletion mutants in gene U_S11 with a view to determining whether specific sequences are important for its nucleolar localisation. In addition, it would be interesting to determine whether this polypeptide, in the absence of other viral products, would localise to nucleoli of uninfected cells. *

The DNase I footprinting experiments employed crude whole cell extracts. It is now important to use more highly purified preparations of protein, to reduce the problem of endogenous nuclease activity and to increase the sensitivity of the assay. This would also establish whether a specific cleavage event is generating the enhanced cleavages observed, or whether an exonuclease footprint is responsible. The relationship between the apparent binding within U_b and the DR_2 repeats remains to be established. The use of synthetic oligonucleotides representing these two regions in competition experiments should clarify this point. Similarly, competition experiments could also help to elucidate the role of the cellular transcription factor, Spl. In addition, the use of temperature sensitive mutants may help to determine which viral polypeptides, if any, are involved in the interactions observed.

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