Transcription of Herpes Simplex Virus Type 2

by

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Thesis submitted in partial fulfillment

for the degree of PhD.

University of Glasgow

July 1981

S U M M A R Y

To investigate the mechanisms by which HSV-2 mRNA synthesis is regulated, *in vivo* ^{32}P-labelled RNA isolated from virus-infected BHK 21 C13 cells was hybridized to virus DNA fragments generated by restriction endonucleases, using the Southern blot hybridization technique (Southern, 1975). This hybridization data, together with physical maps for the DNA fragments generated by the various restriction endonucleases used in this analysis (Cortini and Wilkie, 1978; Wilkie *et al.*, 1979) enabled the position of the RNA transcripts to be located on the virus genome.

RNA isolated at early times (2 hr post absorption, prior to the onset of virus DNA synthesis) hybridized to DNA fragments from all regions of the genome, with the area from 0.57 to 0.62 map units abundantly represented. This conflicted with previous data obtained using liquid hybridization of excess RNA to virus DNA, which indicated that 21% of the genome (42% of the coding capacity assuming assymetric transcription) was represented by early HSV-2 RNA (Frenkel *et al.*, 1973).

Late RNA (isolated 10 hr post absorption, after the onset of virus DNA synthesis), also hybridized to DNA fragments from all regions of the genome, however there were quantitative differences in the relative abundance of RNA sequences hybridizing to individual DNA fragments between early and late RNA. Late RNA sequences from from the inverted repetitive regions, TR_L/IR_L and TR_S/IR_S, were located predominantly within the nucleus which reflects either specific sequestration within the nucleus, or specific degradation in the cytoplasm.

RNA synthesized in cells infected in the continuous presence of the protein synthesis inhibitor cycloheximide, termed immediate

early (IE) RNA, had a restricted hybridization profile, and represented mainly the repetitive regions flanking both the long (U_L) and short (U_S) unique genome regions. Hybridization of IL RNA to certain restricted regions of U_L and U_S was observed. A similar situation was found using *in vitro* labelled IE RNA, however some differences were detected between nuclear and cytoplasmic IE RNA which were not apparent with *in vivo* labelled RNA. Namely, sequences from TR_S/IR_S were located preferentially within the nucleus, while sequences from the junctions of U_S with TR_S/IR_S and a region of U_L adjacent to IR_L were located predominantly in the cytoplasm. The results do not agree with the liquid hybridization data of Frenkel *et al.* (1973) which suggested that 45% of the virus genome was represented in total IE RNA.

The restricted hybridization pattern of IE RNA was reflected in the number of virus polypeptides synthesized *in vitro* using this RNA. Four virus-induced polypeptides with apparent molecular weights of 178,000, 140,000, 116,000 and 65,000 were detected. These values are similar, but not identical to those of the equivalent polypeptides found *in vivo*, and this probably represents the absence of normal post-translational polypeptide modifications.

The blot hybridization and *in vitro* translation data indicated that the HSV-2 transcriptional programme was identical to that of HSV-1 determined using similar methods (Clements *et al.*, 1977; Preston, 1977). Thus, control of virus transcription is exerted by an off/on mechanism, as in the switch from IE to early phase, and by an abundance control, as evidenced by the differences in the relative abundance of RNAs which hybridize to various regions of the genome, both within and between the three replication phases studied.

HSV-2 IE RNA was further characterized by fractionation of polyadenylated in vivo labelled RNA on agarose gels containing CH_3HgOH. Three bands of 4.7 kb, 3.4 kb and 1.75 kb were detected, and these were located on the genome by hybridization to blot strips containing virus DNA fragments. The 4.7 kb mRNA hybridized to sequences from TR_S/IR_S (0.96-1.00 and 0.82-0.86 map units respectively), while the 3.4 kb mRNA hybridized to fragments containing sequences from TR_L/IR_L (0-0.04 and 0.77-0.82 map units respectively). The 1.75 kb RNA band hybridized to three distinct regions of the genome; at U_L near the junction with IR_L (0.73-0.74 map units), and at the junctions of U_S with both TR_S and IR_S (0.85-0.88 and 0.94-0.97 map units respectively). The locations of the major HSV-2 IE mRNAs were identical to those of the equivalent HSV-1 IE mRNAs (Watson et al., 1979; Anderson et al., 1980a).

The genome locations of these mRNAs were confirmed by hybridizing labelled virus DNA fragments to RNA immobilized on activated diazobenzoyloxymethyl paper. This procedure allowed the detection of two additional minor IE mRNAs of 5.0 kb and 4.3 kb located within the region 0.28-0.40 map units, and three mRNAs of 7.0 kb, 1.75 kb and 1.25 kb in the region 0.57-0.62 map units.

Synthesis of cDNA initiated on polyadenylated HSV-2 IE RNA using oligo $(dT)_{10}$ as primer resulted in a product of small size consisting of sequences complementary to the 3'-termini of the mRNAs. Hybridization of this cDNA to blot strips allowed these mRNAs to be oriented on the genome. The 4.7 kb IE mRNA located within TR_S/IR_S was transcribed towards, but not into the terminally redundant "a" sequence, and the 3.4 kb mRNA located in TR_L/IR_L was transcribed towards U_L. The third 1.75 kb IE mRNA, located within U_L, was oriented using a cloned DNA fragment, and was transcribed

towards IR_L. Orientations of several minor IE mRNAs also were determined.

The structure of the 1.75 kb IE mRNA located in U_L was analysed using the Berk and Sharp (1977) nuclease S1 technique with end-labelled, cloned virus DNA fragments. The mRNA was 1640 nucleotides in length, and appeared to be unspliced throughout its entire length. The genomic DNA sequence at the 3'-end of this mRNA was determined, and the end of the mRNA was located precisely. The DNA region sequenced contained two out-of-phase termination codons, one of which formed part of the AAUAAA hexanucleotide found near the 3'-end of most polyadenylated eukaryotic mRNAs.

ABBREVIATIONS

Abbreviations for media and solutions are given in Materials. All temperatures are given in degrees Centigrade.

ATP	adenosine-5'-triphosphate
BHK	baby hamster kidney 21 C13 cells
bis-acrylamide	N, N'-triethylene-bisacrylamide
BMV	bovine mammillitis virus
bp	base pairs
BSA	bovine serum albumin
CCV	channel catfish virus
cDNA	complementary DNA; reverse transcribed DNA.
Ci	curie
cm	centimetre
CMV	cytomegalovirus
cpm	counts per minute
CTP	cytosine-5'-triphosphate
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytosine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
DTT	dithiothreitol
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EHV-1	equine herpes virus type 1 (equine abortion virus)
g	gravity
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

hr	hour
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HVA	herpesvirus ateles
HVS	herpesvirus saimiri
IE	immediate early
kb	kilobase(s)
l	litre
M	molar
MDV	Marek's disease virus
min	minute
ml	millilitre
mm	millimetre
m.o.i.	multiplicity of infection
mRNA	messenger RNA
NP-40	Nonidet P40
NPT	non-permissive temperature
PAGE	polyacrylamide gel electrophoresis
p.f.u.	plaque forming units
PIPES	piperazine-N-N'-bis(2-ethanesulphonic acid)
$^{32}PO_4$	(^{32}P) orthophosphate
POPOP	1, 4 bis (2- (5-phenyloxazoyl) benzene
PPO	2, 5 diphenyloxazole
PrV	pseudorabies virus
PT	permissive temperature
RNA	ribonucleic acid
RNAase	ribonuclease
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SV 40	simian virus 40

TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
ts	temperature sensitive
v/v	volume:volume (ratio)
VZV	varicella-zoster virus
w/v	weight:volume (ratio)

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Barklie Clements for his invaluable guidance throughout this project, and Professor J. H. Subak-Sharpe for his overall supervision. I also wish to thank all of the members of the Institute of Virology, particularly those of lab. 204 for making my time in Glasgow both stimulating and entertaining. Finally I would like to thank my wife, Susan, for her patience and for typing this manuscript.

During this work the author was supported by a Medical Research Council grant for training in research techniques, and all the results reported were obtained by the author's own efforts.

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I N T R O D U C T I O N

1. Herpesviridae

Members of the family Herpesviridae have icosadeltahedral capsids of 120-150 nm in diameter consisting of 162 capsomeres, surrounded by a lipid-containing envelope (Wildy et al., 1960). The capsid contains a single, linear, double-stranded DNA genome, and virus multiplication occurs in the nucleus, with enveloped virus being released into the cytoplasm (Roizman and Furlong, 1974; Fenner, 1976).

Herpesviruses have been observed in a wide variety of hosts, such as fungi (Kazama and Schornstein, 1972), oysters (Farley et al., 1972), fish (Wolf and Darlington, 1971), frogs (Lunger, 1964), birds (Nazerian and Burmester, 1968) and numerous mammals including man (Nahmias, 1972). A single host may be infected with several distinct herpesviruses e.g. man is the host for at least five viruses, herpes simplex virus (HSV) types 1 and 2, EBV, VZV and human CMV.

Classification of a virus as a member of the herpesviridae is based primarily on morphology with the members exhibiting considerable diversity at the biochemical level. The (G + C) content of herpesviruses span a range from 33% for canine herpesvirus to 75% for B-virus, and there is little DNA homology or common antigenicity between most of the viruses (Honess and Watson, 1977). A provisional classification based mainly on biological properties of the viruses has been proposed (Roizman et al., 1978) which provides for three subfamilies.

a) Alphaherpesvirinae

These viruses are characterized by a short replicative cycle and exhibit host ranges extending from very wide to narrow. After an infection of variable severity in the natural host, these viruses often establish latent infections in neural tissue from which they can be reactivated. The prototype of this subfamily is HSV-1, and HSV-2, VZV and PrV are also members.

b) Betaherpesvirinae

Viruses of this subfamily grow slowly with a restricted host range, and on infection cause enlargement of the infected cell. As with members of the alphaherpesvirinae, these viruses often persist for long periods after the initial infection. Most viruses in this group are cytomegaloviruses, and human cytomegalovirus is the prototype.

c) Gammaherpesvirinae

Members of this group can replicate in lymphoblastoid cells, and often cause lymphoproliferative diseases in the natural host, for which they are usually very specific. These herpesviruses often persist in the natural host where they are specific for either T-lymphocytes (MDV) or B-lymphocytes (EBV). These viruses may also cause malignant lymphomas.

Herpes simplex viruses have been shown to exist as two serotypically distinct viruses, HSV types 1 and 2, which differ in their site of infection (Dowdle et al., 1967). HSV-1 is usually transmitted by a nongenital route, occurring frequently during childhood, at sites above the waist, primarily as oral and facial lesions, while HSV-2 is usually transmitted venereally, and occurs most often in adolescents and young adults at sites below the waist, primarily the genitals.

Although HSV-1 and HSV-2 can be distinguished serotypically, they have both type-specific and type-common antigenic determinants (Savage et al., 1972; Sim and Watson, 1973; Melnick et al., 1976). The major capsid protein showed type-common antigenicity (Melnick et al., 1976), as did a protein of approximately 50,000 molecular weight (Honess and Watson, 1974); Cohen et al., 1978), whereas a non-structural early HSV-1 polypeptide of 175,000 molecular weight, and a structural HSV-1 glycoprotein of 123,000 molecular weight showed type-specific

antigenicity (Melnick et al., 1976). Cross-absorption studies using recombinants of HSV-1 and HSV-2 enabled Halliburton et al. (1977) to show a minimum of four HSV-1 and three HSV-2 type-specific antigenic sites. Killington et al. (1977), by cross neutralization studies, showed that BMV was antigenically related to both HSV-1 and HSV-2. No cross reactivity could be found between PrV or EHV-1 and either HSV-1, HSV-2 or BMV.

It is possible to differentiate between HSV-1 and HSV-2 and other herpesviruses on the basis of the different patterns of the virion and infected cell polypeptides after polyacrylamide gel electrophoresis (Cassai et al., 1975; Killington et al., 1977; Powell et al., 1977). In addition, the HSV-1 and HSV-2 genomes differ sufficiently from each other to provide alternative methods of distinguishing between the two viruses. Both virus DNAs have a very high G + C content, of 67% for HSV-1 and 69% for HSV-2 (Russell and Crawford, 1963; Goodheart et al., 1968; Kieff et al., 1971), and the different DNA fragment patterns generated by digestion of HSV-1 and HSV-2 DNAs with various restriction endonucleases are extremely useful for virus typing (Skare et al., 1975; Hayward et al., 1975; Lonsdale, 1979).

HSV-1 has been isolated from elements of the human sensory, autonomic and central nervous systems (Bastian et al., 1972; Baringer, 1975; Lonsdale et al., 1979), and is considered to be primarily associated with the trigeminal ganglia, while HSV-2 which is associated with the sacral ganglia (Baringer, 1974) establishes latent infections in the central nervous system and sensory ganglia of mice and rabbits (Knotts et al., 1973), and virus has been reactivated from tissue explants culture in vitro (Stevens and Cook, 1971). HSV-specific DNA has also been shown to be present in latently infected ganglia (Stevens and Cook, 1974).

2. Structure of the Herpesvirus Genome

Virus genome replication occurs within the nuclei of infected cells (Munk and Sauer, 1964), and commences at about 2 hr post-absorption in HSV-1 strain 17-infected BHK cells, with a peak at 5-6 hr post absorption (Hay, 1979). In cells infected with HSV-2 strain HG-52, replication begins at 2-3 hr post absorption with a peak at approximately 9 hr (Rixon, 1977).

The HSV genome consists of a linear duplex DNA molecule (Russell, 1962; Ben-Porat and Kaplan, 1962). Size estimates obtained by electron microscopy and centrifugation of HSV-1 DNA on neutral sucrose gradients gave molecular weight values of approximately 100×10^6 (Becker et al., 1968; Kieff et al., 1971; Wilkie, 1973). The HSV-2 genome was estimated to have a molecular weight of $99 \pm 5 \times 10^6$ on neutral sucrose density gradients (Kieff et al., 1971). Estimates obtained by summing the molecular weights of DNA fragments generated by restriction endonucleases gave values of 96.8×10^6 for HSV-1 and 98.5×10^6 for HSV-2 (Clements et al., 1976; Cortini and Wilkie, 1978; A. J. Davison, personal communication).

HSV-1 and HSV-2 show DNA homology estimated at between 20-70% as determined by DNA-DNA hybridization (Kieff et al., 1972; Ludwig et al., 1972; Sugino and Kingsbury, 1976), and at 40% by RNA-DNA hybridization (Bronson et al., 1972). A commonly accepted value is 50% homology (Kieff et al., 1972), and this is distributed throughout the genome, with certain localized areas of high homology (A.J. Davison, personal communication).

After release of HSV-1 DNA from virions, on banding in alkaline sucrose gradients, intact single stranded DNA with a molecular weight of 48×10^6 was detected, together with less rapidly sedimenting

material (Frenkel and Roizman, 1972; Wilkie, 1973), indicating the presence of alkali-labile phosphodiester bonds, or single-strand nicks or gaps in the DNA. Frenkel and Roizman (1972) reported that the intact material was non-complementary, and therefore consisted of only one DNA strand, while the smaller material was the product of nicks or gaps at non-random sites on the other strand. Wilkie (1973), however, concluded from liquid hybridization studies that both strands were represented in the intact DNA.

Alkali lability of certain bonds in HSV DNA may be due to the presence of ribonucleotides (Hirsch and Vonka, 1974), however, most labile sites are likely to be due to single-strand nicks and gaps, since 50% can be repaired using a mixture of DNA ligase and Micrococcus luteus DNA polymerase (Hyman et al., 1977).

Partial digestion of HSV-1 DNA with λ-exonuclease or exonuclease III generates linear molecules capable of circularizing on annealing, and these were vizualized by electron microscopy (Grafstrom et al., 1974; Sheldrick and Berthelot, 1974; Wadsworth et al., 1976). This was interpreted to indicate the presence of a direct terminal redundancy at both ends of the genome. The terminal redundancy was termed the "a" sequence, and had an estimated length of 400-800 bp (Grafstrom et al., 1975; Wadsworth et al., 1976). Following treatment with λ-exonuclease, DNA molecules were observed with a small single-stranded loop of approximately 700 nucleotides at one end, suggesting that the terminal redundancy was present as an internal inverted repeat at least at one end of the genome (Hyman et al., 1976; Wadsworth et al., 1976).

Electron microscopy also revealed the presence of much larger inverted repetitions within self-annealed, intact, single-stranded HSV-1 DNA (Sheldrick and Berthelot, 1974; Wadsworth et al., 1975).

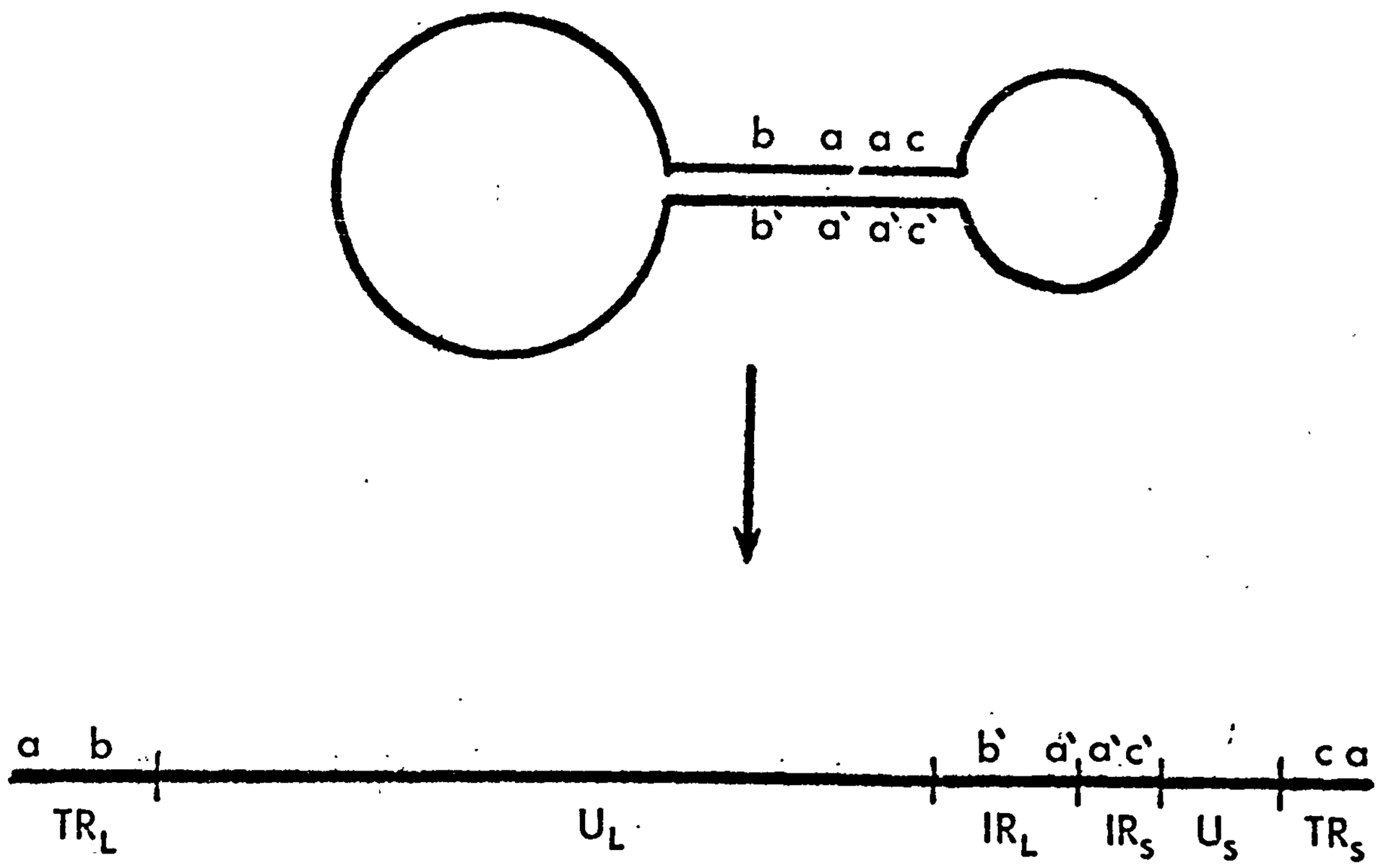


FIGURE 1

Model of HSV genome structure. Taken from Sheldrick and Berthelot (1974).

The structures formed included a large and a small single-stranded circle, joined by a region of double-stranded DNA. From these observations, a model for the structure of the HSV-1 genome was proposed consisting of two unique regions (U_L and U_S) each bounded by repetitive regions (TR_L/IR_L and TR_S/IR_S respectively) with the repetitive regions present as internal conjoint inversions (Sheldrick and Berthelot, 1974; Wadsworth et al., 1975). The repetitive regions are termed b (TR_L) and c (TR_S) and the internal inversions b' and c' respectively. A diagram of this structure is shown in Figure 1.

A prediction of this model was that inversion of the unique U_L and U_S regions could occur by either intramolecular or intermolecular recombination of the homologous repetitive regions (Sheldrick and Berthelot, 1974). These inversions would generate a population of virus DNA molecules with four possible orientations of the two unique DNA regions with respect to each other and these are shown in Figure 2.

A further prediction of this model is that the distance between two loci, one of which lies in U_L and the other in U_S, depends on the relative orientation of the two unique regions (Figure 2). Restriction endonucleases which cleave HSV DNA in either one or both unique regions, and which also cleave within one set of the repetitive regions or not at all in the repetitive regions, will generate certain DNA fragments in less than molar quantities. Similarly, restriction endonucleases which cleave the DNA at least twice in one or both repetitive regions will generate certain DNA fragments in two molar quantities. Fragments with molarities corresponding to these cleavage patterns have been detected by gel electrophoretic analysis of the restriction

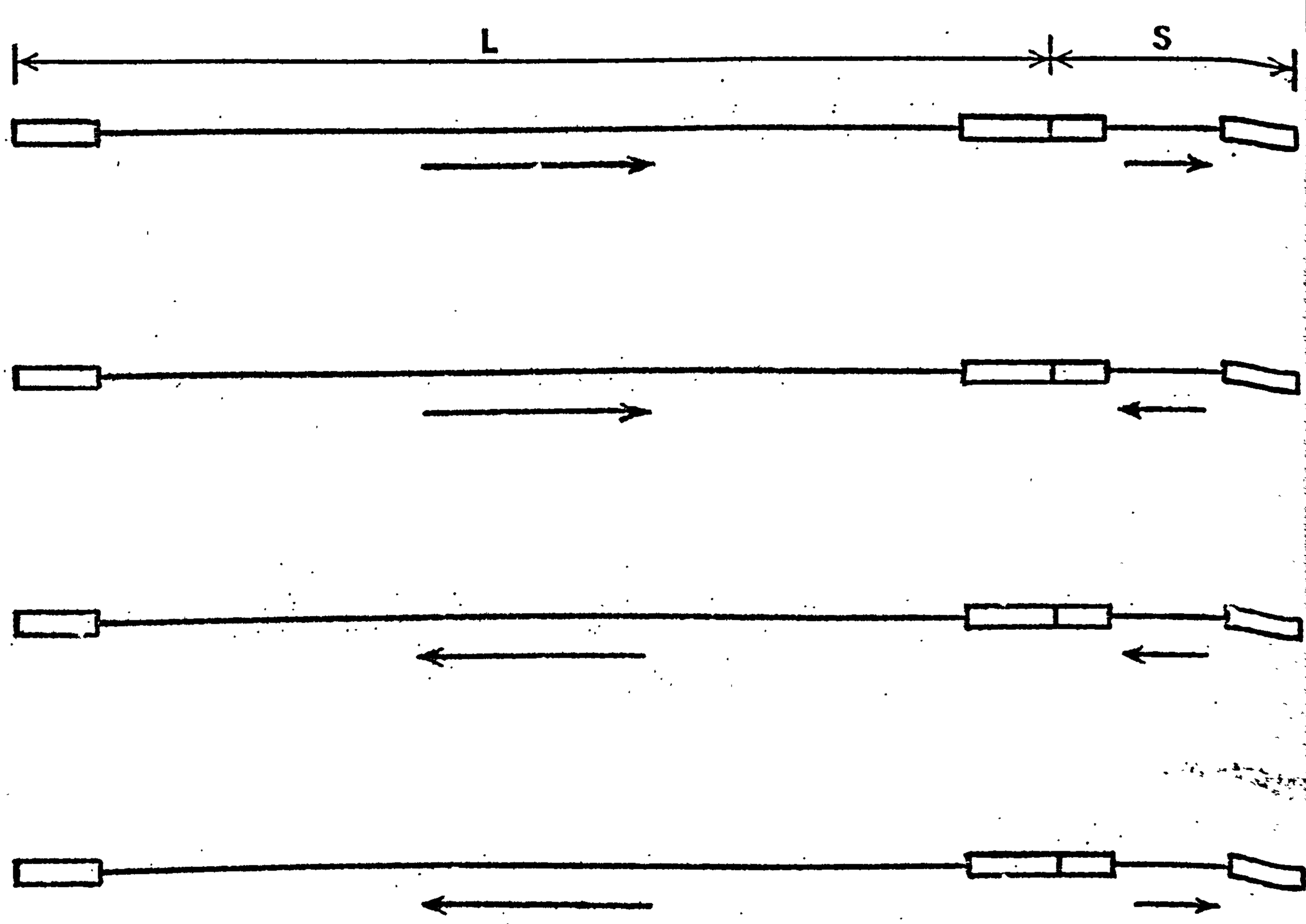


FIGURE 2

Four possible structures of HSV DNA determined by the relative orientations of the L and S segments.

endonuclease digestion products of HSV-1 and HSV-2 DNAs indicating that both viruses share a common genome structure (Hayward et al., 1975; Clements et al., 1976; Cortini and Wilkie, 1978). The relative amounts of the sub-molar fragments indicated that the four genome arrangements shown in Figure 2 were present in equal amounts.

Therefore by virtue of the HSV genome structure several DNA fragments produced by digestion with a restriction endonuclease may contain sequences in common.

This model of the genome structure was confirmed by partial denaturation mapping of HSV-1 DNA, and it was shown that the TR_S/IR_S region was of higher (G + C) than TR_L/IR_L, suggesting that the two sets of repetitive regions are not completely homologous (Delius and Clements, 1976). Physical maps and DNA-DNA hybridization also indicated that TR_L/IR_L and TR_S/IR_S share only limited homology (Skare and Summers, 1977; Wilkie, 1976; Cortini and Wilkie, 1976). An alternative possibility is that the genome arrangements may arise as a consequence of the mechanism of DNA replication (Skare and Summers, 1977).

Although the HSV-1 and HSV-2 genome structures are similar, the molecular weight of HSV-2 DNA is slightly greater than that of HSV-1, and the additional DNA sequences are localized within U_S (A.J. Davison, personal communication). A great amount of heterogeneity in the sizes of certain restriction fragments has been observed between different HSV-1 and HSV-2 isolates, particularly in the repetitive regions (Hayward et al., 1975; Skare et al., 1975; Lonsdale et al., 1979; S.J. Chaney, personal communication). This heterogeneity is likely to be due to the presence of variable copy numbers of short, tandem, direct reiterations found in TR_S/IR_S of the HSV-1 genome (A.J. Davison and N.M. Wilkie, manuscript submitted).

By comparison of the physical maps of the termini and the joint region (the junction between the L and S segments, see Figure 2) for HSV-1 DNA, Wagner and Summers (1978) calculated the terminal redundancy ("a" sequence) to be 265 bp for HSV-1 strain KOS. Size heterogeneity at the joint region was observed, and this was considered to be due to insertion of a 260 bp segment, containing the "a" sequence and a small amount of DNA from TR_L (Wagner and Summers, 1978).

Recent DNA sequence analysis at the joint of HSV-1 strain 17 DNA has shown the "a" sequence to consist of a variable number of tandem repeats of 12 bp units, varying in total size from 327-459 bp (A.J. Davison and N.M. Wilkie, manuscript submitted). The size of the HSV-2 strain HG52 "a" sequence is 252 bp, with no repeating unit, and it is invariant in size (Davison and Wilkie, manuscript submitted). In the HSV-1 and HSV-2 genomes, the "a" sequence can be found in single or multiple copies at the joint region in a stock of plaque-purified virus. This can be seen clearly with the Bam HI digest of HSV-2 strain HG-52 DNA; the joint fragment is Bam HI g, and the fragment Bam HI $\overline{g}$ (Figure 21) has been shown to be the joint fragment containing two copies of the "a" sequence (Davison and Wilkie, manuscript submitted).

Bovine mammillitis virus shares 14% DNA homology with HSV-1 (Stertz et al., 1973/74) and the genome consists of two covalently linked components of 71.5×10^6 and 15.7×10^6, designated L and S respectively (Buchman and Roizman, 1978). The DNA consists of four equimolar populations differing only in the orientation of L and S indicating that the DNA structure is similar to that of HSV DNA (Buchman and Roizman, 1978).

Human cytomegalovirus has a genome of approximately 150×10^6 molecular weight (De Marchi et al., 1978) and consists of a long

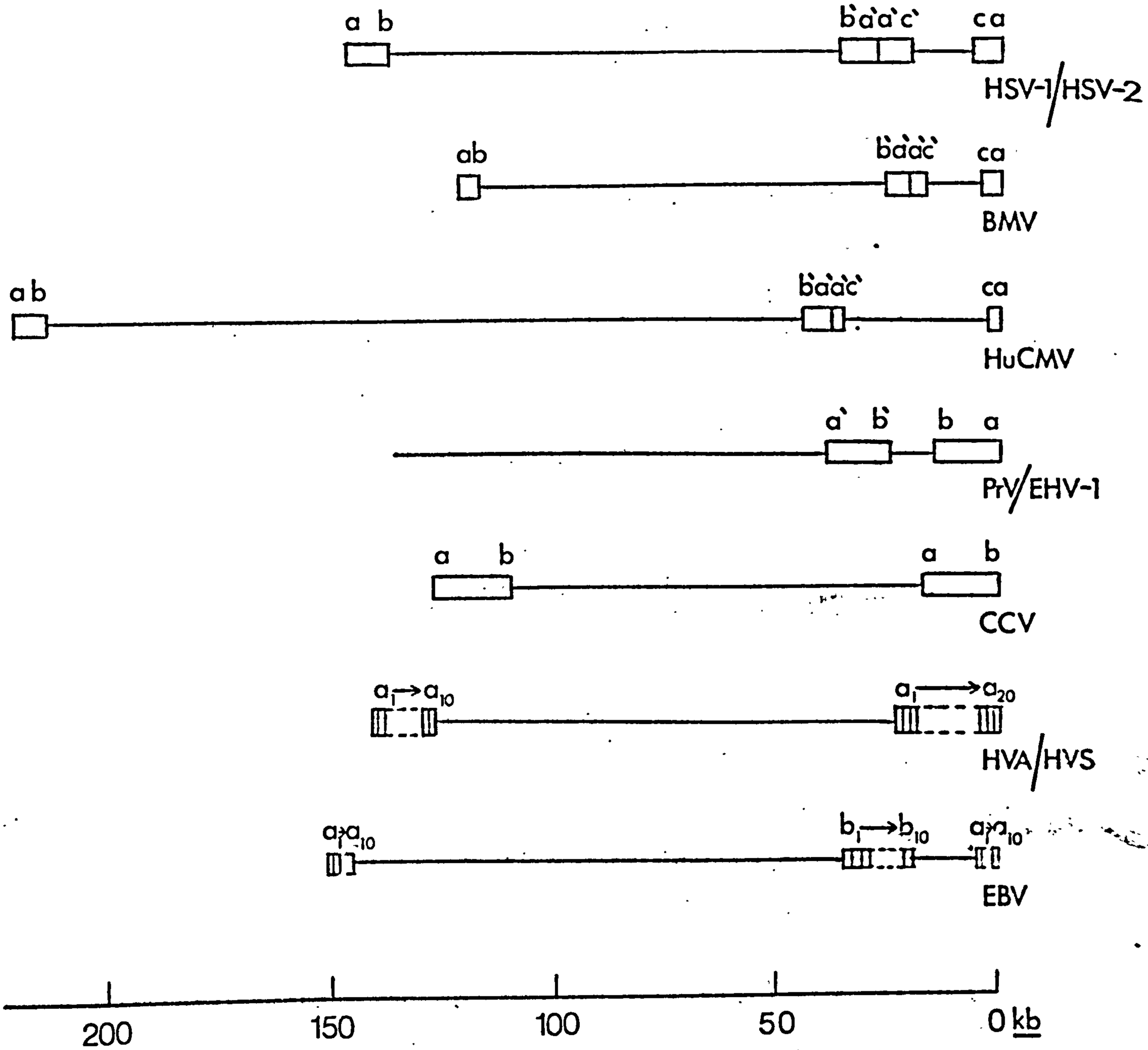


FIGURE 3

Structures of the genomes of various herpesviruses. The dashed lines in the HVA/HVS and EBV genomes indicate variability in the number of the tandem repeat units.

unique sequence of 115 x 10^6 and a short unique sequence of 22 x 10^6. Each unique sequence is bounded by a repetitive region which is present as an internal inversion and four equimolar populations of DNA molecules were observed, similar to HSV DNA (Weststrate et al., 1980).

This genome structure is not, however, common to all herpesviruses. The DNAs of PrV and EHV-1 consist of L and S segments, however both lack TR_L and contain inverted repetitive regions which bound only the U_S region (Stevely, 1977; Ben-Porat et al., 1979; Powell, 1980; J.M. Whalley and A.J. Davison, manuscript submitted). This enables U_S to invert with respect to U_L, but the orientation of U_L is fixed.

Channel catfish virus DNA consists of a large unique region of 62 x 10^6 molecular weight flanked at each end by a 12 x 10^6 molecular weight direct repetition (Chousterman et al., 1979), while the herpesvirus saimiri- and herpesvirus ateles-M genomes each consist of a long unique region bounded by multiple tandem reiterations of a simple, high (G + C) sequence (Bornkamm et al., 1976; Fleckenstein et al., 1978). The number of these tandem reiterations is variable such that the length of the total repeat unit is heterogeneous, and the structure of the repeating unit may be strain specific in herpesvirus ateles (Fleckenstein et al., 1978).

The genome structures of several herpesviruses are compared in Figure 3.

3. Defective Herpesvirus Genomes

Defective interfering particles have been found in most animal virus systems studied (Huang, 1973). The particles usually contain only part of the wild-type virus genome, which is packaged using

normal virus structural proteins, but these are unable to reproduce in the absence of a helper virus, though, as their name suggests, they interfere with the growth of wild-type virus.

Continuous undiluted serial passage of clonally purified HSV-1 and HSV-2 produces an increase of the particle:p.f.u. ratio as a result of a decrease in the yield of infectious virus. Often this is associated with the production of DNA with a greater buoyant density than virus DNA. Diluted passage of these virus preparations increases infectious virus yields, with a concomitant loss of the high density DNA (Bronson et al., 1973; Frenkel et al., 1975; Locker and Frenkel, 1978). Several HSV-1 defective virus preparations have been studied in some detail.

The defective virus preparations generated from HSV-1 strain Justin and HSV-1 strain Patton contained DNA with molecular weights similar to that of standard virus, however the DNAs consisted of multiple tandem repeats of sequences from within TR_S/IR_S together with a small region from one of the junctions of U_S with TR_S/IR_S. These tandem repeats were arranged in head to tail arrays (Frenkel et al., 1976; Graham et al., 1978). The defective stock of HSV-1 strain Justin overproduced the immediate early polypeptide ICP 4 (175×10^3 molecular weight (Frenkel et al., 1975)). Two different defectives have been generated using HSV-1 strain Angelotti (Kaerner et al., 1979). One was similar to those described above, containing sequences from TR_S/IR_S and U_S, while the other contained additional sequences from U_L. This analysis was complicated, however, due to homology between TR_S/IR_S and U_L in the virus strain used.

All of the defective HSV DNAs analysed have contained sequences from TR_S/IR_S and this may be due to the presence of an origin of virus DNA replication in this region (Frenkel et al., 1976).

Two defective preparations of PrV have been well characterized. These were produced independently by undiluted serial passage of the same parental plaque-purified wild-type virus stock, and had different DNA structures (Ben-Porat et al., 1974; Rixon and Ben-Porat, 1979). One of the defective viruses contained multiple copies, possibly tandem repeats, of a sequence from the end of U_L linked to sequences from the middle of U_L. The other contained additional sequences from the inverted repetitive regions, and also from U_S (Rixon and Ben-Porat, 1979). These defectives overproduced certain RNA species and polypeptides, but when mixed with standard virus prior to infection, these appeared at the appropriate time during the infectious cycle, though at elevated levels (Rixon et al., 1980).

EHV-1, when passaged in hamster cells at a moderately high m.o.i., produced defective virus containing high density DNA. Sequences in this defective DNA were a subset of the normal virus DNA, and the defective particle lacked two normal virion polypeptides, one a nucleocapsid component, and the other an envelope protein (Campbell et al., 1976).

In preparations of herpesvirus saimiri, two types of DNA molecule were isolated, H-DNA of very high (G + C), and M-DNA of lower (G + C). Both DNAs were the same length as measured by electron microscopy (Fleckenstein et al., 1975; Bornkamm et al., 1976). Only the M-DNA was infectious, and the H-DNA consisted of multiple reiterations of the repeating unit found at the end of the virus M genome (Fleckenstein et al., 1975).

4. HSV RNA Biosynthesis

a) RNA Metabolism in Infected Cells

After infection with HSV-1, there is an overall decrease in the rate of RNA synthesis (Roizman et al., 1965), with a decrease in the synthesis of 28S and 18S ribosomal RNAs, polydisperse cytoplasmic, and 4S RNAs (Wagner and Roizman, 1969b; Roizman et al., 1970). The rate of processing 45S ribosomal RNA is diminished (Wagner and Roizman, 1969b), as is the stability of pre-existing cytoplasmic mRNA (Nishivka and Silverstein, 1977). Induction of a 380 nucleotide RNA, probably of host origin early during infection has been reported (Talley-Brown and Millette, 1979). The in vitro activity of both α-amanitin-sensitive and -resistant RNA polymerases decreased in nuclei isolated from HSV-1-infected LS cells (Preston and Newton, 1976), although this effect was not observed in nuclei isolated from KB or HEp-2 cells 8 hr after infection (Alwine et al., 1974). Increased in vitro RNA polymerase activity was observed with nuclei from infected as compared to uninfected cells (Ben Zeev et al., 1976).

A virus-induced factor, requiring virus protein synthesis for activity, and which preferentially inhibits the host RNA polymerase I (associated with the expression of ribosomal RNA genes) has been described in extracts of HSV-1-infected KB cells (Sasaki et al., 1974). This inhibitor may be involved in the reduction of rRNA synthesis observed in infected cells.

HSV transcripts are synthesized in the nucleus, and appear in the cytoplasm 10-15 min later (Wagner and Roizman, 1969a). By 2 hr post infection, 5% of the total nuclear RNA, and 25-30% of the polyadenylated nuclear RNA is virus-specific (Stringer et al., 1977); 60-70% of the polyadenylated polyribosome-associated RNA is virus-specific at 2 hr post infection. Late in infection (6 hr post-

infection, after the onset of virus DNA replication), 20% of the total nuclear RNA, and 70-90% of the polyadenylated polyribosome-associated RNA is virus-specific (Stringer et al., 1977).

Most of the nuclear RNA from infected cells was larger on sucrose gradients than polyribosomal RNA (Wagner and Roizman, 1969b), and similar virus sequences were represented in both the high molecular weight nuclear RNA and polyribosomal RNA (Wagner and Roizman, 1969a). However, no direct precursor-product relationship was established for these two classes of RNA by pulse-labelling experiments (Wagner and Roizman, 1969a). This suggested that some virus RNA transcripts were synthesized as small molecules, or that the processing is very rapid.

b) RNA Polymerase(s) Required for Virus Transcription

Herpesvirus particles do not contain any detectable RNA polymerase activity, and transcription of the virus genome immediately following infection is likely to be performed by pre-existing host cell enzymes. This is supported by the finding that naked HSV DNA is infectious (Graham et al., 1973; Sheldrick et al., 1973), as is that of herpesvirus saimiri (Fleckenstein et al., 1975).

Levels of RNA polymerases I, II and III in HSV-1-infected cells appear relatively constant, and no new RNA polymerase activities could be detected (Sasaki et al., 1974; Lowe, 1978). Muller et al. (1978) reported that levels of RNA polymerase I decreased by approximately 70% at 8 hr after infection of rabbit kidney cells with HSV-1, though no new polymerase activity could be detected.

Synthesis of virus-specific RNA in vitro using nuclei from HSV-1-infected cells was sensitive to levels of α-amanitin which specifically inhibit RNA polymerase II (Alwine et al., 1974; Ben Zeev

et al., 1976). The use of ∝-amanitin-resistant cell lines has further implicated RNA polymerase II in transcription of the virus genome (Ben Zeev and Becker, 1977; Constanzo et al., 1977). It is unclear whether this enzyme alone is required, or whether it is modified during infection.

c) Temporal Regulation of HSV Transcription

The herpesvirus replication cycle may be considered as comprising three phases, immediate early (IE), early and late. The IE phase occurs immediately following infection, before the onset of virus protein synthesis (or in the continuous presence of protein synthesis inhibitors). The early phase is that which occurs before the onset of virus DNA replication (or in the continuous presence of DNA synthesis inhibitors). The late phase occurs after the onset of virus DNA replication. None of these phases are necessarily mutually exclusive, events which occur during the IE or early phases may also occur at later times.

HSV transcription has been examined at these three stages primarily using two hybridization techniques:

Liquid hybridization, in which a small amount of radiolabelled virus DNA is hybridized with excess non-radioactive RNA from infected cells, in liquid phase, to give an estimate of the genome proportion represented in RNA sequences.

Blot hybridization as developed by Southern (1975), in which radiolabelled RNA is hybridized to virus DNA fragments generated by restriction endonucleases and bound to a nitrocellulose membrane.

(i) Early RNA

Using the liquid hybridization technique, two abundance classes

of early RNA were detected in HSV-1-infected cells (Frenkel and Roizman, 1972b; Swanstrom and Wagner, 1974; Swanstrom et al., 1975). The abundant RNA class was transcribed from 14% of the virus DNA (equivalent to 28% of the single strand), with the scarce class representing a further 30% of the virus DNA (Frenkel and Roizman, 1972b). Abundant RNA was present in 136-fold excess with respect to the scarcer class (Frenkel et al., 1973). Swanstrom et al. (1975) described an abundant class of early HSV-1 RNA representing 20% of the virus DNA, and which was present in a molar concentration 10-15-fold that of the less abundant class.

Blot hybridization experiments demonstrated that early RNA hybridized to virus DNA fragments located throughout the genome (Clements et al., 1977). The hybridization was not uniformly distributed, with some regions of the genome represented more abundantly than others.

Virus RNA made in the presence of DNA synthesis inhibitors is similar to RNA isolated at early times during infection (Wagner et al., 1972; Murray et al., 1974; Clements et al., 1977; Holland et al., 1980). Jones and Roizman (1979), using liquid hybridization, however, reported that nuclear RNA from cells infected with HSV-1 in the presence of phosphonoacetic acid, an inhibitor of virus DNA synthesis, represented only 39% of the genome, while cytoplasmic RNA represented 26%. Similarly, nuclear RNA synthesized in the presence of canavanine, an argenine analogue, represented 33% of the virus genome, and cytoplasmic RNA, approximately 19% (Jones and Roizman, 1979).

The proportion of the HSV-2 genome transcribed at early times, as analysed by liquid hybridization, was reported to be markedly different to that determined for HSV-1. RNA isolated 2 hr post-infection from HSV-2-infected cells contained a single abundance class

which hybridized to only 21% of the virus DNA (Frenkel et al., 1972; Frenkel et al., 1973). However blot hybridization of HSV-2 early RNA indicated that fragments from all regions of the virus genome were represented to varying amounts in the RNA sequences (Bodemer and Bodemer, 1979).

(ii) Late RNA

At late times during infection with both HSV-1 and HSV-2, the sequences represented in early RNA are still present (Frenkel and Roizman, 1972b; Wagner, 1972; Murray et al., 1974).

RNA isolated late during infection with HSV-1 comprised two abundance classes, the most abundant representing 19% of the virus DNA, and the least abundant, 28% (Frenkel and Roizman, 1972b; Frenkel et al., 1973). The major abundance class was present in a 40-fold (Frenkel et al., 1973) or 3-fold (Swanstrom et al., 1975) molar equivalent excess over the minor class. Fractionation of infected cells showed that while sequences from all regions of the genome were represented at late times (Jones and Roizman, 1979), some sequences were either preferentially retained in the nucleus, or rapidly degraded within the cytoplasm (Kozak and Roizman, 1974; Jones et al., 1977; Jones and Roizman, 1979). Thus, 50% of the virus DNA (the total coding capacity assuming assymmetric transcription) was represented in nuclear RNA, whereas only approximately 40% was represented in cytoplasmic RNA.

Blot hybridization experiments also showed that late RNA hybridized to all of the virus DNA fragments (Oakes et al., 1976; Clements et al., 1977), with different relative amounts of hybridization to fragments than found with early RNA. Clements et al. (1977) also demonstrated that RNA sequences from the joint region of the

virus genome were located preferentially within the nucleus.

RNA isolated late during infection with HSV-2, analysed using liquid hybridization, revealed two abundance classes representing a total of 50% of the virus DNA, similar to the situation described for HSV-1 (Frenkel et al., 1972; Frenkel et al., 1973). The abundant class of HSV-2 late RNA in contrast to the HSV-1 situation, drove 31% of the DNA probe into a hybrid, and was present in an 8-fold molar excess over the scarce RNA class. The remaining 19% of the virus genome was represented within the scarce class (Frenkel et al., 1973). Hybridization of HSV-2 late RNA to isolated virus DNA fragments showed that fragments from all regions of the genome were represented by RNA (Oakes et al., 1976; Bodemer and Bodemer, 1979).

(iii) Immediate Early RNA

The nuclear retention of specific RNA sequences described for HSV-1 late RNA was more dramatically observed with liquid hybridization analysis of IE RNA. Nuclear IE RNA drove approximately 50% of the virus DNA into a hybrid, whereas cytoplasmic IE RNA represented only 10% of the DNA (Kozak and Roizman, 1974). The sequences retained within the nucleus were not transported to the cytoplasm after removal of a cycloheximide block in the presence of Actinomycin D (Kozak and Roizman, 1974). More recent estimates obtained using liquid hybridization have suggested that 30% of the genome is present in nuclear IE RNA, with 12% represented as cytoplasmic IE RNA (Jones and Roizman, 1979). Swanstrom et al. (1975) however, by liquid hybridization detected abundant RNA representing 20% of the virus genome, with less abundant RNA hybridizing to other regions of the DNA.

Estimates of the proportion of the virus genome represented in

cytoplasmic IE RNA determined by the blot hybridization technique were similar to that of Swanstrom et al. (1975) of 20-25% (Clements et al., 1977; Watson et al., 1979). Moreover, the blot hybridization results obtained with nuclear IE RNA did not agree with those derived by liquid hybridization. The location of sequences and proportion of the virus DNA represented in nuclear RNA as determined by blot hybridization appeared to be almost identical to those of IE cytoplasmic RNA (Clements et al., 1977).

Liquid hybridization of total cellular HSV-2 IE RNA indicated that approximately 45% of the virus genome was represented in RNA sequences (Frenkel et al., 1973). The results of blot hybridization presented in this thesis do not support the results of liquid hybridization, and this is discussed later.

Two considerations must be borne in mind when interpreting the results of liquid hybridization experiments. Firstly, the amount of virus-specific RNA in the infected cell, and the degree to which the probe is driven into a hybrid by excess RNA, as an indication of the proportion of the genome present, is dependent on the multiplicity of infection (Kozak and Roizman, 1974; Jacquemont and Roizman, 1975a). Secondly, self-complementary virus RNA sequences are present in HSV-1-infected cells at late times (Kozak and Roizman, 1975; Jacquemont and Roizman, 1975a and b), though not at early times (Swanstrom et al., 1975). The resultant RNA-RNA hybridization reaction will therefore compete with the DNA-RNA reaction. Pre-annealing late nuclear RNA resulted in the loss of sequences available for hybridization, representing 15% of the virus DNA, and sequences representing 5% of the genome were lost on pre-annealing cytoplasmic RNA (Kozak and Roizman, 1975). After denaturation, the double-stranded RNA (prepared by RNA self-annealing followed by RNAase treatment) drove 50-55% of

virus DNA probe into a hybrid. This material consisted of at least two abundance classes representing 29% and 26% of virus DNA, differing 40-fold in molar concentration respectively.

Ts mutants have been used to further investigate the temporal regulation of HSV-1 transcription. Watson and Clements (1978) analysed the RNA synthesized by several ts mutants which were maintained at the NPT throughout infection. The results suggested that the mutants were blocked at various stages of infection, and indicated that more than one virus gene product was required to ensure that transcription progressed to the late stage (Watson and Clements, 1978). One mutant, ts K, at the NPT showed a transcriptional pattern resembling the IE pattern (Watson and Clements, 1978). Following infection with this mutant at the PT, cells were shifted up to the NPT at early and late times, and the transcriptional pattern reverted from the normal early or late pattern to the IE pattern (Watson and Clements, 1980). This indicated that at least one virus polypeptide was required throughout infection to ensure transcription of the whole virus genome. The mutation of ts K has been mapped by marker rescue in the TR_S/IR_S of the virus DNA (Stow et al., 1978; Stow and Wilkie, 1978). This region specifies an IE polypeptide of 175,000 molecular weight (Preston et al., 1978; Watson et al., 1979).

The 175,000 polypeptide (Vmw 175, ICP4) exists in three forms due to post-translational modification, with two forms observed predominantly within the nucleus (Pereira et al., 1977). After infection with ts K at the NPT, this polypeptide was not completely processed, and was located predominantly within the cytoplasm (Preston, 1979). Both of these defects were reversible on downshift to the PT, even in the absence of further protein synthesis (Preston, 1979).

Several ts mutants with lesions located within TR_S/IR_S have been

studied. These did not show complementation with ts K, and all were defective in the transition from IE to early and late stages of infection (Dixon and Schaffer, 1980). One of these mutants, ts B2, showed an IE pattern of transcription at the NPT (Holland et al., 1979).

The pattern of transcriptional regulation of PrV appears to be similar to those of HSV-1 and HSV-2, with restricted transcription from a limited part of the genome at immediate early times, followed by more extensive transcription at later times during infection (Rakusinova et al., 1971; Feldman et al., 1979; Powell, 1980), as does human cytomegalovirus (De Marchi et al., 1980).

d) Size, Genome Map Locations, and Polypeptides Specified by HSV mRNAs

Recently, the genome locations of certain accurately sized mRNAs have been quite precisely mapped, and the polypeptides encoded by some have been identified. The mRNAs have been studied by size fractionation and blot hybridization, selection by hybridization to specific virus DNA fragments, or a combination of these techniques. Following this, the purified mRNAs have been used to direct the translation of a polypeptide product in a cell free system. Hybrid arrested translation has also been used to assign polypeptide products to specific regions of the genome to which mRNAs have been mapped. The orientations of several virus transcripts on the genome have also been determined.

Using liquid hybridization, it was shown that HSV-1 cytoplasmic IE RNA hybridized to several noncontiguous regions of the virus genome, mapping in both L and S segments (Jones et al., 1977; Jones and Roizman, 1979). Some IE transcripts mapped entirely within

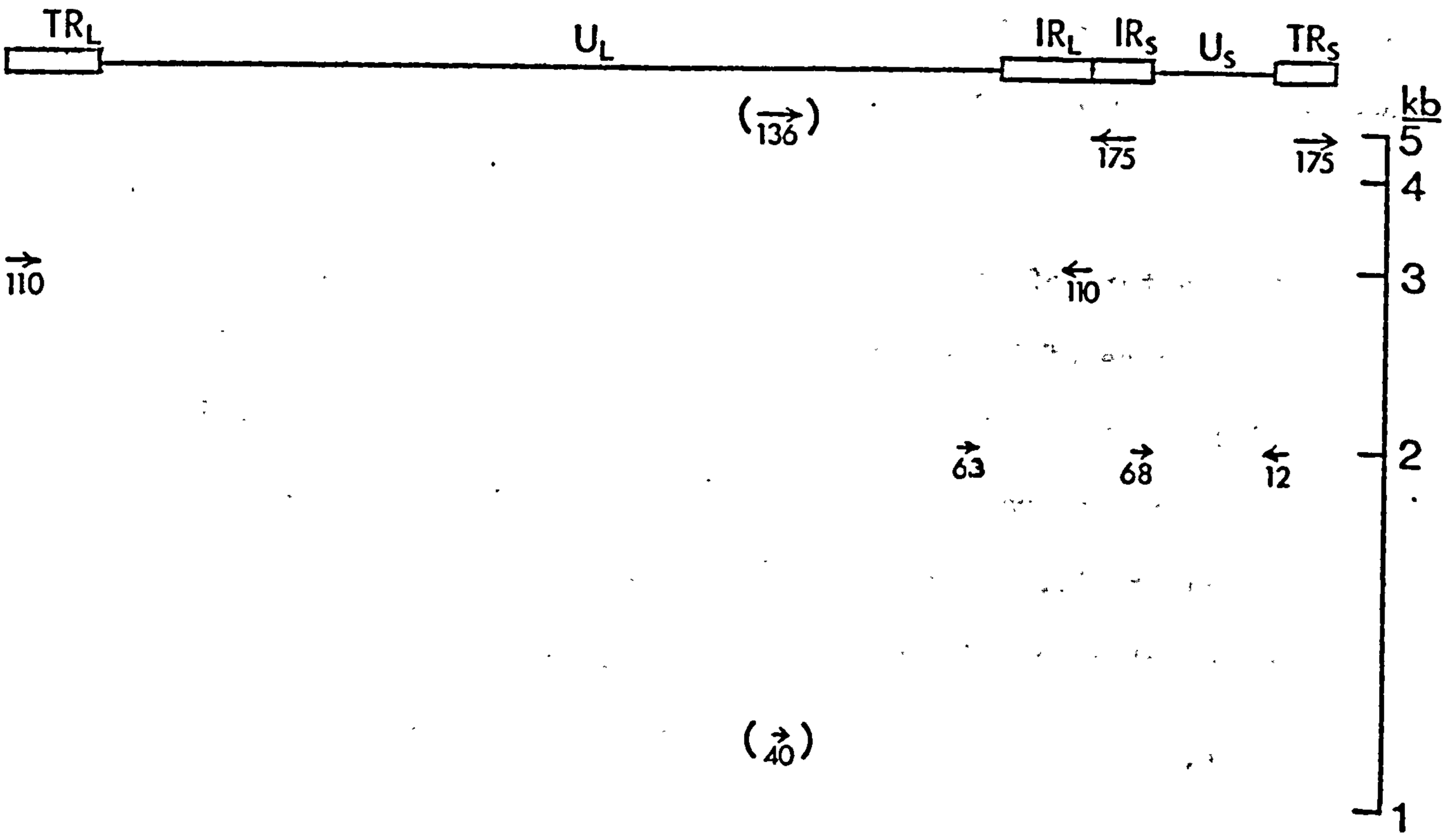


FIGURE 4

Sizes, genome locations and orientations of HSV-1 IE mRNAs. The sizes are indicated by the scale, and the length of the line. The molecular weights (x 10^{-3}) of the polypeptides encoded are given. The mRNAs enclosed by brackets are minor species.

the unique regions, whereas others were located, at least partly, within the repetitive regions (Jones et al., 1977; Jones and Roizman, 1979). Clements et al. (1977) reached similar conclusions using the blot hybridization technique. Subsequently, polyadenylated cytoplasmic IE RNA was size fractionated by electrophoresis through denaturing agarose gels containing methyl mercuric hydroxide. The mRNAs comprise three size classes, of approximately 4.7 kb, 3.0 kb and 2.0 kb (Watson et al., 1979). The RNAs were located on the virus genome by hybridization to nitrocellulose blot strips containing HSV-1 DNA fragments, and were also used to direct translation in vitro. The 4.7 kb mRNA encoded a 175,000 molecular weight polypeptide, and mapped entirely within TR_S/IR_S. The 3.0 kb mRNA encoded a 110,000 molecular weight polypeptide, and mapped entirely within TR_L/IR_L. The 2.0 kb RNA band contained three distinct mRNAs, one mapping in U_L near the junction with IR_L, encoding a polypeptide of 63,000 molecular weight, and two mapping across both junctions of U_S with TR_S and IR_S, one of which coded for a polypeptide of 68,000 and the other a polypeptide of 12,000 molecular weight (Watson et al., 1979).

The genome locations of these IE mRNAs were confirmed by Anderson et al. (1980a) using RNA purified by hybridization to excess HSV-1 DNA immobilized on cellulose, and it was possible to identify the IE mRNA coding for the 68,000 molecular weight polypeptide using this technique.

The orientations of four of the five major, and some of the minor, HSV-1 IE mRNAs were determined by hybridizing radiolabelled cDNA, prepared from a template of either total or size fractionated IE RNA using an oligo d(T) primer, to blot strips. The cDNA prepared in this way preferentially contains sequences complementary to the 3'-end of the mRNA, and thus allowed location of this end of the mRNA

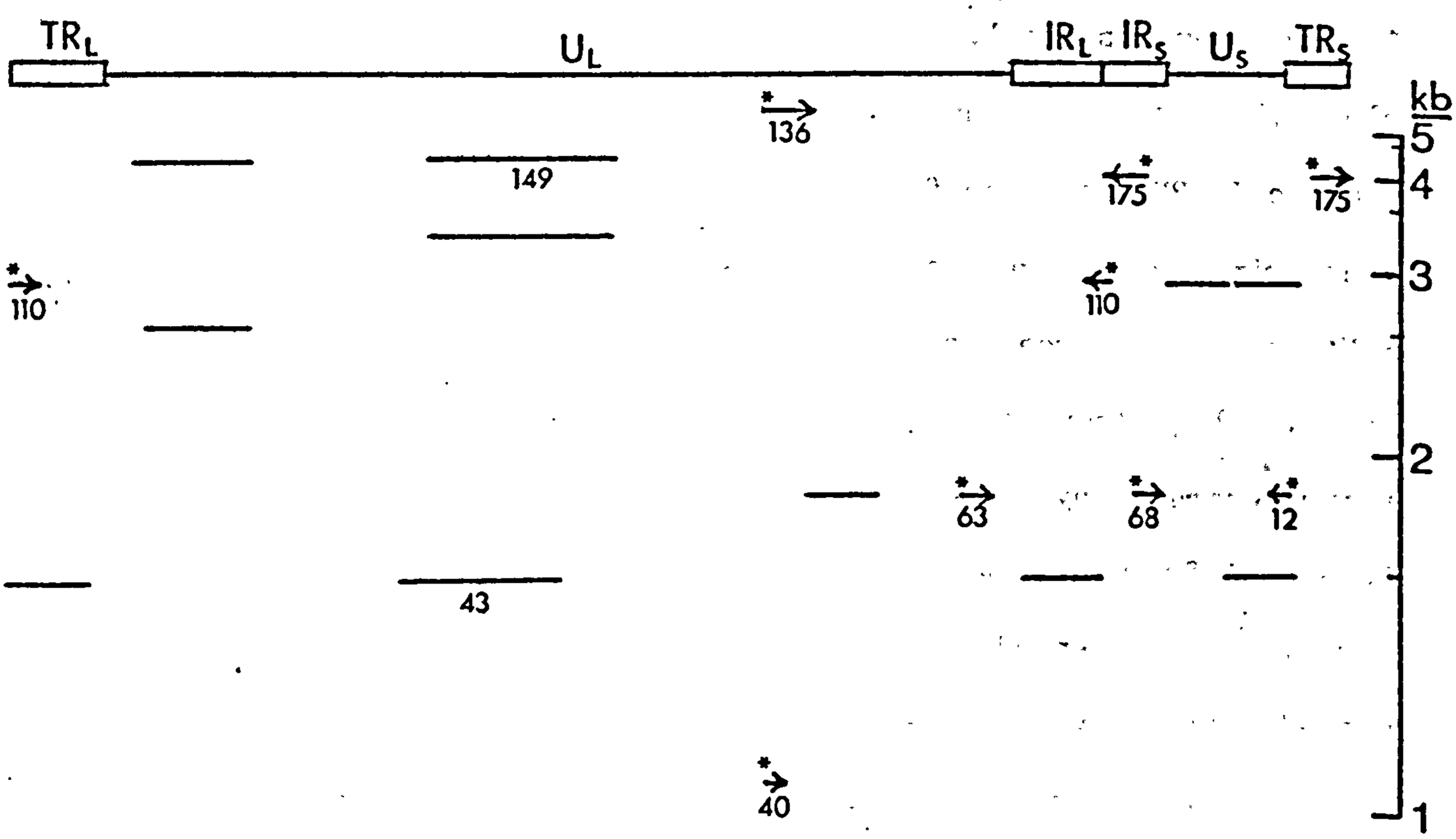


FIGURE 5

Sizes and genome locations of HSV-1 early mRNAs. The sizes are indicated by the scale, and the genome locations by the area shown by the lines. The molecular weights ($\times 10^{-3}$) of some of the polypeptides encoded are given. The mRNAs marked by an asterisk are also present in IE RNA.

(Clements et al., 1979; Anderson et al., 1980a). The mapping data for HSV-1 IE mRNA is summarized in Figure 4.

Evidence concerning the size, genome location and direction of transcription of HSV-2 IE mRNAs is presented in this thesis and is discussed later.

Polyribosome-associated HSV-1-specific RNAs isolated early during infection have been mapped on the virus genome by electron microscopy of RNA-displacement loop (R-loop) structures formed after hybridization of the RNA with virus DNA (Stringer et al., 1978). Early RNAs mapped throughout the genome, as indicated by other hybridization techniques (Clements et al., 1977; Jones and Roizman, 1979), but genome regions not represented in early RNA were observed (Stringer et al., 1978). However, these unrepresented regions were found mainly in areas of high (G + C) content as detected by partial denaturation, and it is unclear whether they are truly not represented by RNA, or whether they were not able to participate in R-loop formation as a consequence of their DNA sequence (Delius and Clements, 1976; Stringer et al., 1978).

Early HSV-1 nuclear and cytoplasmic RNA has been analysed using the techniques applied to IE RNA. Several discrete sizes of RNA species have been reported, varying from approximately 1.0 kb to greater than 6.0 kb (J. McLauchlan and J.B. Clements, personal communication; Holland et al., 1979; Anderson et al., 1980b), and 16 of these mRNAs have been mapped on the virus genome. A summary of the mapping data for HSV-1 early RNA is given in Figure 5.

Transcription from the region of the HSV-1 DNA containing the Hind III k and l fragments (0.53 to 0.59 map units), an area abundantly represented in both early and late RNA has been extensively

studied. Before the onset of virus DNA replication, these fragments are represented by two major mRNA species, of 5.0 kb and 1.8 kb (J. McLauchlan and J.B. Clements, personal communication; Anderson et al., 1980b). After size fractionation, the 5.2 kb mRNA directed the in vitro synthesis of a polypeptide of 140,000 molecular weight, while the 1.8 kb RNA, which may have been contaminated with another mRNA species, directed the synthesis of two polypeptides of 65,000 and 54,000 molecular-weight (Anderson et al., 1980b). These two mRNAs have sequences in common, and are co-terminal at the 3'-end (J. McLaughlan and J.B. Clements, personal communication). The DNA sequence at the 3'-end of these mRNAs is shown in Figure 52, and will be discussed later.

After the onset of virus DNA replication, sequences in the Hind III k fragment are represented by four abundant mRNAs, of 7.0 kb, 5.2 kb, 3.8 kb and 1.8 kb approximately (Anderson et al., 1980b). The 7.0 kb mRNA appears to be co-terminal at the 3'-end with the 5.2 kb and 1.8 kb mRNAs observed at early as well as late times, and directs the in vitro synthesis of a 54,000 molecular weight polypeptide which is indistinguishable from that synthesized using the 1.8 kb mRNA (Anderson et al., 1980b). The size fractionated 3.8 kb RNA, possibly a mixture of two distinct mRNAs, directs the synthesis of two polypeptides with molecular weights of 122,000 and 86,000 (Anderson et al., 1980b).

The DNA sequence for the region containing the HSV-1 thymidine kinase gene, and the location of the RNA which is synthesized at early times, has been determined (McKnight, 1980; Wilkie et al., 1980). The mRNA is approximately 1,300 nucleotides in length, and is capable of specifying a polypeptide of 40,000 molecular weight (McKnight, 1980). The DNA sequence at the 3'-end of the mRNA is shown in Figure 52.

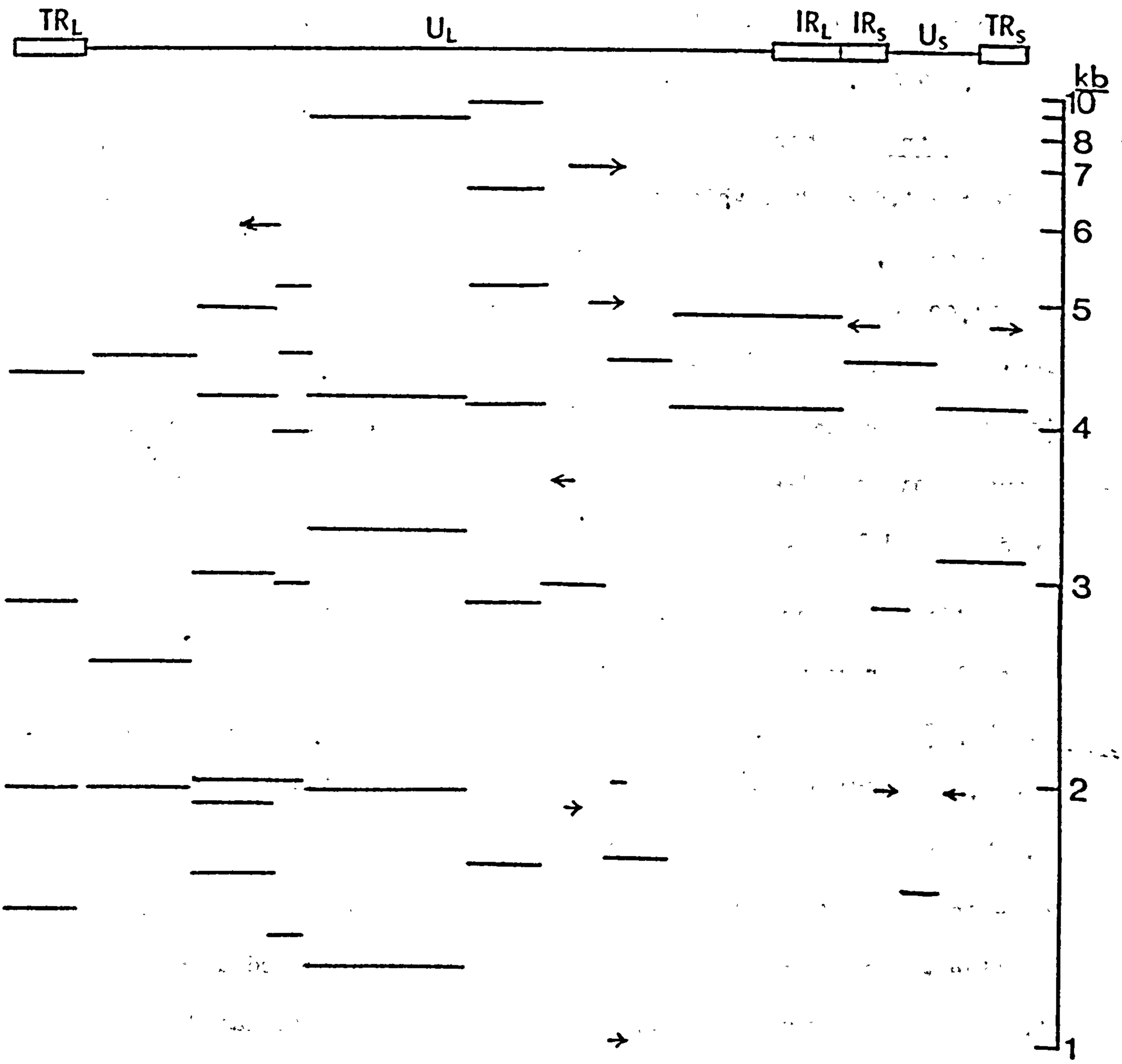


FIGURE 6

Sizes and genome locations of HSV-1 late mRNAs. The sizes are indicated by the scale, and the genome location by the line. Adapted from Anderson et al. (1979).

Late HSV-1 RNA maps throughout the virus genome similar to early RNA, however, differences in the relative degree of representation of some regions were noted between early and late RNA (Clements et al., 1977; Jones et al., 1977; Jones and Roizman, 1979). In addition to the HSV-1 Hind III k DNA fragment discussed above, isolated restriction endonuclease generated HSV-1 DNA fragements from other regions of the genome, covalently linked to cellulose, have been used to select for virus-specific mRNAs within late polyadenylated polyribosome-associated RNA. The selected mRNAs were then fractionated by electrophoresis through denaturing agarose gels, resolving several RNA species with sizes ranging from 1.5 kb to greater than 8.0 kb (Anderson et al., 1979). The results revealed a complex pattern of transcription, with individual DNA fragments represented by several mRNAs. A summary is given in Figure 6.

PrV IE RNA contains two size classes, of 5.3 kb and 2.1 kb (F.J. Rixon, personal communication). The 5.3 kb mRNA is transcribed entirely from the repetitive regions (Figure 3), with the 5'-end of the mRNA located away from the unique short region (F.J. Rixon, personal communication). This is analogous to the situation found with the HSV-1 4.7 kb IE mRNA which maps entirely within TR_S/IR_S (Figure 4). The 2.1 kb class of PrV contains at least two, and possibly three distinct mRNAs. One of these is transcribed from sequences in U_L, while the other(s) run across the junction(s) of the repetitive region and U_S (F.J. Rixon, personal communication). This again resembles the HSV-1 situation.

e) Post-transcriptional Modification of HSV RNA

HSV-1 transcripts undergo similar post-transcriptional modifications to those found with most eukaryotic mRNAs. These

modifications are likely to be performed by pre-existing cellular enzymes.

Virus mRNAs contain poly (A) tracts covalently linked to their 3'-ends, with these polynucleotide tracts being added post-transcriptionally (Bachenheimer and Roizman, 1972). The poly (A) tracts present in infected-cell-RNA contained three distinct size classes of approximately 30, 50 and 155 nucleotides (Silverstein et al., 1976). The average length of the poly (A) tract found in late polyribosome-associated RNA was larger than that in early RNA (Stringer et al., 1977), however, late nuclear RNA contained a greater proportion of the poly-(A)30 tracts than late polyribosome-associated or total cytoplasmic RNA, with 68% of the cytoplasmic RNA containing the largest size class (Silverstein et al., 1976). Similar virus-specific sequences were found in unlabelled cytoplasmic RNA fractionated according to the size of the poly (A) tract (Silverstein et al., 1976).

The 5'-termini of HSV-1 mRNAs contain methylated cap structures of both cap 1 and cap 2 types (Bartkoski and Roizman, 1976; Shatkin, 1976; Moss et al., 1977; Banerjee, 1980). The first methylated bases following the N^7-methylguanosine of the cap were predominantly N^7-methylguanosine and N^6-methyladenosine (Moss et al., 1977). Virus RNA also contains methylated ribonucleosides internally in the mRNA, mainly N^6-methyladenosine, with small amounts of N^5-methylcytosine (Moss et al., 1977). Methylation of internal bases, but not of the cap structure, was reported to be inhibited at late times during HSV-1 infection (Bartkoski and Roizman, 1978), though the effect of this on HSV mRNA stability or control of translation is unclear.

Incubation of infected cells with S-isobutyladenosine, an analogue of S-adenosyl-L-homocysteine the cellular methyl group donor,

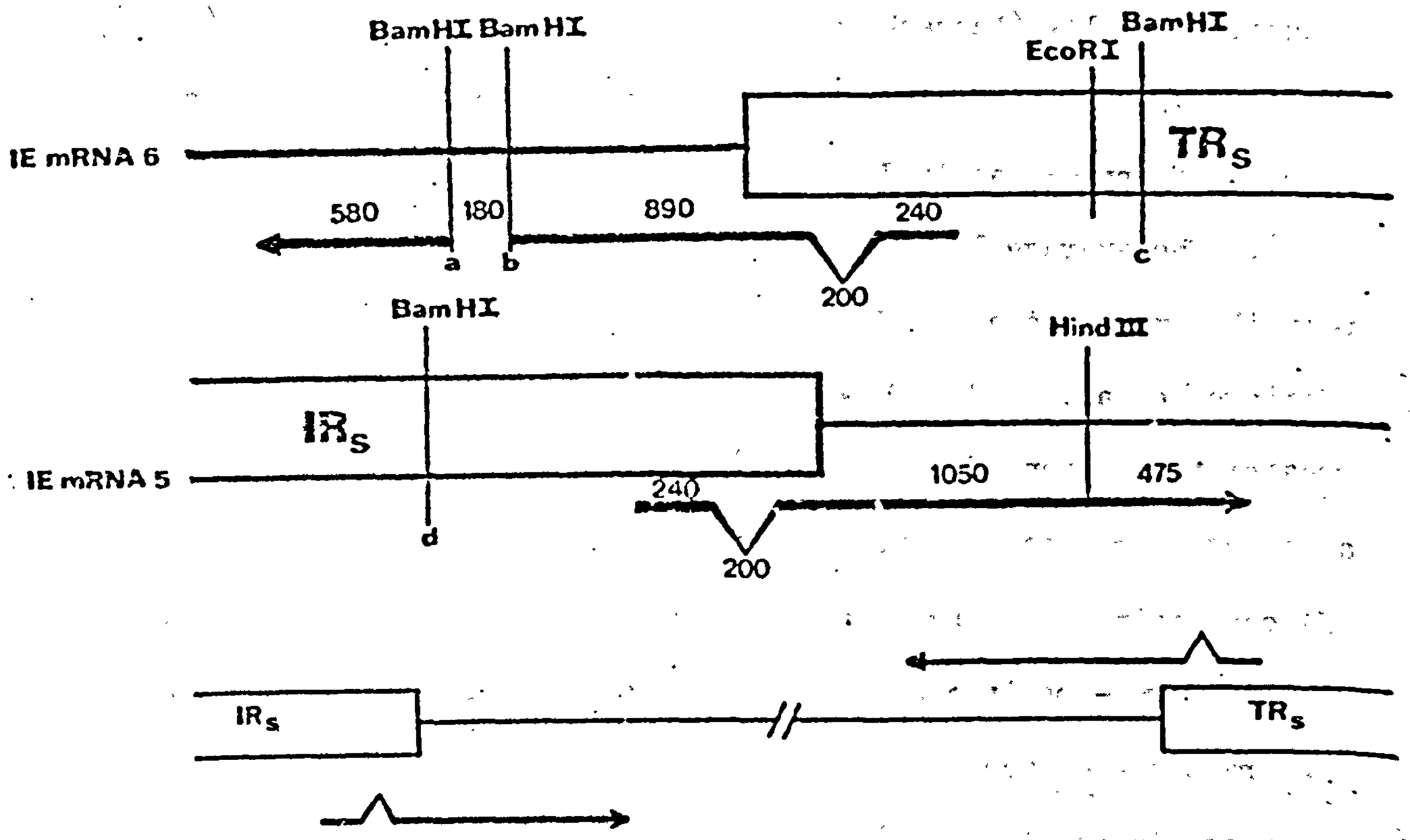


FIGURE 7

Structures of HSV-1 IE mRNA is mapping in U_S and TR_S/IR_S. Taken from Clements et al. (1981; Structure of HSV IE mRNAs in, Herpes DNA: Structure and Replication, Ed. Y. Becker, in the press).

inhibits RNA methylation both internally and at the cap, and was reported to inhibit virus mRNA translation but not synthesis (Jacquemont and Huppert, 1977). However, this drug has several other effects on cellular metabolism which may be the cause of the observed inhibition of virus mRNA translation.

The mRNAs of several eukaryotic cell and eukaryotic virus genes undergo a major post-transcriptional modification process by which RNA sequences transcribed from noncontiguous regions of the genome are joined to form a mature mRNA molecule. The process involves excision of the intervening sequences from a primary transcription product, and the ligation of the resultant RNA segments. The process, termed RNA splicing, may occur several times within one mRNA molecule, even within the sequences coding for the polypeptide product. Examples of mRNAs from other virus systems which undergo this process, and some aspects of the mechanism by which it occurs are discussed later. At least two HSV-1 IE mRNAs are spliced while at least one IE mRNA and several early mRNAs appear not to be spliced.

The two 2.0 kb HSV-1 mRNAs which are transcribed across the U_S/IR_S and U_S/TR_S junctions each contain a single splice (Figure 7). A segment of RNA, approximately 200 nucleotides in length is removed from the mRNAs 240 nucleotides from the 5'-end (Watson et al., 1981; Clements et al., 1981; F.J. Rixon, personal communication). Since a substantial region at the 5'-ends of both of these mRNAs are located within the repetitive regions TR_S/IR_S (Clements et al., 1979), these splices, although in two distinct mRNA species, occur at identical sequences (Figure 7). These splices occur upstream of the coding regions of the mRNAs (R.J. Watson, personal communication).

The third 2.0 kb IE mRNA, which maps in U_L near the junction

with IR_L does not contain any splices (F.J. Rixon, personal communication), in common with the major early 5.2 kb and 1.8 kb mRNAs which map within the HSV-1 Hind III e and k DNA fragments (J. McLauchlan and J.B. Clements, personal communication). The HSV-1 thymidine kinase mRNA also appears to be unspliced (McKnight, 1980).

Data presented in this thesis indicates that at least one HSV-2 IE mRNA appears to be unspliced.

5. Protein Synthesis in HSV-Infected Cells

Protein synthesis in HSV-infected cells occurs in the cytoplasm (Sydiskis and Roizman, 1966). Following infection with either HSV-1 or HSV-2, there is a decrease in the overall rate of protein synthesis, after which, approximately at the time of onset of virus DNA synthesis, the rate of synthesis increases then finally declines (Roizman et al., 1965; Honess and Roizman, 1974; Powell and Courtney, 1975).

Polyacrylamide gel electrophoresis has shown the presence of at least 50 virus-induced polypeptides in both HSV-1 and HSV-2 infected cells (Honess and Roizman, 1973; Powell and Courtney, 1975; Marsden et al., 1976), of which 33 have been identified as structural components of the HSV-1 virion, and 24 of the HSV-2 virion (Heine et al., 1974; Marsden et al., 1976; Strnad and Aurelian, 1976).

By pulse labelling HSV-1 infected cells at various times throughout infection, Honess and Roizman (1973) demonstrated that the synthesis of virus-induced polypeptides was temporally regulated. Four classes of virus-induced polypeptides were defined by their kinetics of synthesis. In general, the rate of synthesis of structural polypeptides increased throughout infection, however non-structural polypeptides were assigned to all four classes (Honess and Roizman, 1973).

Subsequently, it was reported that three groups of virus polypeptides (termed α, β and γ) could be defined by their order of appearance in HSV-1 infected cells (Honess and Roizman, 1974). Polypeptides in these three classes appeared sequentially, and differed in their requirement for prior protein synthesis for their own synthesis.

α-polypeptides, which are translated from IE RNA, did not require prior protein synthesis, being produced after removal of a cycloheximide block present from the time of infection. The rate of synthesis of α-polypeptides peaked at 3-4 hr post-infection in untreated cells, then declined. If actinomycin D was added at the time of removal of cycloheximide, the α-polypeptides continued to be made.

The β-polypeptides did not appear until after α-polypeptide synthesis in untreated cells, and did not appear at all when a cycloheximide block was released in the presence of actinomycin D. Hence, it was suggested that β-polypeptide synthesis required the presence of both new RNA and α-polypeptides. The β-polypeptides then inhibited α-polypeptide synthesis. Similarly, β-polypeptides were proposed to regulate the expression of γ-polypeptides which in turn inhibited the synthesis of β-polypeptides.

In the presence of canavanine, an analogue of arginine whose incorporation renders a polypeptide inactive, from the onset of infection, several β-polypeptides and one γ-polypeptide were synthesized (Honess and Roizman, 1975; Pereira et al., 1977). If canavanine was added 2-3 hr post-infection to allow the synthesis of functional α-polypeptides, all of the β-polypeptides were synthesized (Pereira et al., 1977). This was explained by suggesting that β-polypeptides could be divided into two groups, β_1 and β_2, only one of

which was inhibited by the incorporation of canavanine into α-polypeptides (Pereira et al., 1977). Two possibilities for synthesis of β-polypeptides were proposed; the first a sequential pathway $\alpha \longrightarrow \beta 1 \longrightarrow \beta 2$, and the second, two independent pathways $\alpha 1 \longrightarrow \beta 1$ and $\alpha 2 \longrightarrow \beta 2$ (Pereira et al., 1977). Using the HSV-1 mutant ts K which synthesizes mainly α-polypeptides at the NPT, Preston (1979) presented evidence suggesting that the mechanism involving two independent pathways was the most likely.

Inhibition of virus DNA synthesis in both HSV-1 and HSV-2 infected cells, either by addition of cytosine arabinoside, or using temperature-sensitive mutants which do not synthesize virus DNA at the NPT, resulted in the overproduction of α-polypeptides while synthesis of β-polypeptides continued as usual. In addition, reduced amounts of certain γ-polypeptides were detected (Powell et al., 1975). Other γ-polypeptides which were not detected unless virus DNA synthesis occurred were proposed to represent a true late class of polypeptides (Powell et al., 1975). Alternatively since these true "late" polypeptides undergo post-translational modification, inhibition of their appearance may be due to inhibition of this modification.

Analysis of the polypeptides induced at the NPT by temperature-sensitive mutants of HSV-1 (Courtney et al., 1975, Marsden et al., 1976) and HSV-2 (H.S. Marsden, personal communication) indicate that the regulation of virus protein synthesis is more complex than the α, β, γ cascade system proposed by Honess and Roizman (1974). In general, mutants which have a DNA-synthesis negative phenotype at the NPT exhibit a more restricted polypeptide profile at this temperature than mutants which are capable of replicating virus DNA. Of these mutants, several overproduce the α-polypeptides at the NPT

and one, ts K, induces only a limited number of virus-specific polypeptides (Marsden et al., 1976; Preston, 1979).

6. Post-translational Modification of HSV-induced Polypeptides

Several polypeptides synthesized in vitro using HSV-infected cell RNA have similar, but not identical mobilities on SDS polyacrylamide gels to the polypeptides synthesized in vivo, suggesting that these polypeptides undergo post-translational modification in vivo (Preston, 1977; Cremer et al., 1977; this thesis). Four types of modification of herpesvirus-induced polypeptides have been determined namely cleavage, glycosylation, phosphorylation and sulphation.

Following treatment of HSV-1-infected cells for short periods of time with inhibitors of proteolytic enzymes, such as tosyl phenylalanyl chloromethylketone (TPCK), no evidence of post-translational cleavage of virus-induced polypeptides was detected (Honess and Watson, 1973; Pereira et al., 1977). Recently, however, it has been shown in infected cells treated with TPCK for 18 hr that several virus-induced polypeptides appear to be processed by cleavage (M. Suh, D. MacDonald and H.S. Marsden, manuscript in preparation). Furthermore, analysis of the tryptic peptides of several HSV-1-induced polypeptides has shown that at least three polypeptides are likely to be direct cleavage products of larger precursor polypeptides (MacDonald, 1980).

Certain HSV-1 and HSV-2-induced polypeptides are glycosylated (Spear and Roizman, 1972; Honess and Roizman, 1975; Marsden et al., 1976; Marsden et al., 1978), and most of these are components of the virus envelope (Roizman and Furlong, 1974). The unmodified precursors of the glycoproteins which accumulate in the presence of increasing

amounts of 2-deoxyglucose, have a greater electrophoretic mobility in polyacrylamide gels (Courtney, 1976; Cohen et al., 1980).

Five phosphorylated virion polypeptides have been identified (Gibson and Roizman, 1974; Lemaster and Roizman, 1980) and a virion associated protein kinase has been implicated in the modification of these polypeptides (Lemaster and Roizman, 1980). At least 16 phosphorylated virus-induced polypeptides have been detected in HSV infected cells (Marsden et al., 1978; Wilcox et al., 1980).

Most of the α or immediate early polypeptides are phosphorylated, and one, ICP 4 (HSV-1 Vmw 175), has been shown to consist of at least three forms, all of which are phosphorylated, and two of these forms are found in the nucleus (Pereira et al., 1977). Several polypeptides which exhibit DNA binding properties are phosphorylated (Hay, 1979; Hay and Hay, 1980), and the phosphorylation can affect the affinity of the polypeptide for DNA (Wilcox et al., 1980). The precise nature of the bonding between the polypeptide and phosphate is not known, however two types can be differentiated, one being relatively stable, and the other relatively unstable. The latter lends itself to the suggestion that the function of the phosphorylated polypeptides may be altered or controlled by the degree of phosphorylation (Wilcox et al., 1980).

The major glycoprotein of pseudorabies virus has also been shown to be sulphated (Erickson and Kaplan, 1973).

7. HSV Specified Enzymes

Following infection of cells with HSV-1 or HSV-2, there is an increase in the activity of many enzymes, many of which are involved in the biosynthesis of precursors of DNA synthesis. These enzymes

include pyrimidine deoxynucleoside kinase (dPyK) which has activity as both thymidine kinase (TK) and deoxycytidine kinase (Jamieson et al., 1974; Jamieson and Subak-Sharpe, 1974), ribonucleotide reductase (Cohen, 1972; Ponce de Leon et al., 1977), nucleoside phosphotransferase (Jamieson et al., 1976), deoxycytidine deaminase (Chan, 1977) and deoxycytidine monophosphate deaminase (Rolton and Keir, 1974). In all cases except that of deoxycytidine monophosphate deaminase, there is strong evidence that the enzyme is virus-coded. Langelier et al. (1978) reported that induction of cytidine monophosphate deaminase did not occur on infection of chinese hamster cells that were deficient in this enzyme.

One of the more extensively studied HSV induced enzymes is the dPyK. The TK activity can be differentiated from the host cell enzyme by several biochemical criteria (Klemperer et al., 1967), and the dPyK enzyme specified by different herpesviruses can be differentiated immunologically (Klemperer et al., 1967; Buchan and Watson, 1969; Thouless and Wildy, 1975). Mutant viruses incapable of inducing TK have been isolated (Kit and Dubbs, 1963; Dubbs and Kit, 1964), and the virus structural TK gene has been stably transferred to TK-deficient cells by infection with UV-irradiated virus (Kit and Dubbs, 1963; Munyon et al., 1971) and by transfection using specific HSV genome fragments (Wigler et al., 1977; McDougall et al., 1980).

The HSV-specified dPyK enzymes identified immunologically have molecular weights in SDS polyacrylamide gels of 44,000 for HSV-1 (Honess and Watson, 1974), and 42,500 for HSV-2 (Thouless and Wildy, 1975). The molecular weight of the active enzyme is approximately 80,000 (Cheng and Ostrander, 1976), suggesting that the virus-specified enzyme is comprised of two subunits of similar or identical size (Jamieson and Subak-Sharpe, 1978). The virus-specified dPyK is

non-essential for virus proliferation in growing tissue culture cells, however it is essential in resting cells in which levels of the host-cell enzyme are low (Jamieson et al., 1974).

Following infection with HSV, there is an increase in DNA polymerase activity (Keir and Gold, 1963), and this activity can be distinguished biochemically and immunologically from those of the host (Keir et al., 1966a and b). Purified DNA polymerase of both virus serotypes have molecular weights of approximately 150,000 in SDS polyacrylamide gels, and it has been suggested that the enzymes are active as monomers (Powell and Purifoy, 1977).

Temperature-sensitive mutants of HSV-1 and HSV-2 have been isolated which induce a thermolabile DNA polymerase activity in vivo (Aron et al., 1975; Hay et al., 1976; Purifoy et al., 1977; Joffre et al., 1977).

Herpesvirus-induced DNA polymerases are inhibited by low levels of phosphonoacetic acid (Mao et al., 1975; Mao and Robishaw, 1975). Mutants have been isolated which grow in the presence of phosphonoacetic acid, and these induce a phosphonoacetic acid-resistant DNA polymerase (Hay and Subak-Sharpe, 1976). Genetic and physical mapping studies have shown a very close linkage between the site of phosphonoacetic acid-sensitivity and the DNA polymerase locus (Joffre et al., 1977; Purifoy and Powell, 1977; Chartrand et al., 1979).

Infection with HSV-1 and HSV-2 increases the activity of alkaline DNA exonuclease (Keir and Gold, 1963; Morrison and Keir, 1968; Hay et al., 1971; Hoffman and Cheng, 1978), and a ts mutant of HSV-2, ts 13, specifies an exonuclease enzyme which exhibits ts activity both in vivo and in vitro (Franke et al., 1978). Ts^+

revertants of ts 13 however, remain ts for exonuclease activity (H. Rixon, personal communication), indicating that, as with virus dPyK, this enzyme is not required for virus growth in replicating tissue culture cells.

8. Genome Locations of HSV Polypeptides

At least some of the HSV-1 and HSV-2 gene products are functionally interchangeable as determined by intertypic complementation (Timbury and Subak-Sharpe, 1973; Esparza et al., 1976; Stow and Wilkie, 1978). HSV-1 and HSV-2 are also capable of exchanging genetic material to produce intertypic recombinants which contain genomes partly derived from both parents (Timbury and Subak-Sharpe, 1973; Esparza et al., 1976; Morse et al., 1977; Stow and Wilkie, 1978; Marsden et al., 1978; Preston et al., 1978). By utilizing differences in the patterns of fragments produced by digestion of HSV-1 and HSV-2 DNA with various restriction endonucleases, and slight differences in the electrophoretic mobilities of several equivalent polypeptides between the two serotypes, analysis of intertypic recombinants enabled several virus polypeptides and virus-specific functions to be located on the virus genomes (Morse et al., 1978; Marsden et al., 1978; Preston et al., 1978; Lemaster and Roizman, 1980). The locus for resistance to PAA, which has been associated with the activity of HSV DNA polymerase in vitro (Mao et al., 1975; Hay and Subak-Sharpe, 1976), has been located in a 3.3 kb region of the virus genome near 0.40 map units (Chartrand et al., 1979).

A particularly well studied region of the HSV-1 genome is that containing the virus thymidine kinase. This was located in a 2.0 kb region of HSV-1 DNA (Wigler et al., 1977; Pellicer et al., 1978; Colbere-Garapin et al., 1979). This region has now been

sequenced, and the mRNA has been accurately located as described previously (McKnight, 1980; Wilkie et al., 1980).

The HSV-2 thymidine kinase gene was initially mapped at 0.532 to 0.646 map units (Maitland and McDougall, 1977), however recent studies have placed it at a position colinear on the genome with that of HSV-1 at o.299 to o.326 map units (McDougall et al., 1980).

Several immediate early polypeptides have been located on the virus genome by analysis of intertypic recombinants (Preston et al., 1978), and IE mRNAs as described previously.

Thus far, all of the mapping studies are consistent with the idea that equivalent genes of HSV-1 and HSV-2 are colinear on the genomes.

9. Control of Eukaryotic Transcription

Eukaryotic transcription comprises a complex series of events involving the interaction of a multiprotein enzyme complex with a DNA template which itself is associated with many proteins. Control of the speficifity of this process can be exerted at any one of the many stages which occur before the appearance of the final mRNA product. Expression of the transcribed gene, in the form of a polypeptide product, may also be regulated at one of several stages, before, and after translation. Consideration of some well studied eukaryotic systems furnishes some examples of the ways in which gene expression is regulated. Regulation of virus transcription in productively infected cells has been studied in the hope that the information acquired will illuminate the more complex eukaryotic system. Virus transcription can then possibly be likened to the induction of a tightly regulated, autonomous, gene cluster. In this context, the most extensively studied viruses are adenovirus, SV40

and polyoma virus.

Transcription in eukaryotic cells is performed by three distinct DNA-dependent RNA polymerases, located within the nucleus. The major activities, I and II, are localized within the nucleoli (the site of ribosomal RNA synthesis) and the nucleoplasm, respectively. Polymerase III is also located within the nucleoplasm (Roeder and Rutter, 1970; Zylber and Penman, 1971).

RNA polymerase activities can be identified by their sensitivity to α-amanitin; polymerase I is insensitive, polymerase II is inhibited by low concentrations, and polymerase III is inhibited by high concentrations. Utilizing these differences in sensitivity, the major functions of the enzymes have been described (Zylber and Penman, 1971; Weinmann and Roeder, 1974). Polymerase I transcribes ribosomal RNA, polymerase III the 4S and 5S RNAs, and polymerase II is involved in the transcription of giant heterogeneous nuclear RNA, the putative mRNA precursor (Zylber and Penman, 1971; Weinmann and Roeder, 1974).

As discussed previously, RNA polymerase II is involved in HSV transcription and it has also been implicated in transcription of the polyoma (Amati et al., 1975), SV40 (Jackson and Sugden, 1972) and adenovirus genomes (Price and Penman, 1972; Wallace and Kates, 1972). However, adenovirus also contains genes for two small RNAs ($VA\text{-}RNA_I$ and $VA\text{-}RNA_{II}$), located near position 30 on the genome and within 700 bases of each other (Figure 10; Mathews, 1975) which are transcribed by RNA polymerase III (Weinmann et al., 1976). There is no evidence for such an activity on the SV40 or polyoma genomes or as yet for HSV.

All SV40 (Lavi and Shatkin, 1975; Aloni et al., 1977; Lavi and Groner, 1977), polyoma (Flavell et al., 1979) and adenovirus (Philipson

et al., 1971; Wall et al., 1972; Moss and Koczot, 1976; Ziff and Evans, 1978) mRNAs are modified at the 5'-end by the addition of a cap structure, and at the 3'-end by the addition of a poly (A) tract.

The genomes of both SV40 and polyoma consist of a double-stranded circular DNA molecule of approximately 5,000 base pairs in length. The complete nucleotide sequence of SV40 (Fiers et al., 1978; Reddy et al., 1978b) and polyoma (Friedmann et al., 1978a and b; Deininger et al., 1979; Friedmann et al., 1979) have been determined. The transcriptional programmes of SV40 and polyoma are very similar, being divided into early (before the onset of virus DNA synthesis), and late (after the onset of virus DNA synthesis) phases, with one strand (E-strand) transcribed at early times, and the other (L-strand) transcribed at late times (Sambrook et al., 1972; Khoury et al., 1973; Kamen et al., 1976; Flavell and Kamen, 1977; Condit et al., 1978). The 5'-ends of both early and late RNAs are clustered in an area of the genome which also includes the origin of DNA replication (Figures 8 and 9; Sambrook et al., 1972; Khoury et al., 1973; Kamen et al., 1976), and the 3'-ends of the early and late RNAs overlap by approximately 70 to 100 nucleotides (Fiers et al., 1978; Reddy et al., 1978b).

At early times during SV40 infection, two mRNAs of approximately 19S in size, representing 30% of the E-strand are synthesized (Sambrook et al., 1972; Weinberg et al., 1972). These mRNAs direct the in vitro synthesis of two polypeptides with molecular weights of 97,000 and 17,000. These are designated large T antigen (T) and small t antigen (t) respectively (Prives et al., 1977). The mRNAs for these two polypeptides have similar, if not identical, 5'- and 3'-termini, but differ in structure due to differential splicing (Berk and Sharp, 1978a); Crawford et al., 1978; Fiers et al., 1978;

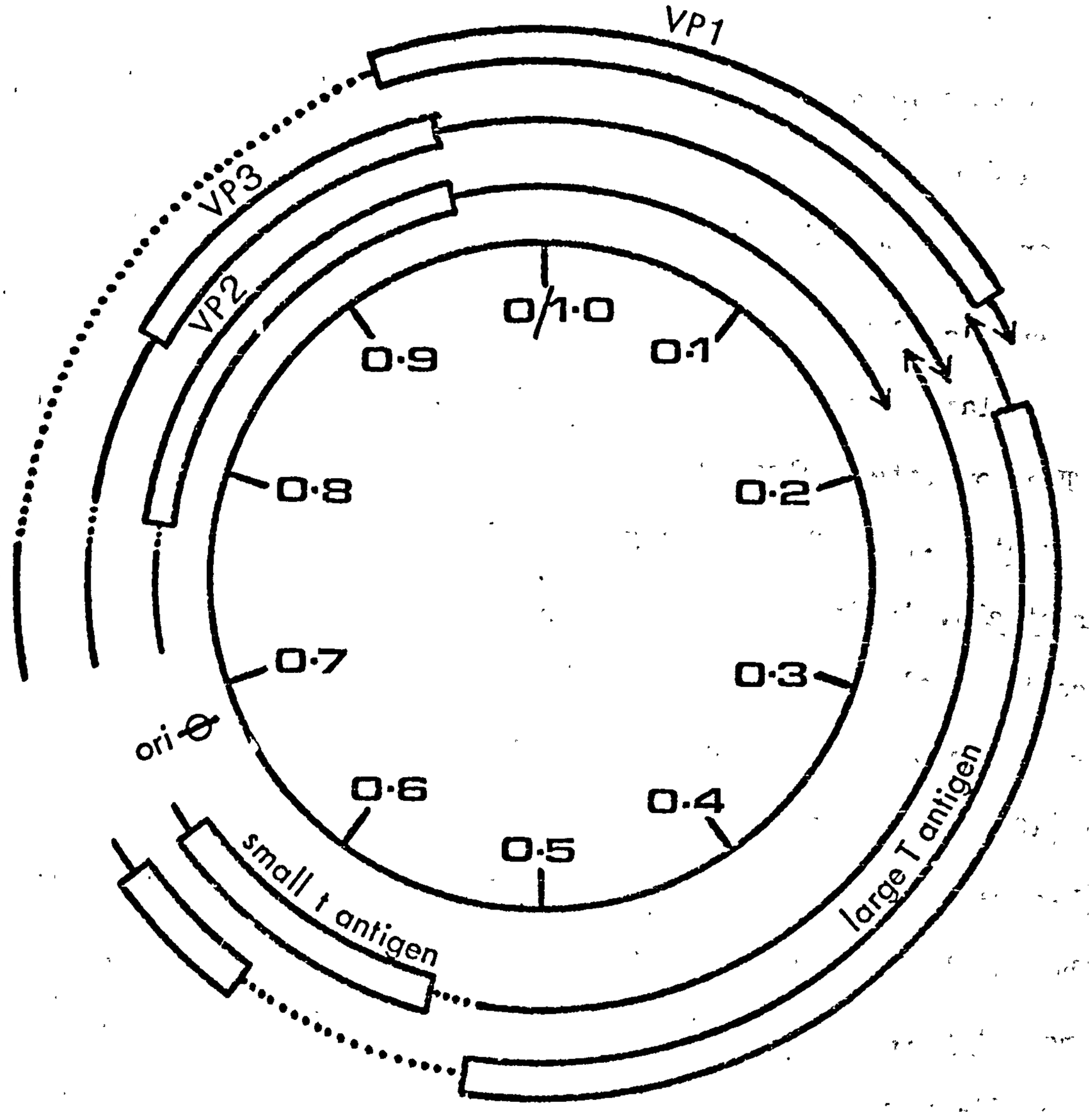


FIGURE 8

Summary of the transcription pattern of SV40 based on Fiers et al. (1978). Segments of RNA removed by splicing are indicated by dotted lines, the coding regions of the mRNAs are shown by the boxed areas and the directions of transcription by the arrows at the 3'-ends of the mRNAs.

Reddy et al., 1978b). The structure of these mRNAs is summarized in Figure 8.

The sequences encoding t antigen are contained entirely within the first RNA segment before the splice, with the splice occurring after the translation stop codon for t. In large T antigen mRNA, however, the single splice donor site occurs "upstream" to that in t antigen mRNA, and splicing removes the small t antigen termination codon allowing translation to continue almost to the end of the mRNA (Reddy et al., 1979). Translation of both polypeptides is initiated at the same codon, and continues in the same way until the splice donor site in T antigen mRNA, and as a consequence, both polypeptides share amino-terminal amino acids, but differ at the carboxy-termini (Paucha et al., 1978; Simmons and Martin, 1978; Reddy et al., 1979). As a consequence of the structure of these mRNAs, the larger polypeptide is encoded by the smaller mRNA, and deletions which affect small t antigen, but which do not extend to the splice donor site of T antigen mRNA have no effect upon T antigen (Thimmappaya and Shenk, 1979).

T antigen binds to, and protects from nuclease digestion, a 120 base pair region of the SV40 genome near the origin of DNA replication and close to the 5'-end of early mRNAs. It has been suggested that this may represent a method by which T antigen may regulate the synthesis of its own mRNA (Tjian, 1978).

Three major SV40 structural polypeptides, VP1, VP2 and VP3 are synthesized at late times. Two size classes of RNA, 16S and 19S, representing approximately 70% of the L-strand, have been detected within the cytoplasm (Sambrook et al., 1972; Weinberg et al., 1972; Weinberg et al., 1974; Hsu and Ford, 1977), and these RNAs share common sequences (Weinberg et al., 1974; Smith et al., 1976).

Synthesis of VP2 and VP3 is directed *in vitro* by 19S RNA, and VP1 by 16S (Rozenblatt *et al.*, 1976). VP2 and VP3 contain a number of common tryptic peptides (Rozenblatt *et al.*, 1976).

At least two forms of 19S mRNA are produced, which differ in their pattern of splicing. These are shown in Figure 8.

The 5'-ends of late SV40 mRNAs are very heterogeneous (Lai *et al.*, 1978; Contreras and Fiers, 1981), and while heterogeneity has been observed at the 3'-ends of late, non-polyadenylated RNA, mature fully processed polyadenylated RNA appears to have a discrete coterminal 3'-end (Lai *et al.*, 1978). The VP2 and VP3 19S mRNAs share a non-coding 5' leader sequence which is heterogeneous in length (Lavi and Groner, 1977; Ghosh *et al.*, 1978; Lai *et al.*, 1978; Contreras and Fiers, 1981), and the VP2 mRNA contains a single splice before the AUG initiation codon (Dhar *et al.*, 1978; Reddy *et al.*, 1978a; Ghosh *et al.*, 1978; Lai *et al.*, 1978). The mRNA for VP3 is not well characterized, but contains a splice which removes VP2 initiation codon. Initiation of VP3 translation occurs at an AUG codon located internally within the VP2 coding sequences, and in phase with them (Rozenblatt *et al.*, 1976; Contreras *et al.*, 1977; Dhar *et al.*, 1978; Reddy *et al.*, 1978a).

The 16S, VP1, mRNA shares the non-coding leader sequence with the VP2 and VP3 mRNAs, however a much larger RNA segment is excised (Figure 8, Aloni *et al.*, 1977; Hsu and Ford, 1977; Lavi and Groner, 1977; Dhar *et al.*, 1978; Lai *et al.*, 1978; Reddy *et al.*, 1978a) such that the AUG initiation codon for VP1 occurs within the coding sequences for VP2 and VP3, but it is out of phase with the VP2/VP3 reading frame (Contreras *et al.*, 1977; Dhar *et al.*, 1978; Ghosh *et al.*, 1978; Reddy *et al.*, 1978a).

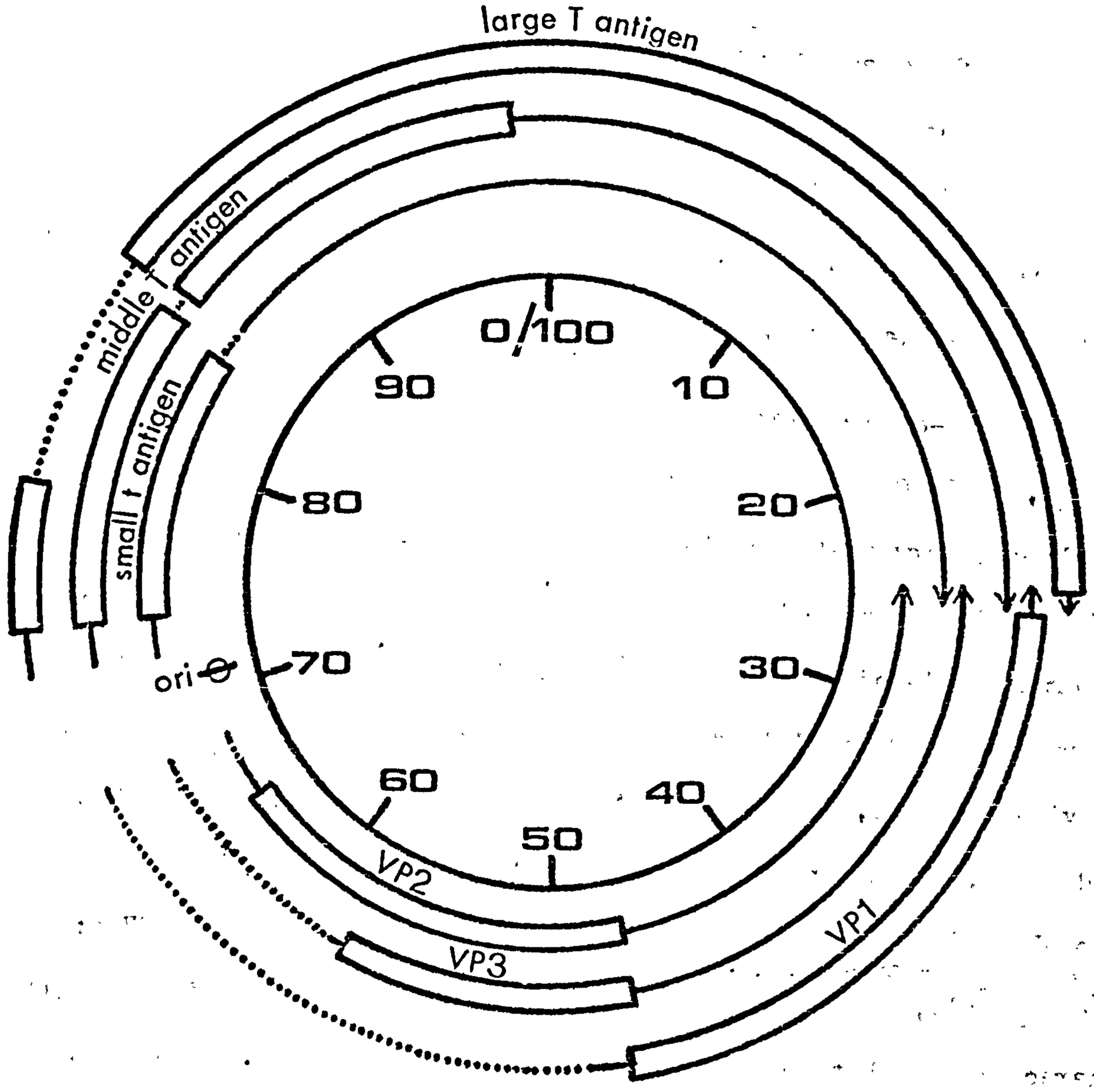


FIGURE 9

Summary of the transcription pattern of polyoma virus.

Segments of RNA removed by splicing are indicated by dotted lines, the coding regions of the mRNAs are shown by the boxed areas and the directions of transcription by the arrows at the 3'-ends of the mRNAs.

The transcriptional pattern of polyoma resembles that of SV40 (Figure 9), and the virus polypeptides share a high degree of amino acid homology with those of SV40, though this homology is not reflected at the level of the DNA sequence (Friedmann et al., 1978; Deininger, 1979; Friedmann et al., 1979). At early times, three polypeptides are synthesized, of approximately 90,000 (large T antigen), 60,000 (middle T antigen) and 22,000 molecular weight (small t antigen; Ito et al., 1977; Hutchinson et al., 1978; Smart and Ho, 1978). All three polypeptides have identical amino-terminal peptides, but differ at the carboxy-termini (Hutchinson et al., 1978; Smart and Ito, 1978).

The structures of the early polyoma mRNAs resemble those of SV40, with the largest polypeptide encoded by the smallest, 16S, mRNA (Figures 8 and 9). Middle T antigen mRNA is generated by a splicing pattern which removes the small t antigen termination codon, allowing translation to continue downstream of the splice, though in a different reading frame that that used to generate large T antigen (Friedmann et al., 1979; Favaloro et al., 1980). As a consequence of the splicing patterns, it is possible to isolate deletion mutants of polyoma which do not synthesize t antigen, but which produce normal T antigen, analogous to the situation found with mutants of SV40 (Hattori et al., 1979; Ito, 1979).

Similar to the SV40 situation, late during polyoma infection, three structural polypeptides of 45,000 (VP1), 34,000 (VP2) and 23,000 molecular weight (VP3) are synthesized (Gibson, 1974; Hewick et al., 1975). VP2 and VP3 have several tryptic peptides in common (Gibson, 1974; Hewick et al., 1975). Three distinct mRNAs, of 16S, 18S and 19S have been isolated. These encode VP1, VP3 and VP2 respectively (Siddell and Smith, 1978). All of the late mRNAs share

a common family of 5'-end leader sequences (Flavell et al., 1979; Legon et al., 1979).

Polyadenylated late nuclear polyoma RNA exists as a giant transcript apparently generated by transcription of the complete L-strand up to as many as four times (Acheson et al., 1971; Birg et al., 1977). Possibly as a consequence of this structure, late polyoma mRNAs typically contain 3-4 reiterations of the leader sequence (Legon et al., 1979; Triesman and Kamen, 1981). The splicing pattern of the three mRNAs resembles that found for SV40 (Figures 8 and 9), with VP3 utilizing an internal, in phase, initiation codon present within the VP2 coding sequences, and VP1 utilizing out-of-phase coding sequences within the region shared by VP2 and VP3 (Deininger et al., 1979; Favaloro et al., 1980).

Transcription in the group C human adenoviruses has been studied using primarily serotypes 2 and 5 (Ad-2 and Ad-5). The genome is a single, linear, double-stranded DNA molecule 35,000 base pairs in length. Conventionally, the genome is divided into 100 units, with 0 at the left hand end of the strand transcribed in the rightwards direction (r-strand; Flint, 1977). The transcriptional programme of the RNA polymerase II-transcribed adenovirus genes is generally divided into two phases, early and late, (Thomas and Green, 1969). At early times, approximately 20% of the virus genome is represented by RNA, while at late times, almost all of the genome is represented (Thomas and Green, 1969). RNA synthesized from regions represented at early times is also present late (Thomas and Green, 1969; Klessig and Chow, 1980).

At early times, five major regions of the adenovirus genome are represented by RNA (E1A, E1B, E2, E3 and E4; Figure 10) together with minor regions (indicated by a question mark in Figure 10;

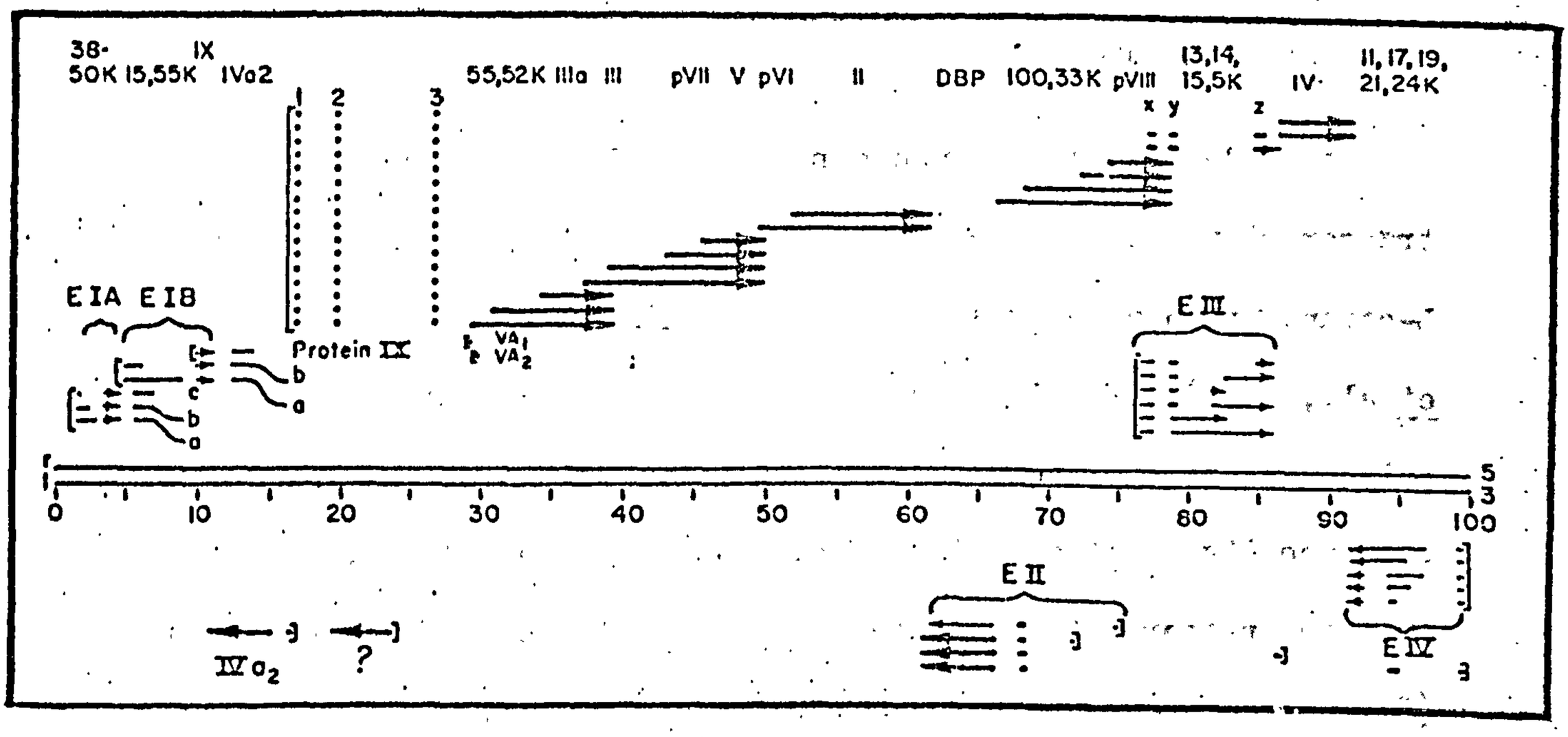


FIGURE 10

Summary of transcription map of adenovirus taken from Ziff (1980). The capped 5'-ends of the mRNAs are indicated by brackets ([and]). The translation products of the mRNAs are shown aligned with their genes.

Kitchingman et al., 1977; Berk and Sharp, 1978b; Chow et al., 1979; Galos et al., 1979). At intermediate times, or in the presence of DNA synthesis inhibitors, the mRNA for polypeptide IX, a structural polypeptide, is transcribed from the E1B region (Persson et al., 1978; Alestrom et al., 1980), and the IVa_2 polypeptide mRNA is also present (Galos et al., 1979; Chow and Broker, 1978). These two mRNAs are also synthesized at late times.

Initiation of early RNA synthesis was observed at each of the early regions, and occurred approximately 30 nucleotides downstream from a TAT(A,T)3 nucleotide sequence in the virus DNA (Evans et al., 1977; Ziff and Evans, 1978; Akusjarvi and Pettersson, 1979; Hashimoto and Green, 1979). Early transcription appears to be temporally controlled, with RNA representing the E1A region appearing in the nucleus 1 hr post-infection, followed by E1B, EIII and EIV, with EII the last early region to be transcribed at 2-3 hr (Nevins et al., 1979).

As summarized in Figure 10, all of the four major early regions are represented by several related mRNA species from both r and l DNA strands, arranged in families which are coterminal at the 3'- and/or 5'-ends. These familial mRNAs differ in structure, determined by the alternative splicing processes undergone by the primary transcript (Berk and Sharp, 1978b; Chow et al., 1979). Translation of these mRNAs produce a group of closely related polypeptides which have common amino- and carboxy-termini (Harter and Lewis, 1978). Throughout infection for each major early region, the relative proportion of each mature, spliced, mRNA alters (Chow et al., 1979). Furthermore, at early times the 5'-end of the EII mRNA is encoded near coordinate 75, however at late times, the 5'-end is encoded primarily near coordinate 72, though minor species with a 5'-end coordinate 86 have been observed. These various leader sequences

are spliced to RNA from coordinate 68, and the main body of the RNA from coordinate 61.6 to 66.5 (Chow et al., 1979).

Polypeptide IX mRNA is not spliced, and is 3'-coterminal with the E1B region transcripts (Alestrom et al., 1980). At late times, nascent transcripts initiated at the 5'-end of polypeptide IX mRNA have been detected, indicating that this mRNA has its own promotor region located within the E1B region (Wilson et al., 1979). The IVa_2 polypeptide mRNA appears to consist of a single species with a single splice which joins a 200 nucleotide leader sequence to the main body (Chow and Broker, 1978; Chow et al., 1979). The mRNAs of polypeptides IX and IVa_2, are transcribed from the r and l strands respectively and overlap by 11 to 16 nucleotides at their non-coding 3'-ends (Alestrom et al., 1980).

A temperature-sensitive mutant of Ad-5, ts 125, is arrested at the early stage when cells are infected at the NPT (Blanton and Carter, 1979). This mutant encodes a heat-labile 72,000 molecular weight DNA binding protein, the E2 region gene product (Blanton and Carter, 1979). As well as having a role in virus DNA replication, the 72,000 polypeptide regulates expression of the EIV region (Ziff, 1980).

At late times, a major r-strand promotor at co-ordinate 16.5 is activated, giving rise to a primary transcript of approximately 25,000 nucleotides (Evans et al., 1977; Ziff and Evans, 1978; Nevins and Darnell, 1978). This primary transcript is processed to produce the major late mRNAs. These mRNAs all contain a similar tripartite leader sequence of 203 nucleotides produced by splicing together RNA representing regions at 16.6, 19.6 and 26.6 (Berget et al., 1977; Chow et al., 1977; Gelinas and Roberts, 1977; Klessig, 1977; Akusjarvi and Pettersson, 1979; Zain et al., 1979). This common

leader is spliced to several sites to form five families of mRNAs which share sequences at the 5'- and 3'-ends, but which differ in the coding sequences (Figure 10).

Additional splicing events occur at late times to produce a heterogeneous population of mRNAs all of which encode a specific polypeptide. For example, the late mRNA transcribed from 30.5 to 39.0 map units can contain an extra segment derived from sequences located between the normal second and third late leader sequences (Chow et al., 1979). The fibre (polypeptide IV) mRNA may contain up to three extra leader sequences, designated x, y and z (Figure 10), between the normal tripartite leader sequences and the main body of the mRNA. Different combinations of the x, y and z leaders on the fibre mRNA have been observed, with the insertion of y sequences being most common (Chow and Broker, 1978; Dunn et al., 1978; Zain et al., 1979). The presence of the y leader does not substantially affect the in vitro translation of the fiber-related mRNA isolated from cells infected with an Ad-2-SV40 hybrid (Dunn et al., 1978).

Recently, evidence for an immediate early phase in adenovirus replication has been reported (Lewis and Mathews, 1980). RNA isolated from Ad-2-infected cells treated with emetine, a stringent inhibitor of protein synthesis, throughout infection was translated in vitro to produce a 13,500 molecular weight polypeptide located at 17.0 to 21.5 map units. Synthesis of this mRNA did not require expression of the E1A region (Lewis and Mathews, 1980). Under similar conditions, the major late promotor was also utilized (Lewis and Mathews, 1980; Shaw and Ziff, 1980; Nevins and Wilson, 1981).

In the systems described above, several aspects of control of gene expression have been reported, which may be generally applicable to other eukaryotic genes. Many such genes contain a sequence with

the canonical structure TATAAA (the Hogness box), approximately 30 nucleotides upstream from the first capped nucleotide. This is found in most Ad-2 genes (Evans et al., 1977; Ziff and Evans, 1978; Askusjarvi and Pettersson, 1979; Hashimoto and Green, 1979), and it has been suggested that this sequence may be a recognition site for the binding of RNA polymerase II. A similar sequence has been found upstream of the 5'-end of the HSV-1 TK mRNA (McKnight, 1980). However, this sequence is not present in the adenovirus EII region (Baker et al., 1979), or the SV40 and polyoma late regions (Fiers et al., 1978; Reddy et al., 1978a; Deininger et al., 1979). The significance of this is not known.

The Ad-2 VA-RNA genes appear to contain regulatory DNA sequences within the gene (Fowlkes and Shenk, 1980), in common with other genes transcribed by RNA polymerase III (Bogenhagen et al., 1980; Engelke et al., 1980; Sakonju et al., 1980).

All of the virus systems discussed, including HSV-1, exhibit temporal regulation of gene expression, with transcription of specific regions of the genome occurring only at late times during infection. This is particularly striking with HSV-1 at the switch from immediate-early to early transcription, and in SV40 and polyoma where transcription on the opposite strand is initiated. This control may occur by negative and/or positive control. In the case of adenovirus, negative control has been demonstrated, with the 72K DNA binding protein apparently causing decreased transcription of the EIV region (Ziff, 1980). As described previously, the SV40 large T antigen may exhibit autoregulation by negative control (Tjian, 1978). An example of positive transcriptional control has been reported during HSV-1 infection, where the ts K gene product is required throughout infection for transcription of both early and late regions (Watson and Clements, 1980).

M A T E R I A L S

1. Virus

The virus used in this study was HSV-2 strain HG-52 (Timbury, 1971).

2. Cells

Baby hamster kidney cells (BHK 21-C13), a continuous cell line (Macpherson and Stoker, 1962), were used in all analyses reported here.

3. Tissue Culture Media and Solutions

Cells were grown in Glasgow modified Eagle's medium (Busby et al., 1964), supplemented with 100 units/ml Penicillin, 100 µg/ml Streptomycin, 0.2 µg/ml n-butyl-p-hydroxybenzoate (an antimycotic), and 0.002% (w/v) phenol red. Low phosphate Eagle's medium (E(LP)) contained 1% of the $Na_2HPO_4.2H_2O$ content of normal Eagle's medium. Low methionine Eagle's medium ($E\frac{met}{5}$) contained 1/5 the normal concentration of methionine. These media were prepared in the Institute of Virology, as were human serum, calf serum, Difco tryptose phosphate broth and trypsin.

Various tissue culture media were used, containing the following constituents:

ETC 10 - 80% Eagle's medium, 10% Difco tryptose phosphate broth, 10% calf serum.

ETHu 2 - 88% Eagle's medium, 10% Difco tryptose phosphate broth, 2% human serum.

E(LP)C 2 - 98% low phosphate Eagle's medium, 2% calf serum.

PBS Ca - 95% phosphate buffered saline, 5% calf serum.

E $\frac{met}{5}$ C 2	-	98% low methionine Eagle's medium, 2% calf serum.
PBS	-	phosphate buffered saline, consisting of 170 mM NaCl, 3.4 mM KCl, 1 mM Na_2HPO_4, 2 mM KH_2PO_4 in distilled water, pH 7.2 (Dulbecco and Vogt, 1954).
Versene	-	6 mM EDTA in PBS, containing 0.0015% (w/v) phenol red.
Trypsin	-	0.25% (w/v) Difco trypsin in Tris-saline (140 mM NaCl, 30 mM KCl, 0.28 mM Na_2HPO_4, 1 mg/ml dextrose, 25 mM Tris-HCl (pH 7.4), 0.0015% (w/v) phenol red, 100 µg/ml Streptomycin, 100 units/ml Penicillin).
Giemsa Stain	-	1.5% (v/v) suspension of Giemsa in glycerol, heated at 56°C for 90-120 min, diluted with an equal volume of methanol.

4. Bacterial Host for Cloning Vector

The host bacterium used in all cloning experiments was E. coli. B K12 strain HB101 isolated by Boyer and Roulland-Dessoix (1969). The genotype of the bacterium is ram C1 F^- Pro^- Gal^- Str^R Rec^-. The ram C1 indicates that the bacterium is deficient in restriction and modification systems for DNA and is therefore susceptible to transfection with exogenous, unmodified DNA.

5. Plasmid Cloning Vector

The plasmid cloning vector used was pAT 153 (Twigg and Sherrat, 1980). This was derived from pBR 322 by partial digestion with Hae II to produce a 0.62 kb deletion spanning the Hae II fragments from nucleotide 1646 to nucleotide 2351 (Sutcliffe, 1978). The plasmid carries ampicillin and tetracycline resistance, and is nic^-, being unable to undergo conjugal transfer. The plasmid contains

single restriction endonuclease cleavage sites for Hind III, Bam HI, EcoR I, Sal I, Cla I, Xma III, Pst I, Bal I and Xor II.

6. Buffer Solutions

The following buffers were routinely used:

RSB	-	10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3).
TNE	-	100 mM NaCl, 10 mM EDTA, 50 mM Tris HCl (pH 7.5).
TNES	-	TNE buffer containing 0.1% (w/v) SDS.
PB	-	equal volumes of equimolar solutions of NaH_2PO_4 and Na_2HPO_4, pH 6.8.
TBE	-	90 mM Tris, 90 mM boric acid, 1 mM EDTA, pH 8.3.
I x E (P)	-	30 mM NaH_2PO_4, 1 mM EDTA, 36 mM Tris, pH 7.8.
SSC	-	150 mM NaCl, 15 mM tri-sodium citrate, pH 7.4.
Isotonic lysis buffer	-	150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.8), 0.65% (v/v) NP40.
Extraction buffer	-	7.0 M urea, 350 mM NaCl, 10 mM EDTA, 1% (w/v) SDS, 10 mM Tris-HCl (pH 7.9).

7. Chemicals

Agarose, SDS, Tris, polyvinylpyrrolidone, PIPES, HEPES, DTT, cycloheximide, yeast RNA, E. coli. rRNA, BSA, and all deoxynucleoside triphosphates were obtained from Sigma Chemical Company, London.

Acrylamide, boric acid, sodium hydroxide, caesium chloride, trichloroacetic acid, PPO and POPOP, were obtained from Koch-Light Laboratories Ltd, Colnbrook, Bucks.

Bis-acrylamide, ammonium persulphate, hydroxylapatite, TEMED, and Coomassie brilliant blue R-250, were supplied by BioRad Laboratories, Richmond, California.

Sephadex G-50, and Ficoll 400, were provided by Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Cs_2SO_4 (ultrapure grade) was provided by Calbiochem-Behring Corporation, San Diego, California.

Ethanol (analytical grade) was supplied by James Burroughs Ltd, London.

Aquasol was supplied by New England Nuclear, Boston, Massachusetts.

Oligo (dT) cellulose (T2 grade) was purchased from Boehringer-Mannheim GmbH, Mannheim, West Germany.

CH_3HgOH was manufactured by Ventron GmbH, Karlsruhe, West Germany, and supplied by Lancaster Synthesis Ltd, Lancaster, U.K.

Ampicillin was supplied by Beecham Research Laboratories, Brentford, U.K.

All other chemicals were of analytical grade, and were obtained from BDH Chemicals Ltd, Poole, Dorset.

8. Radiochemicals

All radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks. $^{32}PO_4$ was carrier free (PBS II). The specific activities of the other radiochemicals used were:

L (^{35}S) methionine	600 Ci/mmol
(5-^{3}H) uridine	25-30 Ci/mmol
5'-α-^{32}P-deoxynucleoside triphosphate	300 Ci/mmol
5'-γ-^{32}P-deoxyadenosine triphosphate	5000 Ci/mmol

9. Enzymes

DNA polymerase 1, proteinase K, and nuclease S1 were obtained from Boehringer Mannheim GmbH, Mannheim, West Germany.

T_4 DNA polymerase large fragment , and restriction endonuclease Pvu II were supplied by New England Biolabs, Beverly, Massachusetts.

T_4 polynucleotide kinase was supplied by P-L Biochemicals Inc., Milwaukee, Wisconsin.

Ribonuclease A (bovine pancreas) was obtained from Sigma Chemical Company.

Restriction endonuclease Xma I was supplied by Worthington Biochemicals Company, Freehold, New Jersey.

All other enzymes were supplied by Bethesda Research Laboratories Inc., Rockville, Maryland.

10. Miscellaneous Materials

Kodirex X-ray film and X-omat H film were supplied by Kodak Ltd, London.

Plastic tissue culture plates were obtained from Flow Laboatories, Irvine, Scotland.

Visking dialysis membrane was supplied by Medicell International Ltd, London.

Kapton tape was obtained from the Minnesota Mining and Manufacturing Company, U.S.A.

Plastic tissue culture tubes (0.6 x 15.5 cm) were obtained from Falcon Incorporated, Oxnard, California.

Nitrocellulose membrane sheets, type BA80, were supplied by Schleicher and Schüll GmbH, Dassel, West Germany.

METHODS

1. Cell Tissue Culture

BHK 21-C13 cells were grown in 80 oz roller bottles containing 200 ml ETC 10 in an atmosphere of 95% air, 5% CO_2 at 37°C. Cells were harvested by washing successively with versene and with trypsin, and were resuspended in medium at a concentration of approximately 5×10^6 cell/ml.

50 mm plastic tissue culture plates were seeded with cells at a density of 2×10^6 cells/plate, and 90 mm plates with 6×10^6 cells/plate in 4 and 10 mls of ETC 10 respectively.

For purification of $^{32}PO_4$-radioactively labelled samples, sub-confluent monolayers were incubated in E(LP)C 2 for 16 hr prior to infection.

2. Production of Virus Stocks

Virus stocks were prepared by infecting almost confluent BHK-21-C13 cells in 80 oz roller bottles containing 20 ml ETC 10, at an m.o.i. of approximately 1 p.f.u./1,000 cells. Infected cells were incubated at 31°C until they could be harvested by shaking into the medium (glass beads were added to bottles, if necessary, to help dislodge cells), and were pelleted by centrifugation at 1,500 g for 15 min at 4°C. The cell pellet was then resuspended in an equal volume of the supernatant, and this suspension was sonicated for 20 sec, using a Dawe Soniprobe. The suspension was centrifuged at 1,500 g for 15 min to pellet cell debris which was again resuspended in an equal volume of medium, and the sonication repeated. The two supernatants were combined, and stored at -70°C in 0.5 ml aliquots. The cell debris pellet was resuspended in an equal volume of medium, and stored at -70°C in 0.5 ml aliquots.

3. Titration of Virus Stocks

Serial dilutions (0.1 ml) of the virus stock in PBS Ca, were added to confluent monolayers of BHK cells in 50 mm tissue culture plates. After absorption of virus for 1 hr, 4 ml of ETHu 2 were added, and infected cells were incubated at 37°C for 2-3 days. After incubation, cells were fixed and stained with Giemsa for 10 min at room temperature, and plaques were counted using a low power microscope.

4. In Vivo Labelling of RNA with $^{32}PO_4$ in Infected Cells

RNA was labelled in cells grown as monolayers in 90 mm tissue culture plates, unless otherwise stated. After incubation with E(LP)C2, cells were infected at a m.o.i. of 100 p.f.u./cell, and virus was absorbed for 1 hr at 37°C.

To label early RNA, cells were incubated with 4 ml of medium containing 1 mCi/ml $^{32}PO_4$ for 2 hr prior to infection. After the virus absorption period, unabsorbed virus was removed by washing five times with pre-warmed E(LP)C2, and 4 ml of medium containing 1.25 mCi/ml $^{32}PO_4$ was added. Incubation was for a further 2 hr at 37°C.

Late RNA was prepared by infecting cells, allowing virus to absorb for 1 hr, and removing unabsorbed virus as before. The cells were then incubated with 4 ml of E(LP)C2 containing 1.25 mCi/ml $^{32}PO_4$, for a further 10 hr, at 37°C.

IE RNA was labelled in cells which were incubated in E(LP)C2 containing 200 µg/ml cycloheximide for 30 min prior to infection. Cells were infected, washed and maintained in this medium. Cell monolayers in 90 mm tissue culture plates were incubated in 4 ml

medium containing 1.25 mCi/ml $^{32}PO_4$ and 200 ug/ml cycloheximide for 6 hr after the virus absorption period.

IE RNA was prepared from cells in 80 oz roller bottles essentially as above. Cells were infected with virus at a m.o.i. of 100 p.f.u./cell in 20 ml E(LP)C2 medium containing 200 µg/ml cycloheximide. After the virus absorption period, cells were incubated in 20 ml medium containing 0.5 mCi/ml $^{32}PO_4$ and 200 µg/ml cycloheximide. RNA was isolated 6 hr after the virus absorption period.

5. Labelling Infected Cells with ^{3}H-uridine

Confluent cell monolayers in 30 mm plastic tissue culture plates were incubated in media containing various concentrations of cycloheximide for 30 min prior to infection. The cells were infected with HSV-1 Glasgow strain 17 syn^{+} (Brown et al., 1973), at a m.o.i. of 50 p.f.u./cell, at 37°C. After a 1 hr absorption period, unabsorbed virus was washed off, and 1 ml medium containing the appropriate concentration of cycloheximide, and 100 µCi of 5-^{3}H-uridine was added. After a further incubation of 6 hr at 37°C, RNA was prepared as described below.

Duplicate 50 µl aliquots of the cytoplasmic extracts, and 25 µl of the nuclear extracts were spotted onto Whatman glass fibre GF/C filter discs. These were then washed at 4°C twice in 10% (w/v) TCA, twice in 5% (w/v) TCA, and twice in absolute alcohol. The dried filter discs were then counted by liquid scintillation spectrometry.

6. Labelling Infected Cells with (35S) methionine

Confluent cell monolayers in 30 mm plates were incubated in E $\frac{met}{5}$C2 for 2 hr prior to infection and 30 min before infection the

medium was replaced with E $\frac{met}{5}$C2 containing various concentrations of cycloheximide. The cells were infected with HSV-1 as above. After virus absorption, and removal of unabsorbed virus, 0.5 ml of medium containing the appropriate concentration of cycloheximide and 5.7 µCi (^{35}S) methionine was added.

After 5.5 hr after the virus absorption period, the cells were harvested by scraping into the medium with 2 ml of PBS and the cells were pelleted by centrifugation at 900 g for 3 min. 400 µl of 2% (w/v) SDS, 0.7 M 2-mercaptoethanol, 10% (v/v) glycerol, was added to the cell pellets and the samples were incubated at 100°C for 2 min to degrade DNA and RNA, and to reduce viscosity. Duplicate 25 µl aliquots were washed as above, using TCA containing 1% (w/v) methionine.

7. Cell Fractionation and RNA Extraction

Cell fractionations were performed essentially as described by Kumar and Lindberg (1972). All manipulations were performed at 4°C.

Cells were scraped into the medium and pelleted by centrifugation at 1,500 g for 3 min. The cell pellet was resuspended in 2.5 ml isotonic lysis buffer and left on ice for 10 min, the nuclei were pelleted by centrifugation at 1,500 g for 3 min, and the supernatant cytoplasmic fraction was decanted and retained. The nuclei were resuspended in 2.5 ml isotonic lysis buffer, left on ice for 10 min, and centrifuged as before. The supernatants were combined and 5 ml extraction buffer was added. Isotonic lysis buffer (5 ml) and 5 ml extraction buffer were added to the nuclear pellet, and this was thoroughly mixed to ensure lysis. Nuclear and cytoplasmic fractions were extracted twice with phenol/chloroform (1:1 V/V) which had been saturated with TNE. Nucleic acids were precipitated by the addition of

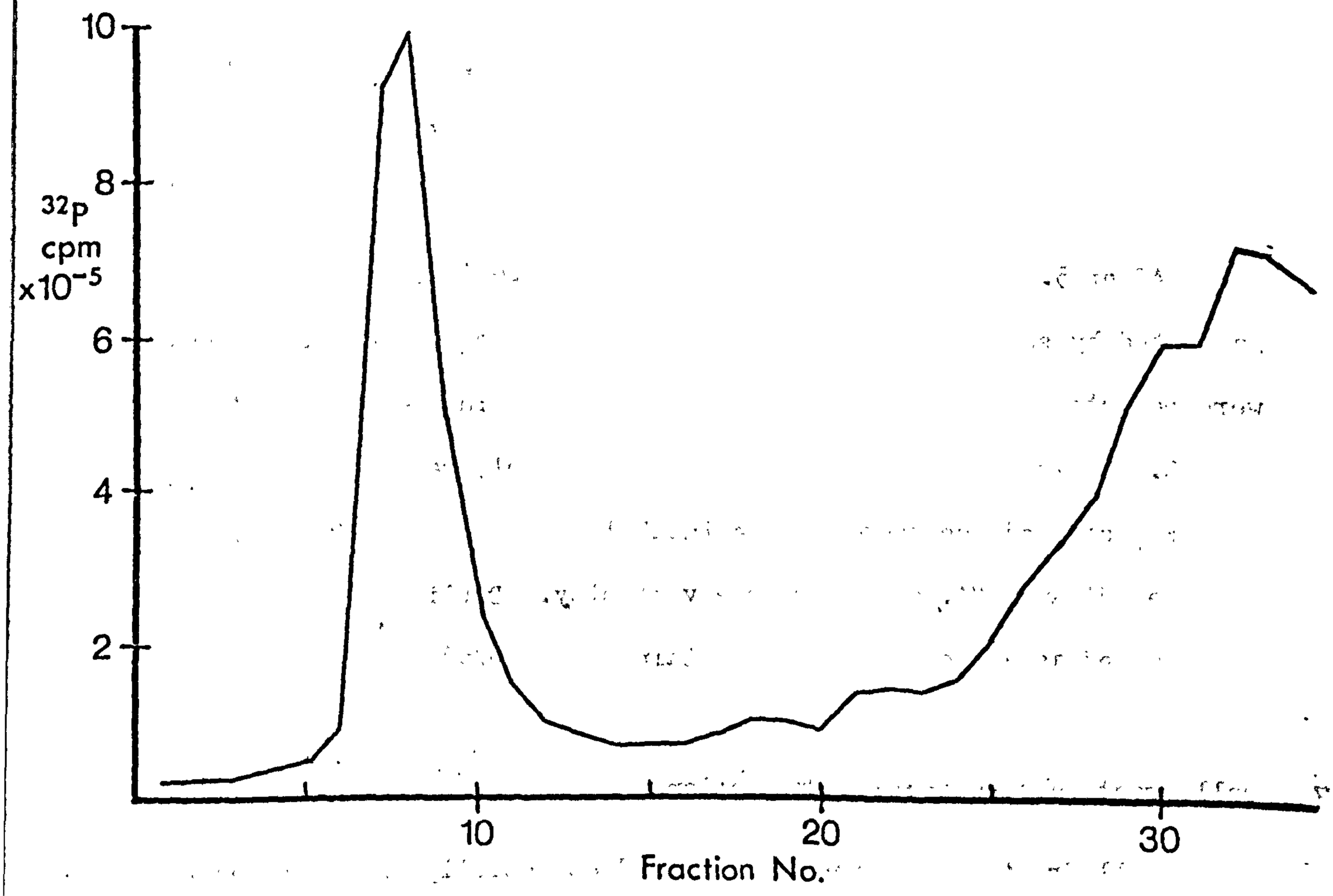


FIGURE 11

Profile of a Cs_2SO_4 gradient of *in vivo* ^{32}P-labelled HSV-2 late nuclear RNA.

three volumes of ethanol and overnight storage at -20°C, and were recovered by low speed centrifugation.

In general, RNA preparations to be hybridized to blot strips were further purified by isopycnic centrifugation in $Cs2SO_4$ gradients. Pelleted RNA samples were dissolved in TNE, and Cs_2SO_4 was added to give a final density of 1.60 g/ml. Samples were centrifuged at 218,000 g using a TV 865 vertical rotor in a Sorval OTD 65 ultracentrifuge. Under these conditions, single-stranded RNA forms a sharp band which allows the complete separation of RNA from DNA (Szybalski, 1968). The profile of a typical Cs_2SO_4 gradient of late nuclear RNA is shown in Figure 11.

8. Selection of Polyadenylated RNA

Polyadenylated cytoplasmic RNA was selected as described by Scolnick et al. (1973). Pelleted RNA was dissolved in 1 ml of binding buffer (500 mM KCl, 10 mM Tris-HCl pH 7.5). The RNA solution was passed three times through a column of oligo(dT)-cellulose which had previously been equilibrated with binding buffer, and the column was washed with 1 ml aliquots of binding buffer. Polyadenylated RNA was eluted with 10 mM Tris-HCl (pH 7.5), made to 200 mM with respect to sodium acetate, and precipitated with three volumes of ethanol by overnight storage at -20°C. The elution profile for a typical ^{32}P-labelled RNA sample is shown in Figure 12. Where necessary, 10 µg of yeast tRNA was added to polyadenylated RNA as a carrier before ethanol precipitation.

9. Preparation of Virus DNA

Almost confluent 80 oz roller bottles were infected with HSV-2 at a m.o.i. of 1 p.f.u./1000 cells in 20 ml ETC 10 medium, and were

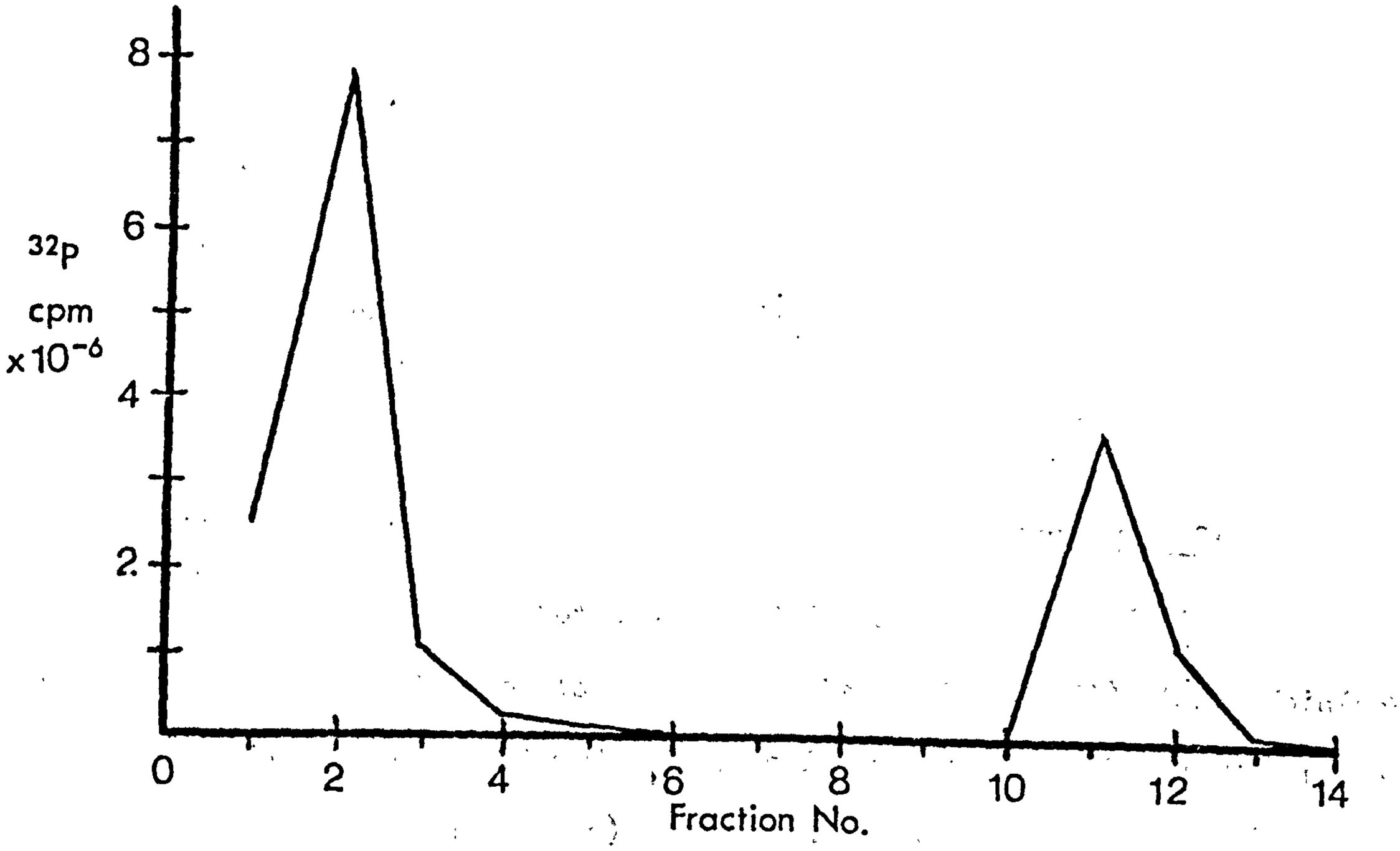


FIGURE 12

Elution profile of <u>in vivo</u> ^{32}P-labelled HSV-2 IE cytoplasmic RNA from an oligo(dT) cellulose column.

incubated at 31°C. The cells were harvested by shaking into the medium, pelleted by centrifugation at 1,500 g for 15 min, and resuspended in 5 ml RSB containing 0.5% (v/v) NP-40. The suspension was left on ice for 10 min for cell lysis to occur, and was then centrifuged at 1,500 g for 15 min as before. This was repeated twice. The combined supernatants were centrifuged at 100,000 g for 45 min, and the supernatant discarded.

RSB (5 ml) was added to the virus pellet and this was disrupted by sonication in a Cole-Palmer ultrasonic cleaning bath. EDTA and SDS were added to 10 mM and 2% (w/v) respectively, and the solution was mixed by gentle rotation with an equal volume of RSB saturated phenol. The aqueous phase was collected following centrifugation at 2,000 g for 15 min. The extraction was repeated twice, and the DNA was then extracted with chloroform containing 2% (v/v) isoamyl alcohol. The DNA solution was dialysed against three changes of 0.1 x SSC, and then CsCl was added to a density of 1.72 g/ml. Virus DNA was banded by centrifugation at 130,000g for 24 hr in a TV.865B rotor using a Sorval OTD 65 ultracentrifuge, and the gradient was collected dropwise from the bottom of the centrifuge tube using a MSE gradient fractionator. Virus DNA, was usually well separated from any small amounts of contaminating cell DNA on these gradients. Fractions containing virus DNA were pooled and dialysed against 0.1 x SSC. DNA concentrations were estimated by spectrophotometry.

10. Estimation of Nucleic Acid Concentration by Spectrophotometry

Nucleic acid preparations were diluted with distilled water, and absorption at 260 nm was determined using quartz microcuvettes and a Perkin-Elmer 124 double beam spectrophotometer. DNA and RNA concentration was estimated assuming one optical density unit is

equivalent to 40 µg/ml.

11. Restriction Endonuclease Digestion of DNA

Endonuclease digestions were performed in 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 6 mM Tris-HCl (pH 7.9), 0.02% (w/v) BSA, and sufficient endonuclease to produce a limit digest. Reactions were terminated after 4 hr at the appropriate incubation temperature for the enzyme, either 37°C or 60°C, by addition of 0.25 volumes of 5 x gel electrophoresis buffer containing 50% (v/v) glycerol and 0.02% (w/v) bromophenol blue.

12. In Vitro Labelling Procedures

a) Labelling of Total DNA

HSV-2 DNA was labelled in vitro to high specific activity by nick translation, essentially as described by Rigby et al. (1977). 1 µg of HSV-2 DNA in 50 µl of 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 1 mM DTT containing 2.5 µg BSA, 130 pmol of each of α-^{32}P-dATP, α-^{32}P-dGTP, α-^{32}P-dCTP and α-^{32}P-TTP, and 2 units of DNA polymerase I was incubated at 15°C for 1.5 hr. Reactions were terminated by addition of EDTA to 10 mM, and glycerol to 20% (v/v). Reaction mixtures were fractionated on a Sephadex G-50 column (0.8 x 25 cm) equilibrated with TNES, and the peak of radioactivity eluted just after the void volume was collected. The specific activity of nick translated DNA preparations was approximately 10^8 c.p.m./µg.

b) 5'-end Labelling of DNA Fragments

Restriction endonuclease generated DNA fragments were 5'-end labelled using T4 polynucleotide kinase essentially by the method of Maxam and Gilbert (1980). The 5'-terminal phosphate of DNA fragments

generated by restriction endonuclease digestion were removed using bacterial alkaline phosphatase.

After enzyme digestion, the reaction mixture was incubated at 65°C for 10 min to inactivate the endonuclease. An equal volume of 40 mM Tris-HCl, pH 8.0, containing 2 units/pmol DNA fragment ends of bacterial alkaline phosphatase was added and the reaction mix was incubated at 65°C for 1 hr, followed by phenol extraction, and precipitation with 3 volumes of ethanol at 20°C.

The DNA, recovered by centrifugation, was washed with ethanol and taken up in 25 µl of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermidine, 15 pmol of γ-^{32}P-ATP and 4 units of T4 polynucleotide kinase. The mixture was incubated at 37°C for 1 hr and the reaction was terminated by addition of EDTA to 20 mM and glycerol to 20% (v/v). The labelled DNA was separated from the unincorporated γ-^{32}P-ATP by chromatography on a Sephadex G-50 column (0.8 x 25 cm) in TNES. The DNA was then precipitated with 3 volumes of ethanol at -20°C.

c) 3'-end Labelling of DNA Fragments

DNA fragments with recessed 3'-ends generated by cleavage with restriction endonucleases were labelled at the 3'-end using T4 DNA polymerase and one or more α-^{32}P-deoxynucleoside triphosphate to fill out the cleavage site (Maxam and Gilbert, 1980).

The DNA fragment, or mixture of fragments, to be labelled were precipitated from ethanol, and taken up in 20 µl of 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 1 mM DTT containing 1 µg BSA, 25 pmol of the appropriate α-^{32}P-deoxynucleoside triphosphate(s) and 5 units of T4 DNA polymerase. The mixture was incubated at 15°C for 3 hr. The reaction was terminated either by addition of EDTA to 10 mM and

glycerol to 20% (v/v) followed by Sephadex G-50 fractionation, or by incubation at 65°C for 10 min followed by cleavage with a restriction endonuclease.

d) Preparation of cDNA's

A reaction volume of 50 µl contained 5 µg of HSV-2 IE cytoplasmic RNA, 200 pmol of α-^{32}P-dCTP, and α-^{32}P-dGTP, 0.04 mM each of unlabelled dCTP, and dGTP, 0.4 mM dATP, and TTP, 5 µM primer oligo$(dT)_{10}$, 50 mM Tris-HCl (pH 8.3), 140 mM KCl, 7mM $MgCl_2$, 10 mM DTT, and 8 units of avian myoblastosis virus reverse transcriptase. The reaction mixture was incubated at 42°C for 90 min, and reactions were terminated by addition of EDTA to 10 mM followed by phenol extraction. The nucleic acids were precipitated with 3 volumes of ethanol and overnight storage at -20°C. Precipitates were recovered by centrifugation, washed with alcohol, dried, dissolved in 100 µl of 0.3 M NaOH, 1 mM EDTA and incubated at 65°C for 20 min to hydrolyse IE mRNA. Incubations were cooled, neutralised with acetic acid, and labelled cDNA was purified by filtration through a Sephadex G-50 column (0.6 x 14 cm) in TNES. The specific activity of the cDNA was 2-6 x 10^6 c.p.m./µg.

e) 5'-end Labelling of HSV-2 IE RNA

5 µg of total IE nuclear and IE cytoplasmic RNA from HSV-2 infected cells was dissolved in 50 µl of 50 mM Na_2CO_3 and incubated at 50°C for 20 min. Reactions were terminated by the addition of Tris-HCl (pH 7.5) to 100 mM, and the RNA was recovered by ethanol precipitation. The partially degraded RNA was labelled using T4 polynucleotide kinase as described above, the RNA was fractionated on Sephadex G-50, and recovered by ethanol precipitation.

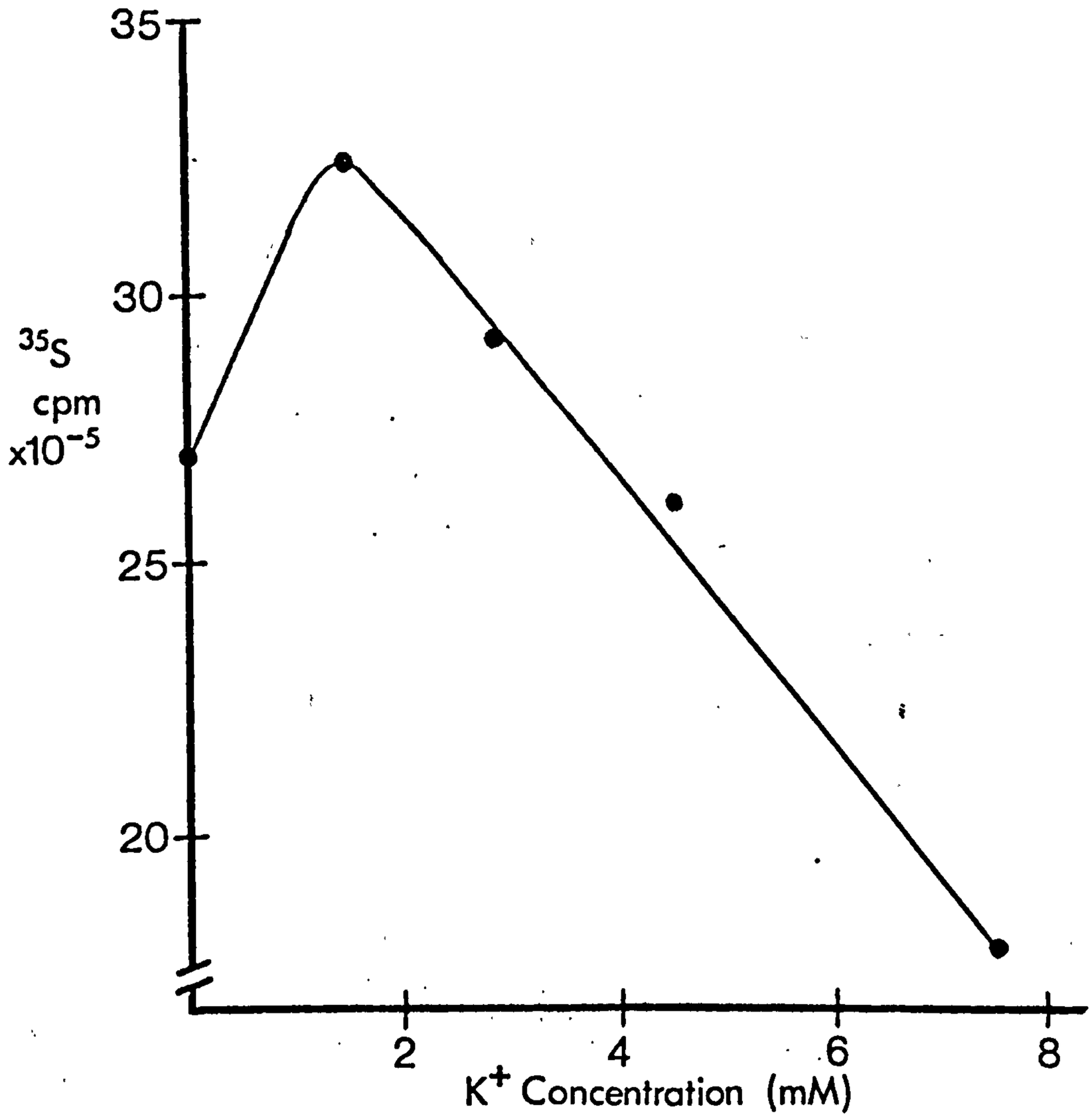


FIGURE 14

Effect of K^+ concentration on incorporation of ^{35}S-methionine into TCA precipitable material in a rabbit reticulocyte in vitro translation system.

13. In Vitro Protein Synthesis using a Rabbit Reticulocyte Lysate

The system used for in vitro protein synthesis was essentially that described by Preston (1977a).

To induce reticulocytosis, rabbits, weighing approximately 2.5 kg, were subcutaneously injected on the neck with 1 ml of a neutral 2.5% (w/v) phenylhydrazine solution for four consecutive days. After a further two days, the rabbits were killed and the blood was immediately collected into a beaker containing 1,000 units of heparin.

The blood was filtered through muslin and centrifuged at 1,500 g for 15 min. The pellet was resuspended in approximately 2 volumes of 130 mM NaCl, 7.4 mM $MgCl_2$, 5 mM KCl, 10 mM Tris-HCl (pH 7.4), centrifuged, and the pellet resuspended, as above. This was repeated three times. After the final centrifugation, the pellet was resuspended in an equal volume of 1 mM DTT, mixed for 1 min, centrifuged at 15,000 g for 20 min, and the supernatant (lysate) was retained.

The "S100" fraction was prepared by centrifuging the lysate at 100,000 g for 2 hr. The supernatant comprised the S100 fraction.

The "pH5" fraction was prepared by centrifugation of the lysate at 100,000 g for 30 min followed by addition of 1 M acetic acid until the pH of the supernatant had dropped to pH 5.0. The formed precipitate was pelleted by centrifugation and dissolved in 200 mM sucrose, 100 mM NH_4Cl, 5 mM $MgCl_2$, 1 mM DTT, 0.2 mM EDTA, 20 mM Tris-HCl (pH 7.4).

Ribosomal pellets were obtained from uninfected BHK 21-C13 cells by sonication of the cells in 100 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 20 mM HEPES (pH 7.4), followed by centrifugation at 15,000 g for

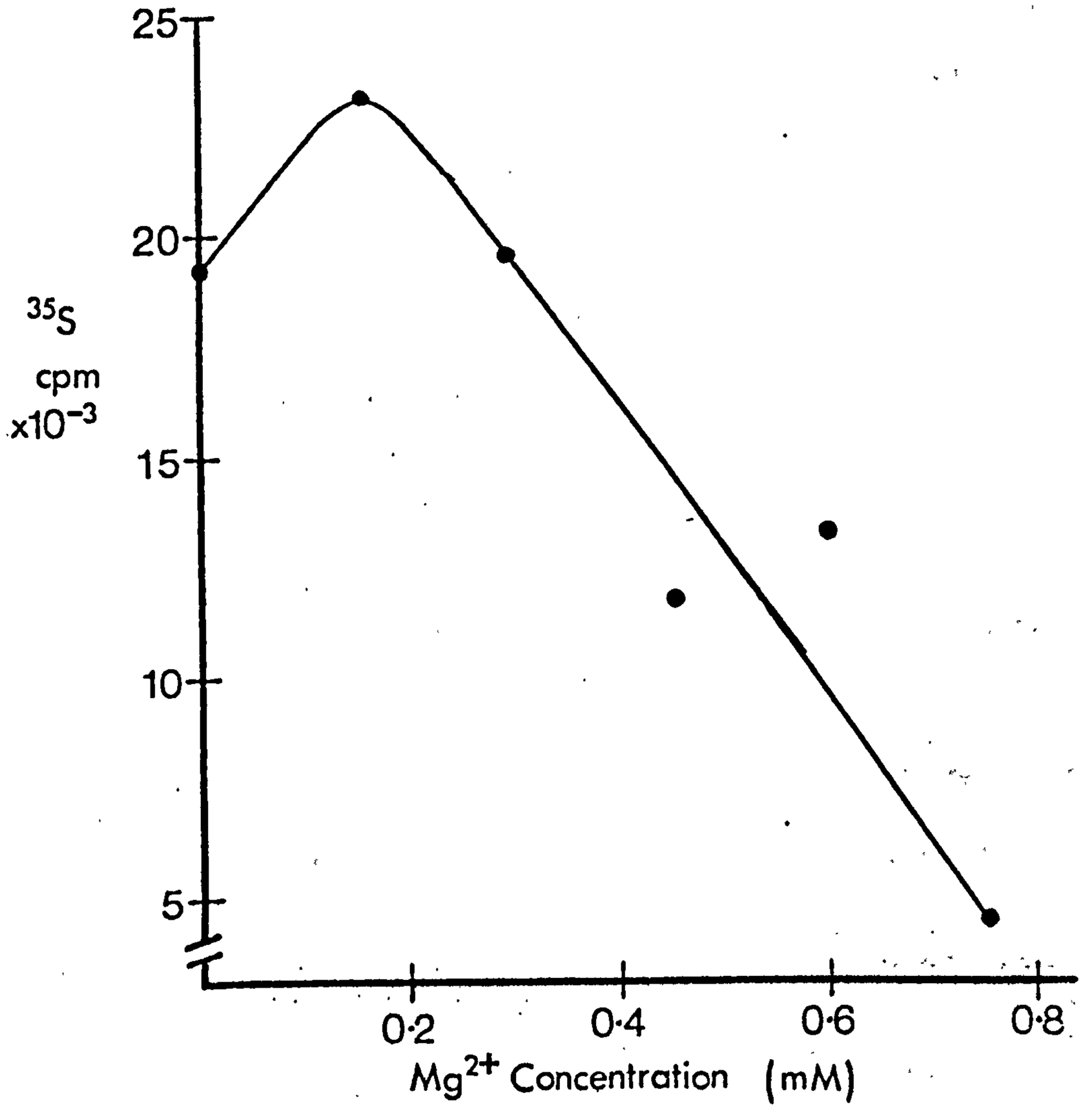


FIGURE 13

Effect of Mg^{2+} concentration on incorporation of ^{35}S-methionine into TCA precipitable material in a rabbit reticulocyte <u>in vitro</u> translation system.

15 min over a cushion of 250 mM sucrose in the same buffer. The supernatant was further centrifuged at 100,000 g for 2 hr. The ribosomal pellet was stirred in 250 mM sucrose, 540 mM KCl, 5 mM magnesium acetate, 0.1 mM EDTA, 20 mM Tris HC-l (pH 7.4), at 0°C for 6 hr followed by centrifugation at 100,000 g for 2 hr. The supernatant represented the ribosomal salt-wash fraction.

The in vitro protein synthesis system was optimised for Mg^{2+} concentration, K^{+} concentration, and pH, using endogenous mRNAs. The graphs of optimization are shown in Figures 13, 14 and 15.

Before translation of virus RNA, the reticulocyte lysate was first treated with 10 µg/ml micrococcal nuclease in the presence of 1 mM CaCl at 20°C for 15 min by the method of Pelham and Jackson (1976) to destroy endogneous mRNAs. Nuclease activity was then inhibited by the addition of ethyleneglycol-bis (2-aminoethylene)-N-N'-tetraacetic acid to 2 mM. In vitro translations were performed in a volume of 25 µl containing 50 mM HEPES (pH 7.9), 0.5 mM spermidine, 0.3 mM CTP, 1 mM ATP, 0.1 mM GTP, 4 mg/ml creative phosphate, 0.2 mg/ml creatine phosphokinase, 0.05 mM of each of the common amino acids excluding methionine, 700-1,000 µCi/ml L-(^{35}S)-methionine, 1.5 µl of the ribosomal salt-wash fraction, 8.75 µl of the reticulocyte S100 fraction, and 2.5 µl of the reticulocyte pH 5 fraction. RNA was added in a volume of 5 µl to give a final concentration of approximately 200 µg/ml. Incubation was for 2 hr at 30°C, after which 25 µl of 100 mM EDTA, 100 mM L-methionine, 300 µg/ml RNAase A was added. Incubation was continued for a further 15 min at 30°C after which 25 µl of a solution of 6% (w/v) SDS, 2 M 2-mercaptoethanol, 30% (v/v) glycerol, 0.02% (w/v) bromophenol blue, was added. After heating at 100°C for 2 min, samples were analysed by PAGE.

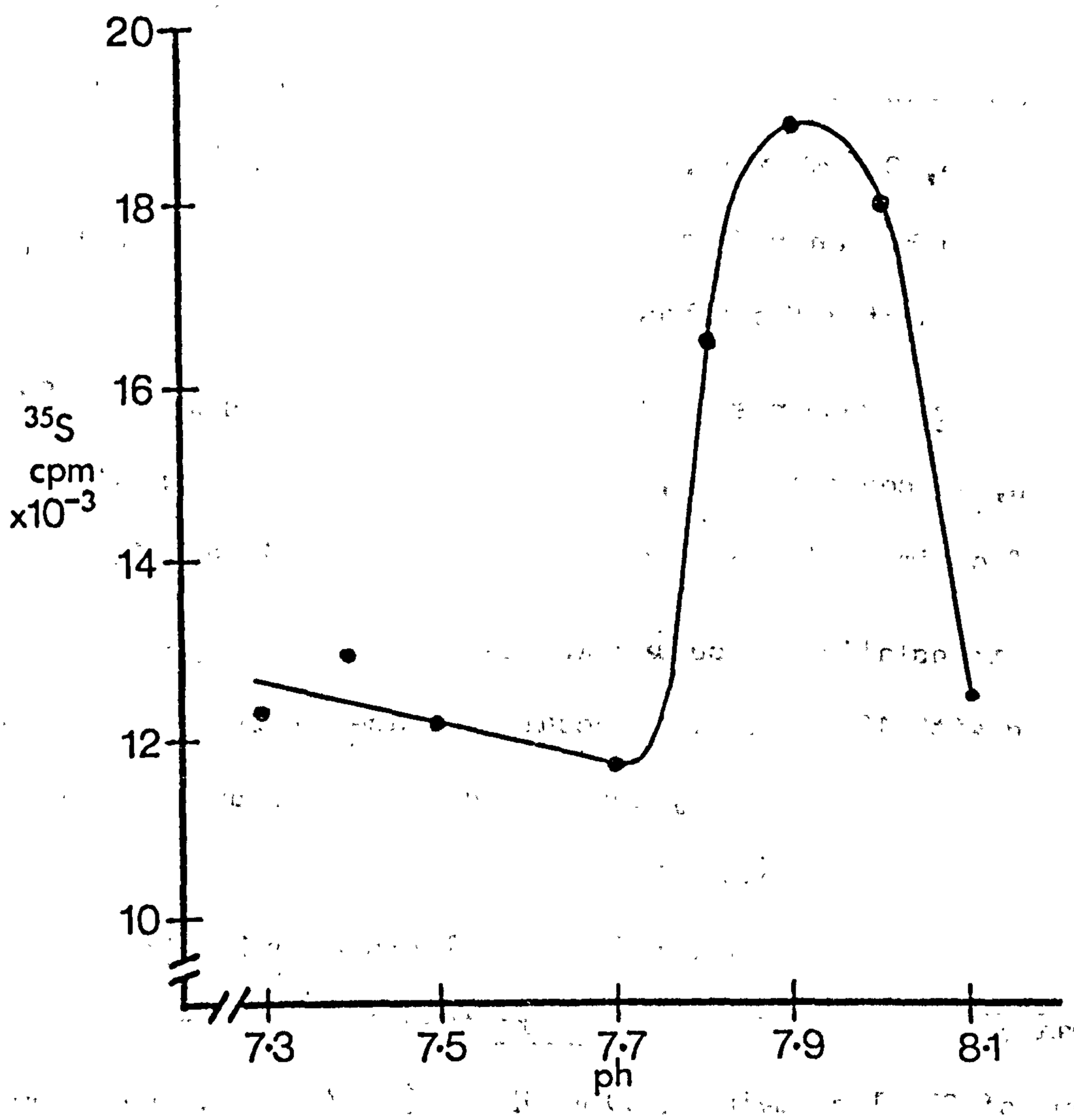


FIGURE 15

Effect of pH on incorporation of ^{35}S-methionine into TCA precipitable material in a rabbit reticulocyte *in vitro* translation system.

14. Gel Electrophoresis

a) Electrophoresis in Non-denaturing Agarose Gels

Electrophoresis was through horizontal slab gels. Gels (26 cm x 16 cm) comprised 200 ml of 0.4 - 2.5% (w/v) agarose in 1 x E(P) or 1 x TBE buffer, usually containing 0.5 µg/ml ethidium bromide. Electrophoresis was at 40 V/gel for 16-30 hr at room temperature. After electrophoresis, gels were viewed under UV light to detect DNA bands. Photographs were made using a Polaroid CU 5 camera with an orange filter, and positive/negative film type 105.

b) Electrophoresis in Denaturing Agarose Gels

Electrophoresis was through horizontal slab gels (26 cm x 16 cm) consisting of 200 ml of 1.5% (w/v) agarose in 30 mM NaOH, 2 mM EDTA, according to the method of McDonnell et al. (1976). Samples were loaded in 1 x alkali running buffer containing 10% (v/v) glycerol and 0.02% bromocresol green after incubation at room temperature for 10 min. Electrophoresis was at 40 V/gel for 16-30 hr at room temperature.

c) Electrophoresis in Two Dimensional Agarose Gels

A horizontal 200 ml slab gel (20 cm x 20 cm) of 1.5% (w/v) agarose in 1 x TBE buffer was cast according to the method of Favaloro et al. (1980), with a single circular 1.5 mm diameter well in one corner, equidistant from two sides. The first dimension was electrophoresed using 1 x TBE buffer in both buffer tanks, at 40 V/gel for 16-20 hr at room temperature. The gel was then removed, and equilibrated with alkaline running buffer (30 mM NaOH, 2 mM EDTA) by shaking with two changes of buffer for a total of 90 min. The gel was then replaced in the gel kit at 90°C relative to the first

orientation, and was electrophoresed with alkali running buffer in the buffer tanks, at 40 V/gel for 16-30 hr at room temperature. Before electrophoresis in the alkaline dimension, a sample of known radiolabelled DNA size markers in alkali running buffer containing 10% glycerol and 0.02% bromocresol green was introduced into the well.

d) Electrophoresis in Non-denaturing Polyacrylamide Gels

Polyacrylamide gels were prepared according to Maniatis et al. (1975). Vertical gel sandwiches (26 cm x 16 cm) were prepared using 1 mm thick spacers, with the sides and bottom sealed with Kapton tape. A 5% polyacrylamide gel was prepared from a stock solution of 30% acrylamide (1 g of N;N'-methylenebis-acrylamide + 29 g of acrylamide/100 ml H_2O). The stock solution was diluted to 5% acrylamide in 1 x TBE buffer, degassed, and mixed with 40 µl TEMED and 400 µl of 10% (w/v) ammonium persulphate/100 ml. The gel was poured, and allowed to set for at least 90 min before electrophoresis. Electrophoresis was at 300 V/gel for 3 hr at room temperature. Samples were applied to the gel in 1 x TBE containing 10% (v/v) glycerol and 0.02% (w/v) of either bromophenol blue or xylene cyanol.

e) Electrophoresis of RNA in Denaturing Agarose Gels

Preparative and analytical gel electrophoresis was performed using 1.5% (w/v) agarose gels containing 5 mM CH_3HgOH in vertical gel kits (15.5 x 19 cm), essentially by the method of Bailey and Davidson (1976).

Precipitated poly (A) RNA was redissolved in 50 µl E buffer (50 mM boric acid, 5 mM $Na_2B_4O_7.10H_2O$, 10 mM Na_2SO_4, 1 mM EDTA, pH 8.2) containing 25 mM CH_3HgOH and 0.02% bromophenol blue. RNA was incubated for 10 min at room temperature before electrophoresis.

Preparative electrophoresis was performed on a gel with a

single wide slot. Electrophoresis was at 30 V for 16 hr. On completion, gels were soaked in 500 mM ammonium acetate containing 1 μg/ml ethidium bromide for 30 min. RNA bands were visualized by UV fluorescence or by autoradiography of dried gels. Preparative gels were covered with cling-film, and autoradiographed directly.

f) Electrophoresis of RNA in Denaturing Polyacrylamide Gels

12% polyacrylamide gels containing 7 M urea were prepared according to the method of Maniatis et al. (1975), by mixing 31.5 g of urea with 30 ml of stock 30% acrylamide solution, 7.5 ml 10 x TBE, and sufficient H_2O to a volume of 75 ml. After degassing the solution was mixed with 30 μl TEMED and 300 μl of 10% (w/v) ammonium persulphate, the gel poured, and polymerized for 4 hr before electrophoresis.

RNA samples were treated with 25 mM CH_3HgOH as described above, prior to electrophoresis. After electrophoresis, the gels were shaken in two changes of 1 l H_2O for 1 hr, dried down, and autoradiographed.

g) Separation of In Vitro Translation Products by PAGE

Proteins were analysed by PAGE using 5.5-16% polyacrylamide gradient gels in the presence of SDS, as described by Marsden et al. (1976). A stacking gel of 5% polyacrylamide in 125 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS was used. The resolving gel was 14.6 cm long with a volume of 38 ml, and the gel buffer was 375 mM Tris-HCl (pH 8.9), 0.1% (w/v) SDS. The ratio of acrylamide to bis-acrylamide was 19:1, and polymerization was assisted by the additional 20 μl TEMED and a concentration gradient of ammonium persulphate which was 0.08% (w/v) at the top of the gel and 0.03% (w/v) at the bottom. This gradient ensured that polymerization occurred from the top of the gel to the bottom, and a glycerol gradient of 0-10% (v/v) was also

incorporated to minimise disturbance of the gel gradient. Polymerization was carried out in a water tank to aid heat dissipation.

Electrophoresis was at 40 mA for approximately 4 hr at 4°C until the bromophenol blue dye front reached the bottom of the gel. Electrophoresis buffer containing 50 mM Tris, 5 mM glycine, 0.1% (w/v) SDS was used in both upper and lower buffer tanks. Following electrophoresis, gels were fixed and stained in methanol:acetic acid:water (50:50:7, v/v) containing 0.2% (w/v) Coomassie brilliant blue. Gels were destained in several changes of methanol:water:acetic acid (50:880:70, v/v), dried and autoradiographed.

h) DNA Sequencing Gels

The products of the four sequencing reactions were resolved using 8% and 20% polyacrylamide gels containing 10 M urea, as described by Maxam and Gilbert (1980).

The gels (0.035 x 23 x 45 cm) were prepared in 0.55 x TBE by mixing appropriate proportions of stock solutions of deionized 20% acrylamide, 10 M urea, and 10 x TBE. The ratio of acrylamide to bis-acrylamide was 30:1. The solution was degassed, mixed with 30 µl TEMED and 400 µl of 10% (w/v) ammonium persulphate, and cast in the gel sandwich by passage through a 21 G syringe needle. Polymerization was for 1 hr after which the gel was pre-electrophoresed at 40 W for a minimum of 1 hr (8% gels) and 1.5 hr (20% gels).

The 20% gels were electrophoresed until the bromophenol blue dye front had migrated approximately 20 cm, while the 8% gels were electrophoresed until the xylene cyanol dye front had migrated 17 cm. An aliquot of sample was applied, and electrophoresis was continued until either the first xylene cyanol dye front, or the second bromophenol blue dye front, was at the bottom of the gel.

After electrophoresis, the gel sandwich was opened, and the gel was covered with a thin plastic sheet, and subjected to fluorography at -70°C for 4-14 days.

15. Elution of DNA Fragments from Agarose Gel Slices

Fragments of DNA were isolated from agarose gel slices as described by Wilkie and Cortini (1976). Gel slices were dissolved in 5 M sodium perchlorate at 60°C, and adsorbed to hydroxylapatite columns (1 cm x 1 cm). Columns were washed with portions of 5M sodium perchlorate and then with 140 mM PB. DNA was eluted with 400 mM PB and dialysed extensively against 0.1 x SSC. DNA was concentrated by ethanol precipitation.

16. Elution of DNA Fragments from Polyacrylamide Gel Slices

Gel slices were macerated using a siliconized glass rod, suspended in elution buffer (500 mM ammonium acetate, 1 mM EDTA, 0.1% EDTA, 0.1% (w/v) SDS and incubated at 4.5°C overnight. The suspension was filtered firstly through glass wool, and then through a sintered glass disc. The DNA was recovered by ethanol precipitation.

17. Elution of RNA from Agarose Gel Slices

Gel slices were macerated in 2 ml of formamide. The homogenates were adjusted to 50% formamide, 3 x SSC, 1 x Denhardt's solution, 100 µg/ml yeast RNA, and were incubated overnight at 45°C with shaking prior to hybridization.

18. DNA Blot Hybridization

The method used for DNA blot hybridization was essentially that of Southern (1975).

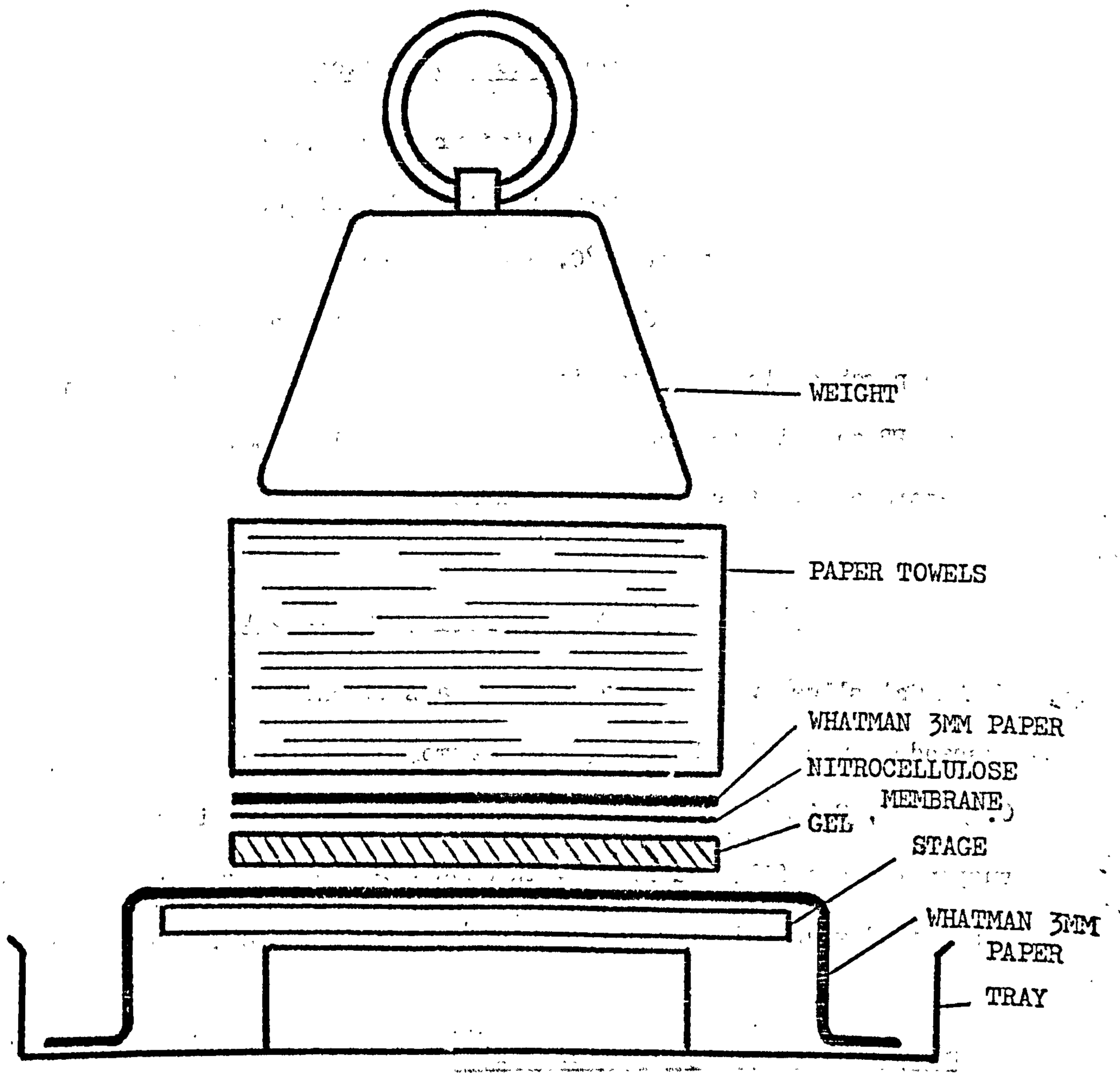


FIGURE 16

Diagram of the procedure used for blotting DNA from agarose gels, by the method of Southern (1975).

Virus DNA fragments generated by restriction endonuclease digestion were seperated by electrophoresis on wide (14.2 cm) one-track agarose gels. After gel electrophoresis, the DNA was visualized under UV light, and excess agarose was removed. The gels were shaken gently in 1 l of gel soak 1 (300 mM NaOH, 600 mM NaCl), at room temperature for 1 hr. Gels were neutralized by removing the gel soak 1, and replacing it with 1 l of gel soak 2 (600 mM NaCl, 1 M Tris, pH 8.0).

The procedure used for DNA blot transfer is shown diagramatically in Figure 16. Gels were placed on two sheets of Whatman 3 MM paper soaked with 6 x SSC supported on a glass plate. The glass plate was supported in a tray containing 2 l of 6 x SSC. A piece of nitrocellulose membrane, pre-soaked in buffer, and cut to the same dimensions as the gel was placed on the gel surface. On the surface of the membrane were placed two sheets of pre-soaked Whatman 3 MM paper, and a pile of dry paper towels cut to the same size as the gel (see Figure 16). The overlapping edges of the supporting filter paper were immersed in the buffer in the tray, and the buffer was drawn up through the gel by the paper towels. DNA fragments resolved in the gel were transferred to the surface of the nitrocellulose membrane, where they were retained in the same spatial relationship as in the gel. DNA transfer was carried out overnight, after which the nitrocellulose membrane was removed, dried in air, and baked at 80°C under a vacuum for 2 hr. Blot strips (5 mm) were cut lengthways from the large nitrocellulose membrane.

Before hybridization, the blot strips were incubated at 45°C overnight in 50% (v/v) formamide, 3 x SSC, 1 x Denhardt's solution (Denhardt, 1966; 0.02% (w/v) Ficoll, 0.02% polyvinylpyrrolidone,

0.02% BSA), containing 100 µg/ml yeast RNA. Hybridizations were performed under the same conditions at 45°C for 48 hr, in plastic tissue culture tubes (0.6 x 15.5 cm), sealed with water-tight metal caps.

The temperature of incubation was determined using the equation of Schildkraut and Lifson (1965), assuming a decrease in T_m of 0.7°C for 1% (v/v) formamide (McConaughy et al., 1969).

Following hybridization, blots were washed in five changes each of 400 ml of 3 x SSC, at 60°C for 1 hr with shaking. Blots to which RNA was hybridized were incubated for 1 hr at 37°C with 25 µg/ml RNAase A in 3 x SSC after the second wash. Washed blots were dried, and subjected to autoradiography or fluorography.

19. RNA Blot Hybridization

The method used for RNA blot hybridization was essentially that of Alwine et al. (1977), in which RNA is transferred from agarose gels onto activated diazobenzoyloxymethyl-paper, where it is immobilized by being covalently bound to the paper.

A sheet of Whatman 540 paper (28 cm x 28 cm) was soaked with 20 ml of a solution containing 1.6 g of N-(3-nitrobenzoyloxymethyl) pyridinium chloride and 0.5 g of sodium acetate, in an enamel tray, and dried at 60°C. It was then incubated at 135°C for 35 min. The paper was then washed twice for 20 min with H_2O at room temperature with shaking, dried at 60°C, washed twice with acetone at room temperature with shaking, and air dried. The paper was reduced by shaking in 300 ml of 20% (w/v) sodium dithionite at 60°C for 30 min, followed by washing with H_2O for 20 min at room temperature with shaking. The paper was then washed with 30% (v/v) acetic acid for

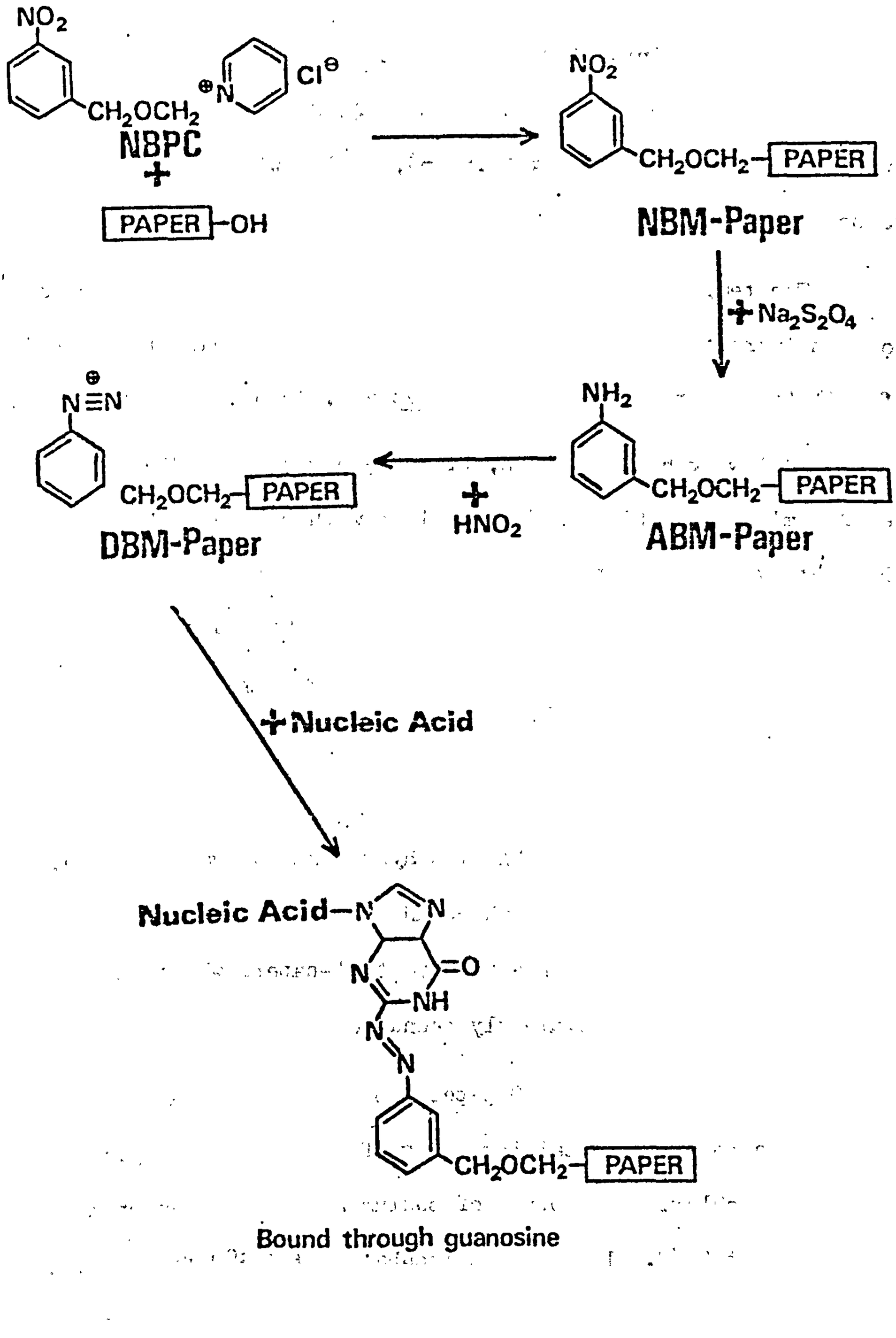


FIGURE 17

Reactions involved in the preparation of diazobenzoyloxymethyl paper.

20 min, followed by several washes with H_2O until the smell of H_2S disappeared.

The activation step was carried out at 4°C. 100 ml of 1.2 M HCl was mixed with 3.2 ml of a freshly prepared 10 mg/ml solution of sodium nitrite, and the paper was soaked in this for 30 min. The nitrous acid was removed by washing the paper in H_2O with five changes. The paper was washed twice, with shaking, in 100 ml of transfer buffer (1 M sodium acetate, pH 4.0) for 20 min; followed by single washes in 1.2 M HCl, ethanol, and ether. The dried paper was stored at -20°C until use. A summary of the chemical reactions involved is given in Figure 17. At 15 min before RNA transfer, the paper was washed twice in transfer buffer.

Virus mRNA species were separated by electrophoresis in denaturing agarose gels containing 5 mM CH_3HgOH, either in parallel gel tracks, or on wide one-track gels. After electrophoresis; the gels were shaken in 50 mM NaOH, 5 mM 2-mercaptoethanol, at room temperature for 40 min, followed by two 10 min washes in transfer buffer.

The procedure for transferring RNA was as described for DNA blot transfer, as shown in Figure 16, with activated paper in place of the nitrocellulose membrane, and transfer buffer replacing 6 x SSC. The transfer was left overnight at 4°C. After transfer, the paper was air dried, and cut lengthways into 5 mm strips.

Hybridizations were performed as described for nitrocellulose blot strips.

20. Characteristics of the Blot Hybridization Reaction

Although hybridization between RNA and DNA is a bimolecular reaction, in situations of excess DNA, the kinetics of the reaction can be described as pseudo first-order (Kennell, 1971). This should be particularly true in the case of a hybridization reaction involving denatured DNA immobilized on a nitrocellulose membrane, since annealing of the DNA is not possible. Under conditions of excess DNA, the rate of the reaction is dependent on the DNA concentration, however, the proportion of an individual RNA species driven into a hybrid increases with increase in input concentration (Kennell and Kotoulas, 1968), with maximal hybridization attained with ratios of DNA to RNA of 10:1 or greater (Kennell and Kotoulas, 1963). Thus, in situations in which filter-bound DNA sequences are present in ten-fold or greater excess over each RNA species, the blot hybridization technique could, in principle, be quantitative.

It has been reported, however, that hybridization of RNA to DNA bound to nitrocellulose exhibit non-ideal kinetics, probably due to the formation of reaction intermediates in a rate-limiting step (Spiegelman et al., 1973). Three points particularly pertinent to the blot hybridization studies of HSV-2 RNA may also be considered:

1. The RNA samples used in this analysis consist of mixed populations of both abundant and scarce mRNA species, such that for certain mRNA species the relevant DNA sequences may not be in sufficient excess to ensure maximal hybridization.

2. Due to the structure of HSV-2 DNA, some fragments, produced by restriction endonuclease digestion, are present in sub-molar quantities (Cortini and Wilkie, 1978). Also, DNA

sequences from the inverted repetitive regions are present in twice-molar amounts with respect to unique sequences, and these repetitive sequences may be present in more than one DNA fragment.

3. The amount of hybridization to a particular DNA fragment is a function of both the number of individual mRNA species, and the number of copies of each mRNA species which map within that fragment.

The blot hybridization profiles will therefore be the end products of a mixture of reactions of various equilibria, each occurring at various rates, according to the relative concentrations of the reactants.

The blot hybridization technique can not, therefore, in general, and particularly in the case of HSV-2, be used to precisely quantitate the relative amounts of RNA sequences representing different DNA fragments, because of the non-ideal kinetics, and also because the proportion of input RNA which hybridizes with excess DNA is concentration dependent. However, reasonable qualitative estimates of the relative degree of representation of a DNA fragment within an RNA sample may be made.

21. Nucleotide Sequencing of End-labelled DNA

The sequencing method used was that of Maxam and Gilbert (1980), which requires that the DNA to be sequenced is labelled with ^{32}P at one end of one strand (either 5'- or 3'-end). Four separate chemical reactions are performed which cleave the DNA at specific nucleotides, with the reaction conditions adjusted so that on average, only one nucleotide is reacted per DNA molecule. Cleavage of the

FIGURE 18 Summary of the protocols for DNA sequencing, based on the method of Maxam and Gilbert (1980).

G	T + C	C	G + A
200 µl DMS buffer	10 µl H_2O	15 µl 5M NaCl	^{32}P-DNA
1 µl carrier DNA	1 µl carrier DNA	1 µl carrier DNA	lypholize
5 µl ^{32}P-DNA	10 µl ^{32}P-DNA	5 µl ^{32}P-DNA	15 µl DPU mix
Chill to 0°C	Chill to 0°C	Chill to 0°C	
1 µl DMS	30 µl hydrazine	30 µl hydrazine	
			20°C for 10 min
20°C for 4 min	20°C for 5 min	20°C for 5 min	
			45 µl H_2O
50 µl DMS stop	200 µl hydrazine stop	200 µl hydrazine stop	500 µl ether
750 µl ethanol	750 µl ethanol	750 µl ethanol	mix
			remove ether
	-70°C for 5 min		repeat ether extraction twice
	Centrifuge for 5 min		
	Discard supernatant		freeze
	250 µl 0.3M NaAc		
	750 µl ethanol		lyophilize
	-70°C for 5 min		
	Centrifuge for 5 min		
	Discard supernatant		
	Ethanol rinse		
	Vacuum Dry		
	100 µl 1.0 M piperidine		
	90°C for 30 min		
	lyophilize		
	10 µl H_2O		
	lyophilize		
	repeat		
	10 µl formamide-buffer-dye		
	90°C for 1 min		
	Chill		
	Load on gel		

DNA will produce a family of radioactive DNA fragments extending from the labelled end to each of the positions of the specific nucleotide(s). The four reactions are cleavage at; guanines alone (G), guanines and adenines (G + A), cytosines alone (C), and cytosines and thymidines (C + T). The reaction products are then resolved on denaturing polyacrylamide gels and the DNA sequence read from an autoradiogram by noting which reaction cleaved at each successive nucleotide along the strand. In the experiments to be described, DNA labelled at the 3'-end was used.

The procedure for G, C + T, and C reactions was exactly as described by Maxam and Gilbert (1980) with reaction times of 4, 5 and 5 min respectively at 20°C. The G + A reaction used was based on the diphenylamine reaction of Dische (1930) in which the deoxyribose sugar attached to purine nucleotides is cleaved (R. Thomson, personal communication). The lyophilised DNA was dissolved in 15 µl of a solution containing 2% (w/v) diphenylamine, 66% (v/v) formic acid, 1 mM EDTA, and incubated at 20°C for 10 min. The reaction was terminated by addition of 45 µl H_2O followed by three extractions with ether. The solution was then lyophilised, and the DNA taken up in 100 µl of 1 M piperidine. The solution was incubated at 90°C for 30 min lyophilized, and the DNA taken up in formamide containing 0.02% (w/v) bromophenol blue, 0.02% (w/v) xylene cyanol, 0.02% (w/v) Orange G. Denaturation before application to polyacrylamide gels was performed by incubation at 90°C for 1 min followed by chilling on ice. The sequencing procedures are summarized in Figure 18.

22. Nuclease S1 Analysis of RNA Structure

The technique used for the analysis of RNA structure was based on the method of Berk and Sharp (1977). Cloned restriction endo-

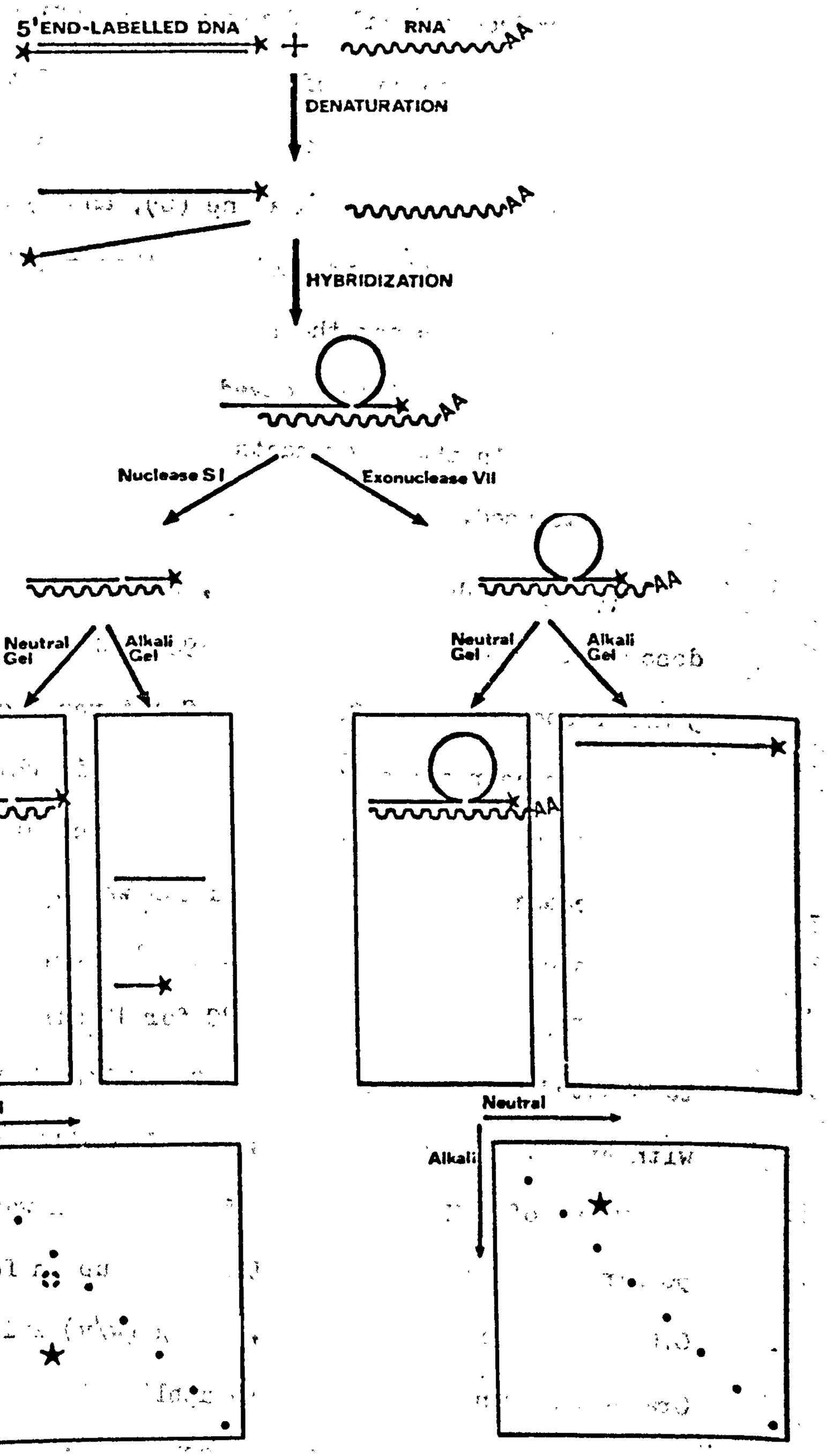


FIGURE 19

Procedure for the analysis of the structure of a mRNA by the S1 nuclease technique of Berk and Sharp (1977) using a 5'-end-labelled DNA probe.

nuclease generated DNA fragments were 5'-^{32}P-end-labelled using T4 polynucleotide kinase, or were 3'-end-labelled using T4 DNA polymerase as described above. The end-labelled fragments were then either cleaved with a restriction endonuclease having only one site in the DNA, and the two labelled ends isolated, or were used as a mixture of labelled fragments, as indicated.

Approximately 0.1-0.5 μg of the DNA fragment(s) was mixed with 15 μg of total HSV-2 IE cytoplasmic RNA in 90% (v/v) formamide, 400 mM NaCl, 1 mM EDTA, 40 mM Pipes (pH 6.4). The solution was incubated at 80°C for 2 min to denature the DNA, and then incubated at 57.5°C for 6 hr. After hybridization, the solution was diluted ten-fold, and adjusted to 250 mM NaCl, 30 mM sodium acetate, 1 mM $ZnSO_4$. Nuclease S1 (5×10^3 units) was added, and the reaction mixture was incubated at 37°C for 1 hr, after which it was extracted with an equal volume of phenol/chloroform (1:1), the aqueous phase adjusted to 300 mM with sodium acetate, and the nucleic acid precipitated with 3 volumes of ethanol. Precipitated samples were recovered by centrifugation, and electrophoresed on native, denaturing, and two dimensional agarose gels.

A summary of S1 nuclease analysis using a 5'-end labelled DNA fragment is shown in Figure 19.

23. Growth and Maintenance of Bacterial Cultures

E. coli. B K12 strain HB101 was grown in L-broth medium (Boyer and Roulland-Dussoix, 1969; 170 mM NaCl, 10 g/l Difco bacto-tryptone, 5 g/l yeast extract) at 37°C with vigourous shaking to ensure good aeration.

Transformed bacteria containing pAT 153 were grown in L-broth containing 100 µg/ml ampicillin.

Seed stocks were prepared by growing a single inoculum of bacteria in 20 ml of the appropriate medium overnight at 37°C. The bacteria were pelleted by centrifugation at 8,000 g for 10 min, and resuspended in 10 ml of a solution containing 50% of 2% (w/v) Difco bacto-peptone, 40% (v/v) glycerol. The bacterial suspension was stored in 1 ml aliquots at -20°C.

24. Preparation of Plasmid DNA

An overnight culture was used to seed 1 l of L-broth containing 100 µg/ml ampicillin. The culture was then shaken vigourously at 37°C for 20 hr, and bacteria were pelleted by centrifugation at 8,000 g for 15 min. The bacteria were then lysed using a neutral detergent, based on the method of Komano and Sinsheimer (1968), and the plasmid DNA isolated essentially as described by Clewell and Helinski (1970).

The bacteria were resuspended in 20 ml of 25% (w/v) sucrose, 50 mM Tris-HCl (pH 8.0), 10 ml of lysozyme (5 mg/ml) was added and the mixture left at 4°C for 30 min. Then 10 ml of 0.2 M EDTA was added, and incubation at 4°C was for 30 min, after which 20 ml of 1.5% (v/v) NP 40 was added and the solution was mixed thoroughly. After 10 min at 4°C, the solution was centrifuged at 20,000 g for 90 min to pellet cell debris and most of the chromosomal DNA. The supernatant was extracted extensively with phenol, and the nucleic acids were precipitated with 3 volumes of ethanol.

The nucleic acid pellet recovered by low speed centrifugation was resuspended in 5 ml of 0.1 x SSC containing 25 µg/ml RNAase A,

and the solution was dialysed against three changes of 0.1 x SSC for 3 hr, phenol extracted, and precipitated with ethanol as before. The precipitate was resuspended in 0.3 M sodium acetate, and the DNA precipitated with 0.54 volumes of isopropanol for 1 hr at room temperature to remove any residual polysaccharide material. The DNA was finally resuspended in 0.3 M sodium acetate and precipitated with 3 volumes of ethanol at -20°C.

25. Ligation of DNA Fragments to Plasmid DNA

The HSV-2 DNA fragments generated by restriction endonuclease Bam HI, designated Bam HI f and Bam HI p (0.73-0.78 map units and 0.78-0.80 map units, respectively) were prepared by cleaving 50 µg of HSV-2 DNA with Bam HI, and separating the fragments by electrophoresis on a wide single-track (14.2 cm) agarose gel in the absence of ethidium bromide. Strips of agarose were cut from both sides of the gel, and were soaked in 1 x E(P) containing 10 µg/ml ethidium bromide for 40 min. The strips were realigned with the gel, the DNA fragments visualized by UV fluorescence, and the appropriate sections of agarose containing the required fragments were excised. DNA was purified from the agarose using hydroxylapatite chromatography as described previously.

The ligation reaction was performed essentially as described by Tanaka and Weisblum (1975). The purified DNA fragments (approximately 2 µg of Bam HI f and 1.2 µg of Bam HI p) were each mixed with 3 ug of pAT 153 DNA which had been digested with Bam HI, in 20 µl of 20 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 0.5 mM ATP, 10 mM DTT, containing 0.5 units of T4 DNA ligase. The mixture was incubated at 4°C for 20 hr, and the reaction terminated by incubation at 65°C for 10 min.

Before transfection of the DNA, the mixture was adjusted to 30 mM with respect to $CaCl_2$.

26. Transformation of E. coli. with Plasmid DNA and Colony Hybridization

Transformation was performed essentially as described by Cohen et al. (1972). E. coli. BK 12 strain HB101 (400 ml) was grown until the culture had an absorbance of 0.6 optical density units at 630 nm. These exponentially growing cells were pelleted by centrifugation at 8,000 g for 10 min, resuspended in 400 ml of 10 mM NaCl, and pelleted as before. The pellet was resuspended in 400 rl of 30 mM $CaCl_2$, and the suspension was left on ice for 20 min before being centrifuged as above. The pellet was finally resuspended in 8 ml of 30 mM $CaCl_2$, glycerol was added to 15% (v/v) (1.4 ml), and aliquots were stored at -70°C until required.

$CaCl_2$ treated bacteria (200 µl) were mixed with the DNA solution in 30 mM $CaCl_2$, and left at 4°C for 30 min. The suspension was then incubated at 37°C for 5 min, 3 ml L-broth (with no ampicillin) was added, and the suspension was incubated at 37°C, with vigorous shaking, for 90 min, after which 0.1 ml inocula were spread on L-broth agar plates (90 mm) containing 100 µg/ml ampicillin. The plates were incubated at 37°C overnight. Bacterial colonies were then analysed by the colony hybridization technique of Grunstein and Hogness (1975).

Sterile gridded nitrocellulose membrane filters which had been washed three times in boiling H_2O, and autoclaved, were placed on L-broth agar/ampicillin plates. Individual bacterial colonies were then transferred onto the membrane, and also into a corresponding linbro well containing 200 µl L-broth/ampicillin, using a sterile

toothpick. The colonies were grown on the membranes overnight at 37°C. The linbro wells were maintained at room temperature.

To prevent movement of the bacteria or DNA from their colonial sites during lysis, denaturation and fixation, the solutions used during these manipulations were allowed to diffuse into the colonies from the underside of the membrane, by placing the nitrocellulose on a filter paper which had been saturated with the appropriate solution. The membrane was placed in 0.1 M HCl for 1 min at room temperature, and then transferred into 0.5 M NaOH for a further incubation of 15 min. After denaturation, the membrane was placed in 1.0 M Tris-HCl (pH 7.5) for 5 min, followed by 1.2 M NaCl for a further 15 min. The membrane was then placed in 10 ml of 0.1 x SSC containing 100 µg/ml proteinase K, and incubated at 37°C for 1 hr. Cellular debris was removed by dipping the membrane into 0.1 x SSC. The nitrocellulose was then baked at 80°C under vacuum for 2 hr.

The membrane was pre-incubated, hybridized using a nick-translated total HSV-2 DNA probe and washed by the method described for DNA blot strips. Colonies containing HSV-2 DNA inserts were detected by autoradiography alongside known HSV-2 clones.

The positive colonies were streaked on to a L-broth agar/ampicillin plate, an isolated colony was picked, grown up as a seed stock, and the plasmid DNA was analysed by: (i) colony hybridization; (ii) nick-translation of plasmid DNA and hybridization to DNA blot strips; (iii) excision of the DNA insert and comparison on agarose gels with HSV-2 DNA fragments before and after cleavage with various restriction endonucleases.

The average efficiency of transformation of bacteria with pAT 153 ligated to the HSV-2 Bam HI f and p fragments was approximately 4%

for each fragment.

27. Liquid Scintillation Counting

Aqueous samples were mixed with 6 ml Aquasol in plastic vials, and glass fibre discs were placed in toluene/PPO (0.4% PPO, 0.005% POPOP in toluene), and counted using the appropriate channel of an Intertechnique SL30 or SL40 liquid scintillation counter. Counting ^{32}P in aqueous solution (Cerenkov emission) was approximately 30-35% as efficient as counting ^{32}P β-particle emission in scintillation fluid.

28. Procedures for Autoradiography and Fluorography

Autoradiographs were exposed using Kodirex X-ray film for up to 1 month with ^{35}S or three weeks with ^{32}P, at ambient temperature.

Fluorographs were made with ^{32}P using a single Du Pont Cronex Lightning Plus intensifying screen behind the film. X-omat H film, pre-sensitised using the methods of Lasky and Mills (1977), was exposed for up to three weeks at -70°C. Using this technique, the sensitivity of detection of radioactivity was five times greater than that of autoradiography.

RESULTS

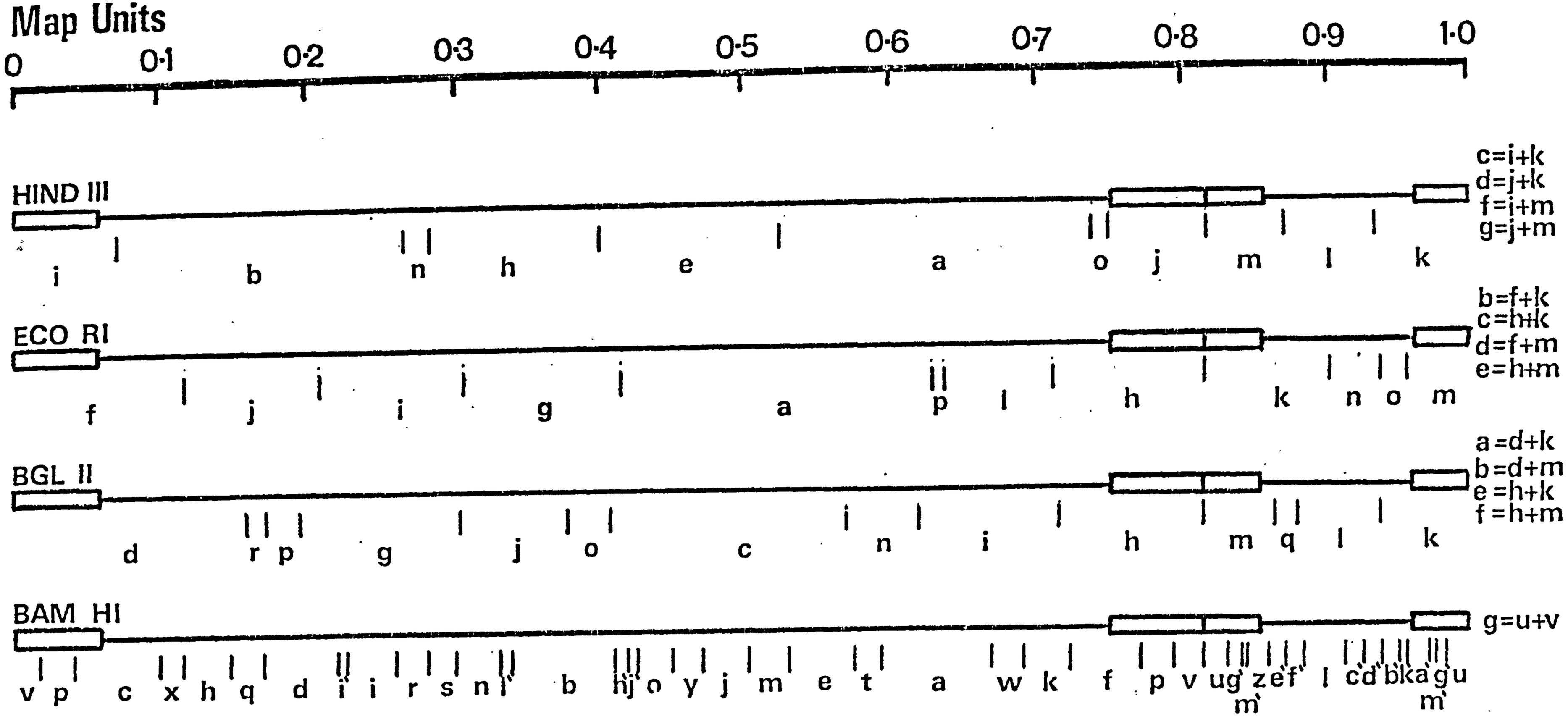


FIGURE 20

Physical maps for the fragments of HSV-2 DNA generated by restriction endonucleases Hind III, Eco RI, Bgl II and Bam HI (Cortini and Wilkie, 1978; Wilkie et al., 1979). These maps are shown for the prototype orientation of both unique regions. Fragments which span the joint region are represented as fusion fragments of the terminal fragments generated on inversion.
A copy of this figure is provided as a supplement at the back of this thesis.

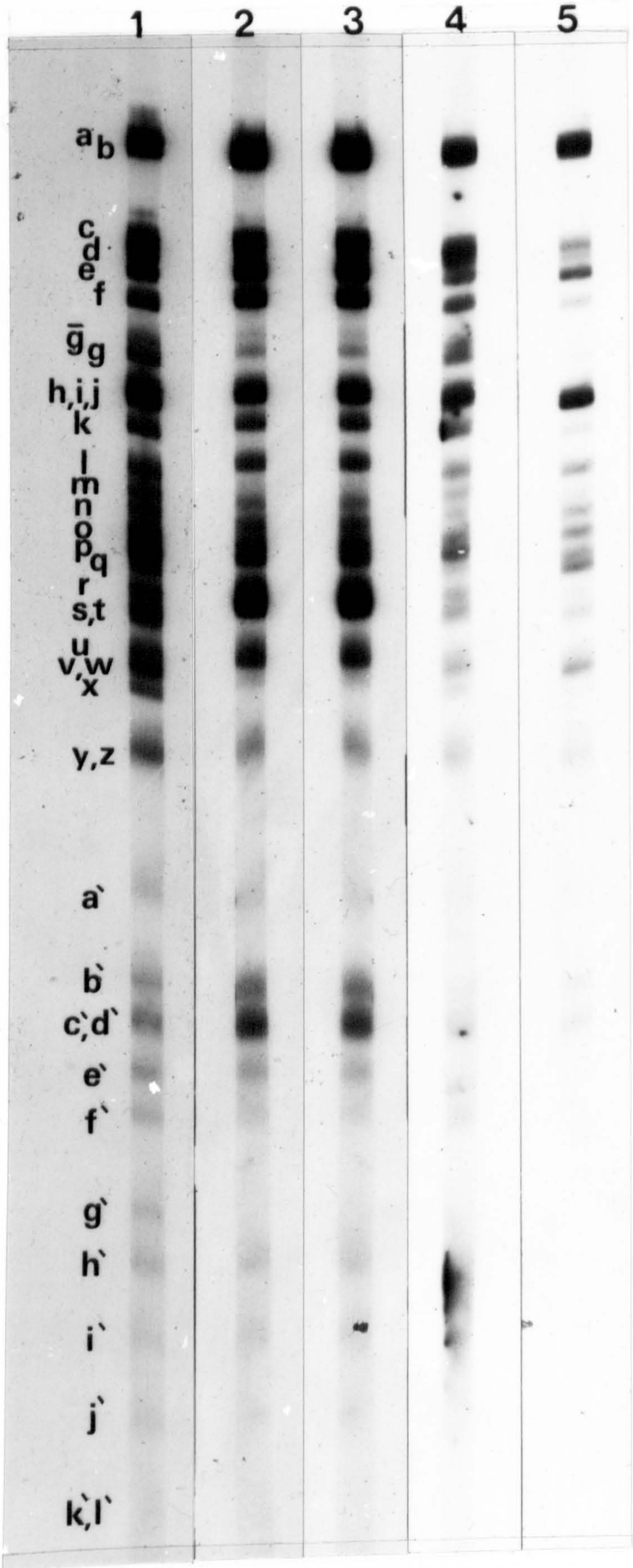


FIGURE 21

Autoradiographs of ^{32}P-*in vivo* labelled nuclear and cytoplasmic early and late RNA hybridized to blot strips containing the Bam HI generated fragments of HSV-2 DNA.

1. Nick-translated total HSV-2 DNA
2. Early nuclear RNA
3. Early cytoplasmic RNA
4. Late nuclear RNA
5. Late cytoplasmic RNA

1. Genome Location of HSV-2 Early and Late RNA

Physical maps for HSV-2 DNA fragments generated by digestion with several restriction endonucleases have been obtained (Cortini and Wilkie, 1978; Wilkie et al., 1979). The Hind III, Eco RI, Bgl II, and Bam HI cleavage maps used in this analysis are shown in Figure 20, and are also included as a separate supplement sheet. These are shown for only the prototype orientation of U_L and U_S. Using these physical maps, hybridization of ^{32}P-labelled RNA from cells infected with HSV-2 to blot strips containing denatured DNA fragments, and visualization of bound RNA by autoradiography or fluorography allowed the locations of the labelled RNA on the virus genome to be determined.

In vivo ^{32}P-labelled nuclear and cytoplasmic RNA samples were isolated from HSV-2-infected cells at 2 hr (prior to virus DNA synthesis; early RNA), and 10 hr (after virus DNA synthesis; late RNA) after the virus absorption period. The RNA was hybridized to blot strips containing HSV-2 DNA fragments generated by digestion with Bam HI. As a control, the total DNA fragment pattern was visualized by hybridizing nick-translated total virus DNA to a blot strip. Autoradiographs of the blot strips are shown in Figure 21.

Nuclear and cytoplasmic early RNA hybridized to all of the DNA fragments generated by Bam HI and no major consistent differences were observed between the hybridization patterns of nuclear and cytoplasmic RNAs. The relative amounts of hybridization to the various DNA fragments was markedly different, presumably reflecting different abundances of transcripts from various regions of the genome. There was greater relative hybridization to Bam HI e, f, st and c'd', whereas little hybridization to Bam HI d, g (and $\bar{g}$) and m was observed (Figure 21).

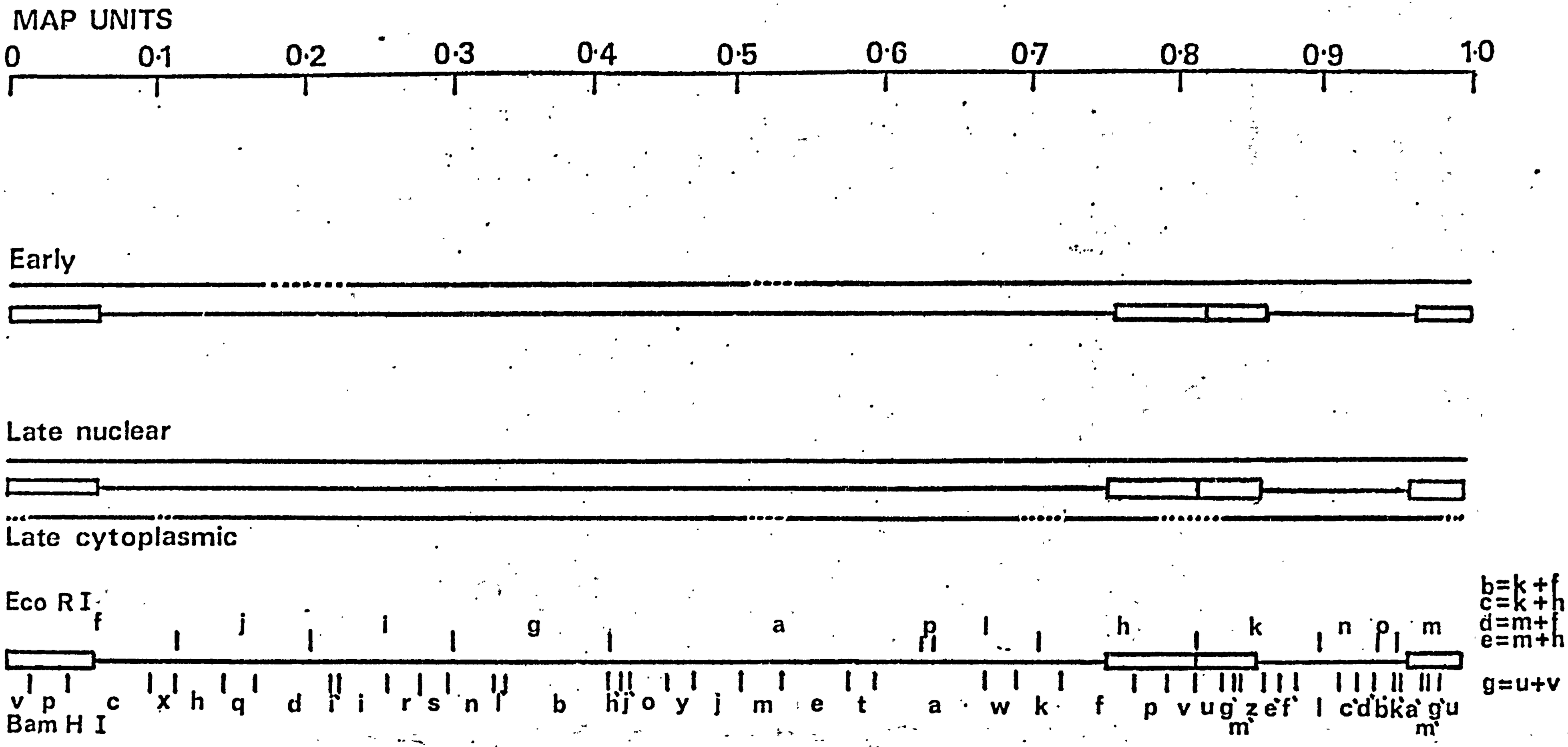


FIGURE 22

Summary of the genome location of early and late RNA. The physical maps for HSV-2 DNA generated by Bam HI and Eco RI restriction endonucleases are also shown. Regions of low levels of hybridization are represented by broken lines.

As with early RNA, late nuclear and cytoplasmic RNA hybridized to all the DNA fragments generated by Bam HI (Figure 21). However, the hybridization patterns of late RNA differed quantitatively from the pattern obtained with early RNA. Comparison of the hybridization patterns of late nuclear and late cytoplasmic RNA revealed differences in the relative amount of hybridization to several DNA fragments. The major hybridization of late nuclear RNA was to Bam HI c, d and hij. Late nuclear RNA consistently showed greater hybridization to Bam HI c, f and g (and $\overline{g}$) which contain sequences primarily from the repetitive regions, and to Bam HI-k, m and x which map in U_L, as compared with late cytoplasmic RNA.

Thus at both early and late times, fragments from all regions of the virus genome were represented by RNA transcripts. At early times the most abundantly represented region was 0.54-0.60 map units (Bam HI e and t). The relative abundance of transcripts differed from early to late times, and at late times there were differences in the hybridization patterns of nuclear and cytoplasmic RNAs.

It should be borne in mind that the location of regions represented at early and late times is constrained by the physical maps available. As has been shown for HSV-1 (Anderson et al., 1979; Anderson et al., 1980; J. McLauchlan and J.B. Clements, personal communication) and for HSV-2 (this thesis), a DNA fragment may be represented by several different or several related mRNAs. Changes in the relative abundance of RNA sequences homologous to any one fragment may therefore reflect quantitative changes of one or more transcripts. Also, the differences observed are relative to the total amount of RNA hybridized to the blot strips, and may not reflect absolute changes in abundance of individual transcripts.

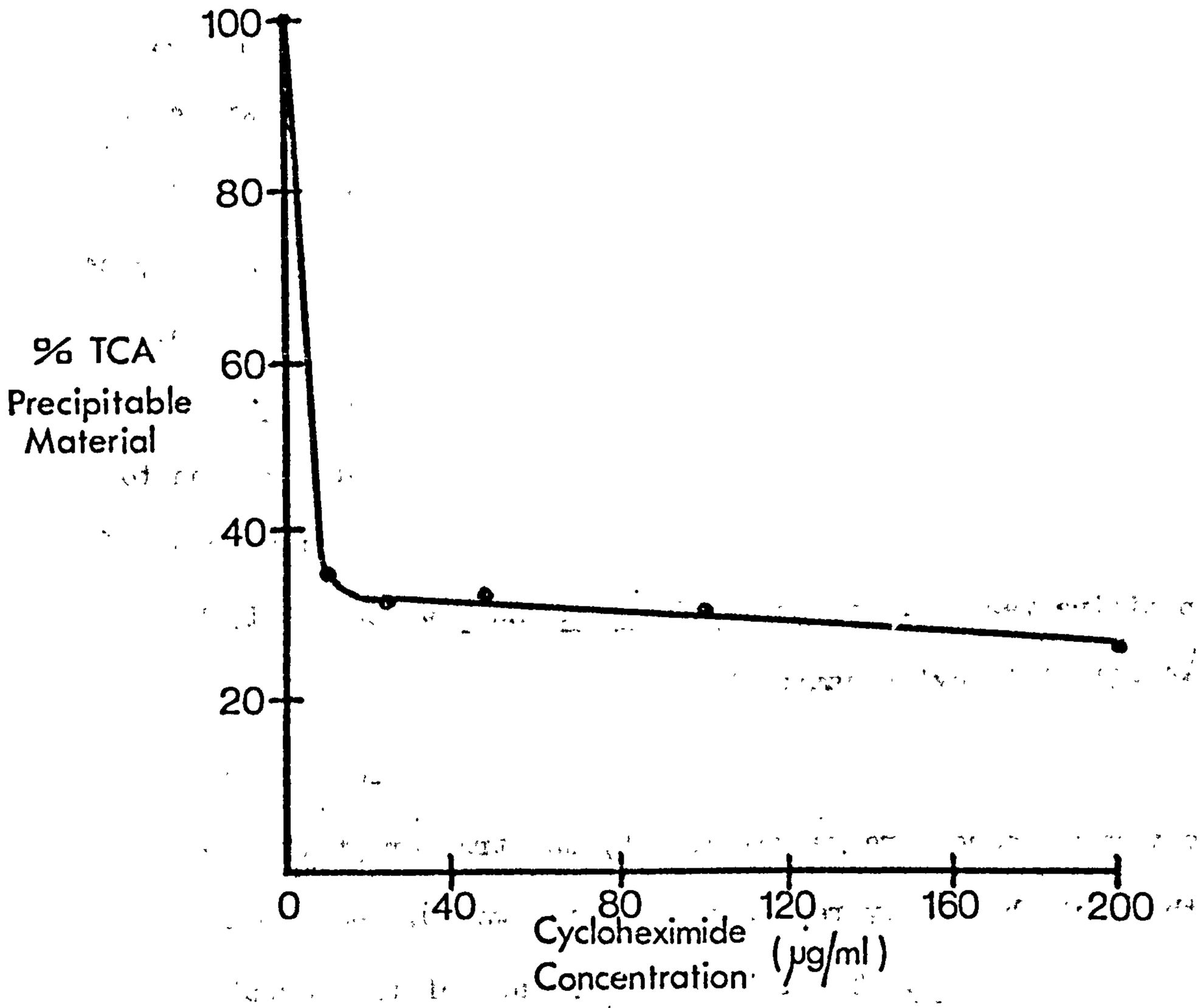


FIGURE 23

Effect of cycloheximide concentration on the incorporation of ^{3}H-uridine into TCA-precipitable material in HSV-1 infected cells.

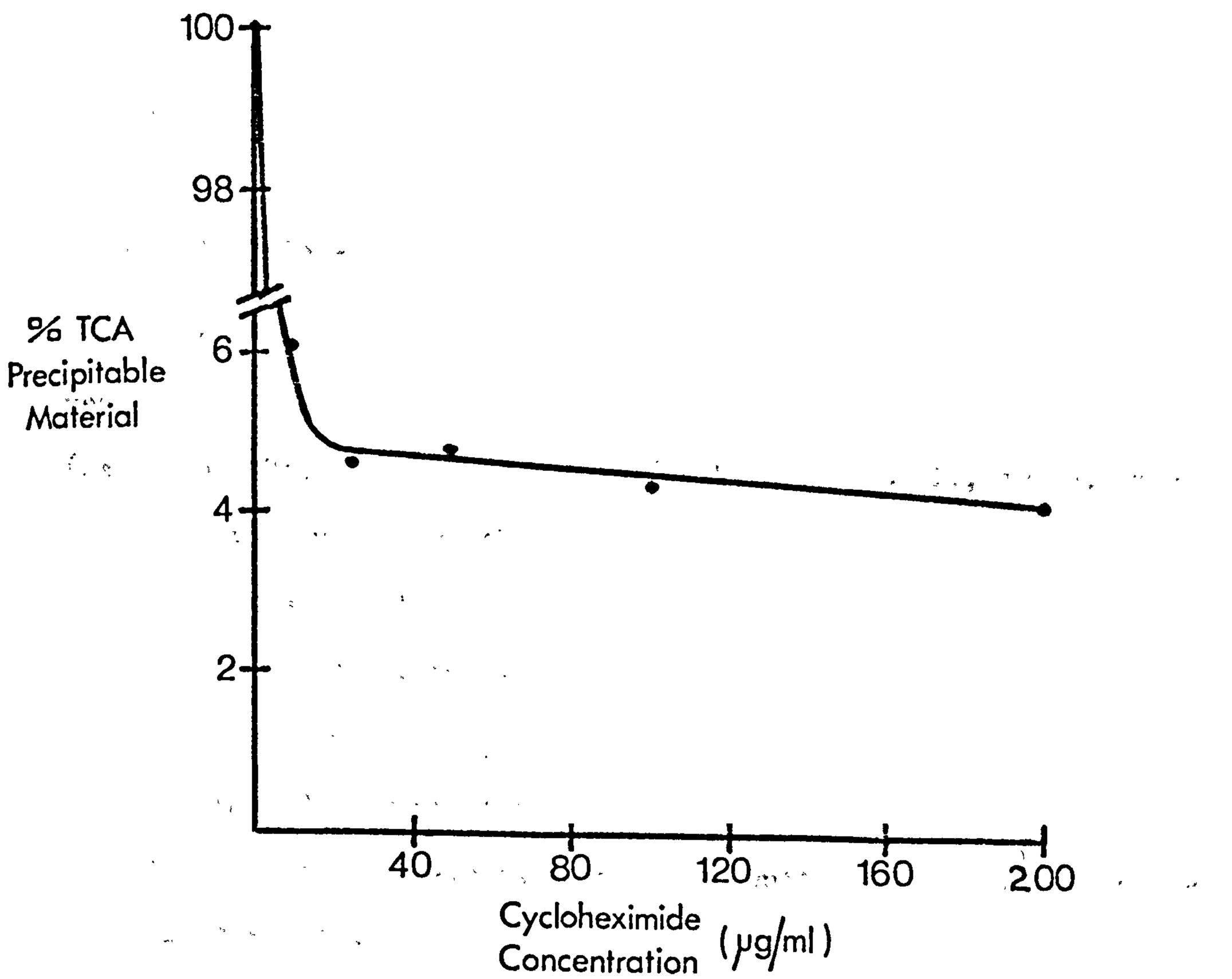


FIGURE 24

Effect of cycloheximide concentration on the incorporation of ^{35}S-methionine into TCA-precipitable material in HSV-1 infected cells.

A summary of the genome locations of early and late RNA is given in Figure 22. The dotted lines indicate regions of the virus genome which are poorly represented in the respective RNA samples.

2. Effect of Cycloheximide on Protein and RNA Synthesis in Infected Cells

Cycloheximide has been shown to inhibit protein synthesis in yeast, rabbit reticulocyte lysates and reóvirus-infected L cells (Kerridge, 1958; Colombo et al., 1965; Watanabe et al., 1967). The effect of various cycloheximide concentrations on cells infected with HSV-1 was investigated. Cells were infected in the continuous presence of the drug, and labelled continuously with either ^{3}H-uridine or ^{35}S-methionine. The levels of incorporation of radioactivity into TCA-precipitable material are shown in Figures 23 and 24. Incorporation is expressed as a percentage of that found in the absence of the drug.

At a concentration of 200 µg/ml cycloheximide, incorporation of ^{35}S-methionine into TCA-precipitable material was inhibited by approximately 96%. The remaining protein synthesis is likely to occur largely in the mitochondria. Incorporation of ^{3}H-uridine into TCA-precipitable material isolated from both the nucleus and cytoplasm was reduced by approximately 65% of that in untreated cells, at 200 µg/ml cycloheximide. This reduction may reflect a true inhibition of RNA synthesis in the cycloheximide-treated, infected cells, however it may also reflect an inhibition of ^{3}H-uridine uptake mediated by cycloheximide (Preston, 1974).

3. Genome Locations of HSV-2 IE RNA

IE RNA was prepared by labelling infected cells with ^{32}P-orthophosphate for 6 hr in the continuous presence of 200 µg/ml

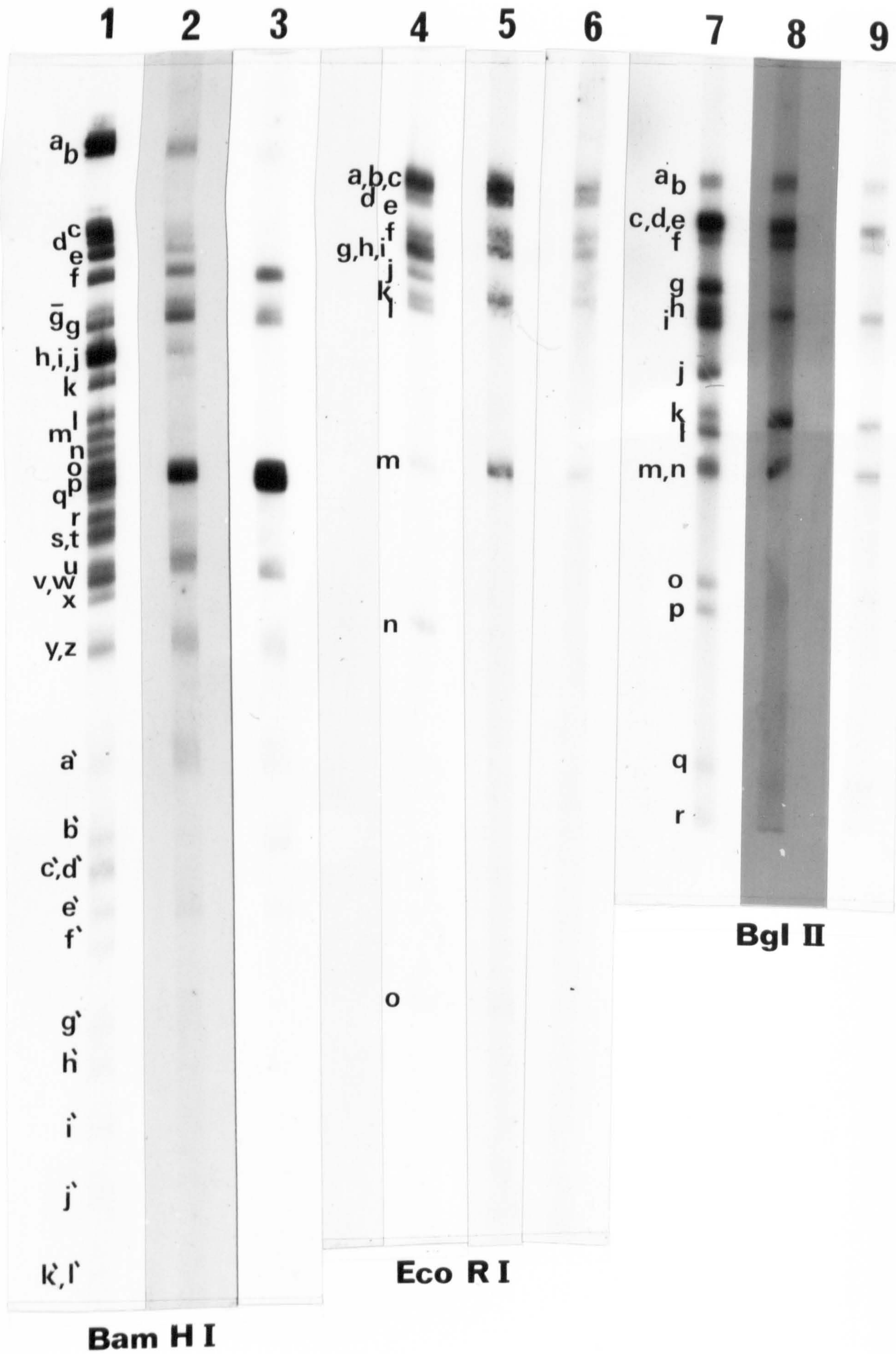


FIGURE 25

Autoradiographs of ^{32}P-in vivo labelled nuclear and cytoplasmic IE RNA hybridized to blot strips containing the Bam HI (tracks 1-3), Eco RI (tracks 4-6) and Bgl II (tracks 7-9) generated fragments of HSV-2 DNA.

1, 4, 7 Nick-translated total HSV-2 DNA
2, 5, 8 Nuclear IE RNA
3, 6, 9 Cytoplasmic IE RNA

cycloheximide. The RNA was hybridized to blot strips containing HSV-2 DNA fragments generated by cleavage with Bam HI, Eco RI and Bgl II restriction endonucleases. Autoradiographs of the blot strips are shown in Figure 25.

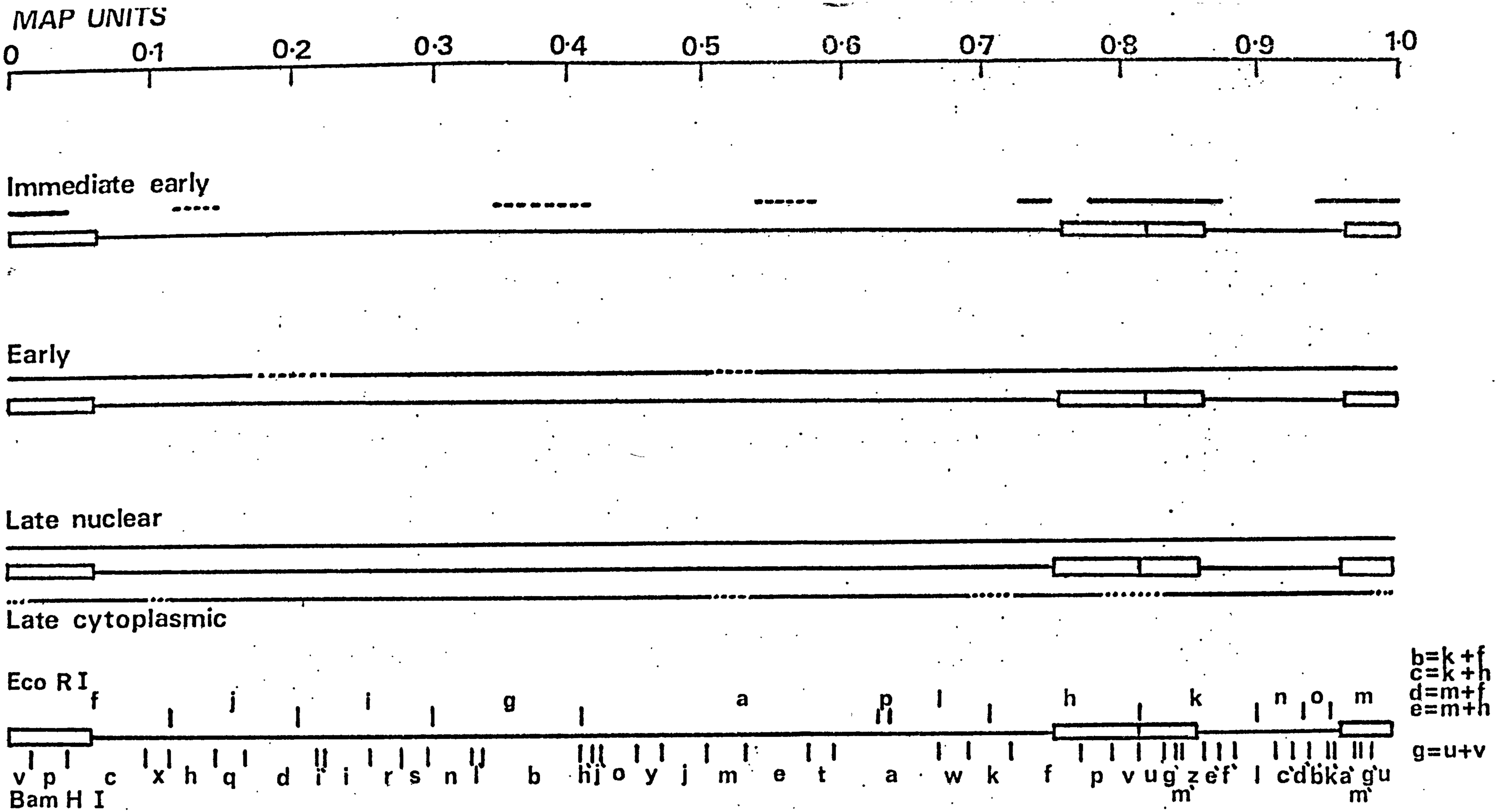
In comparison with early and late RNA (Figure 21), the hybridization pattern of IE nuclear and cytoplasmic RNA is severely restricted. IE nuclear RNA hybridized only to Bam HI b, e, f, g (and $\overline{g}$), hij, k, p, u, v, w, y z, a', b', c', g', k', l' and m'. No hybridization was observed to the other Bam HI fragments, to Bgl II g, i, j, l, p and r, or to Eco RI j, l and n.

The fragments Bam HI c and Bam HI f contain common sequences from TR_L/IR_L (Wilkie et al., 1979; Figure 20), together with sequences from U_L. Since both nuclear and cytoplasmic IE RNA hybridized only to Bam HI f, the RNA must be located in U_L near to the junction with IR_L.

The RNA hybridization to Bam HI hij could not be unambiguously assigned to either Bam HI h or j. However, cDNA prepared using IE RNA as a template hybridized to Eco RI j (Figure 37) indicating that an IE RNA was located in Bam HI h.

Small differences were observed between the hybridization patterns of nuclear and cytoplasmic IE RNA; there was little apparent hybridization of total cytoplasmic RNA to Bam HI hij, or k. The amount of hybridization of nuclear IE RNA to Bam HI k varied between samples. However, these RNA species must be represented in cytoplasmic IE RNA, as they were readily detected in RNA selection on oligo(dT) cellulose (Figure 31), and as minor species in IE cDNA (Figure 37).

As shown in the summary (Figure 26) only certain regions of



Summary of genome locations of HSV-2 RNA transcripts

FIGURE 26

Summary of the genome locations of immediate early, early and late RNA. The physical maps for HSV-2 DNA fragments generated by restriction endonucleases Bam HI and Eco RI are also included. Regions of low levels of hybridization are represented by broken lines.

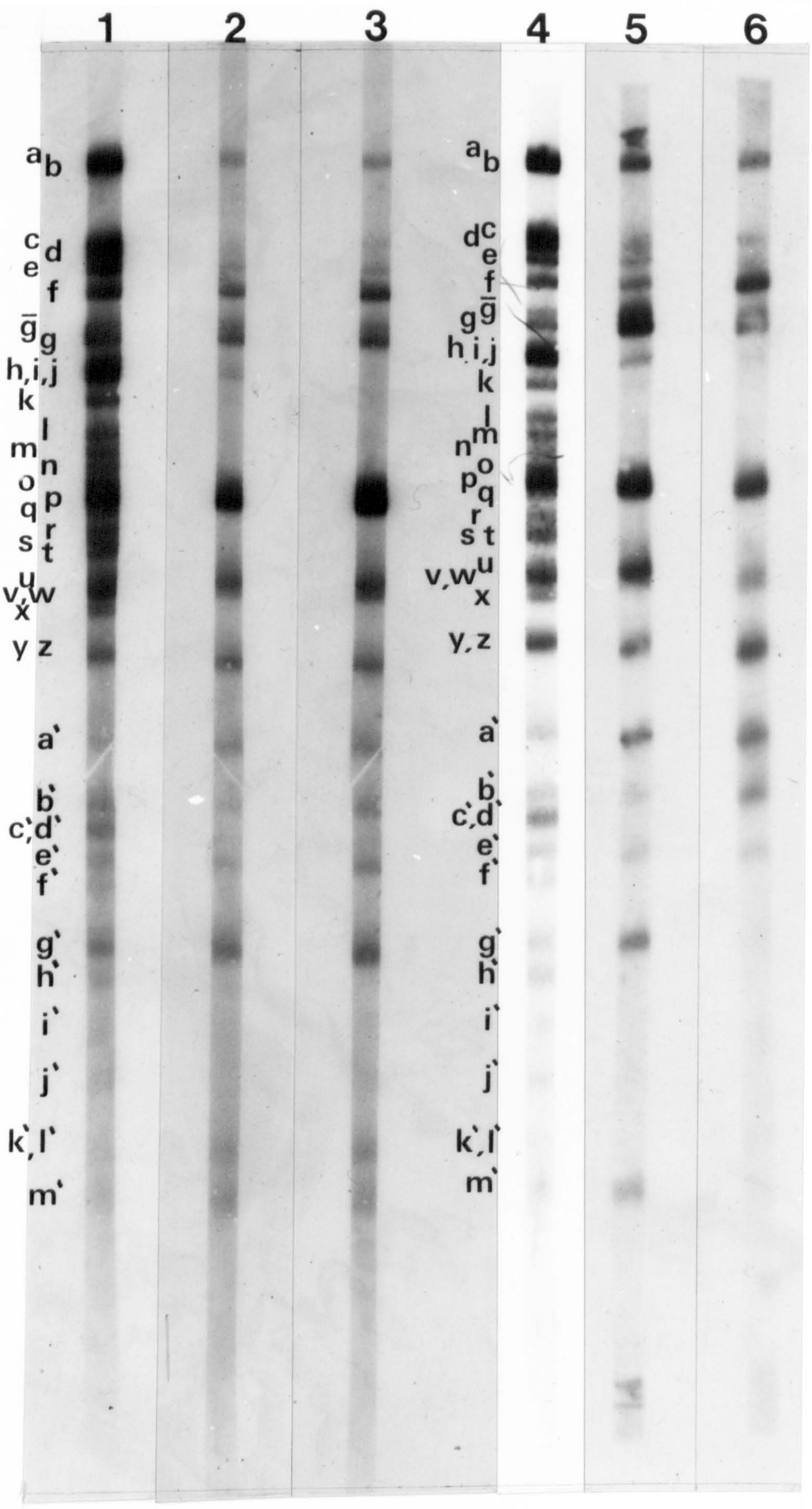


FIGURE 27

Autoradiographs of ^{32}P-*in vivo* and *in vitro* labelled nuclear and cytoplasmic IE RNA hybridized to blot strips containing the Bam HI generated fragments of HSV-2 DNA.

1. Nick-translated total HSV-2 DNA
2. *In vivo* labelled nuclear IE RNA
3. *In vivo* labelled cytoplasmic IE RNA
4. Nick-translated total HSV-2 DNA
5. *In vitro* labelled nuclear IE RNA
6. *In vitro* labelled cytoplasmic IE RNA

the HSV-2 genome are represented by nuclear and cytoplasmic IE RNA as detected using the blot hybridization technique. The most abundant IE transcripts were located predominantly within, or immediately adjacent to both sets of repetitive regions. Three minor regions of IE transcriptions were located within U_L. These were 0.12-0.13, 0.34-0.41 and 0.54-0.60 map units. The latter region is also abundantly represented in early RNA.

4. In Vitro Labelling of IE RNA

To ensure that the restricted hybridization pattern of IE RNA was not due to preferential labelling of certain RNA species in vivo, or to selective fractionation of labelled RNAs as suggested by Jones and Roizman (1979), IE nuclear and cytoplasmic RNA samples were labelled with ^{32}P in vitro using polynucleotide kinase, and hybridized to blot strips. Autoradiographs of the hybridization patterns to Bam HI generated HSV-2 DNA fragments are shown in Figure 27. Qualitatively, the patterns of both nuclear and cytoplasmic IE RNAs were identical to those observed with in vivo labelled RNA. No additional RNA sequences were detected. However, there were several quantitative differences between the patterns of the in vitro and in vivo labelled samples, and also between the hybridization patterns of in vitro labelled nuclear and cytoplasmic RNA. The hybridization patterns of in vitro labelled nuclear and cytoplasmic RNAs indicated that RNA sequences hybridizing to certain DNA fragments were preferentially located within either the nucleus or the cytoplasm. RNA hybridizing to Bam HI g (and $\overline{g}$), u, g' and m', which map in TR_S/IR_S, was present predominantly in the nucleus, while RNA hybridizing to Bam HI f, yz, a', b' and k'l' was located mainly within the cytoplasm (Figure 27). Some differences between the hybridization patterns of nuclear and cytoplasmic IE RNA observed

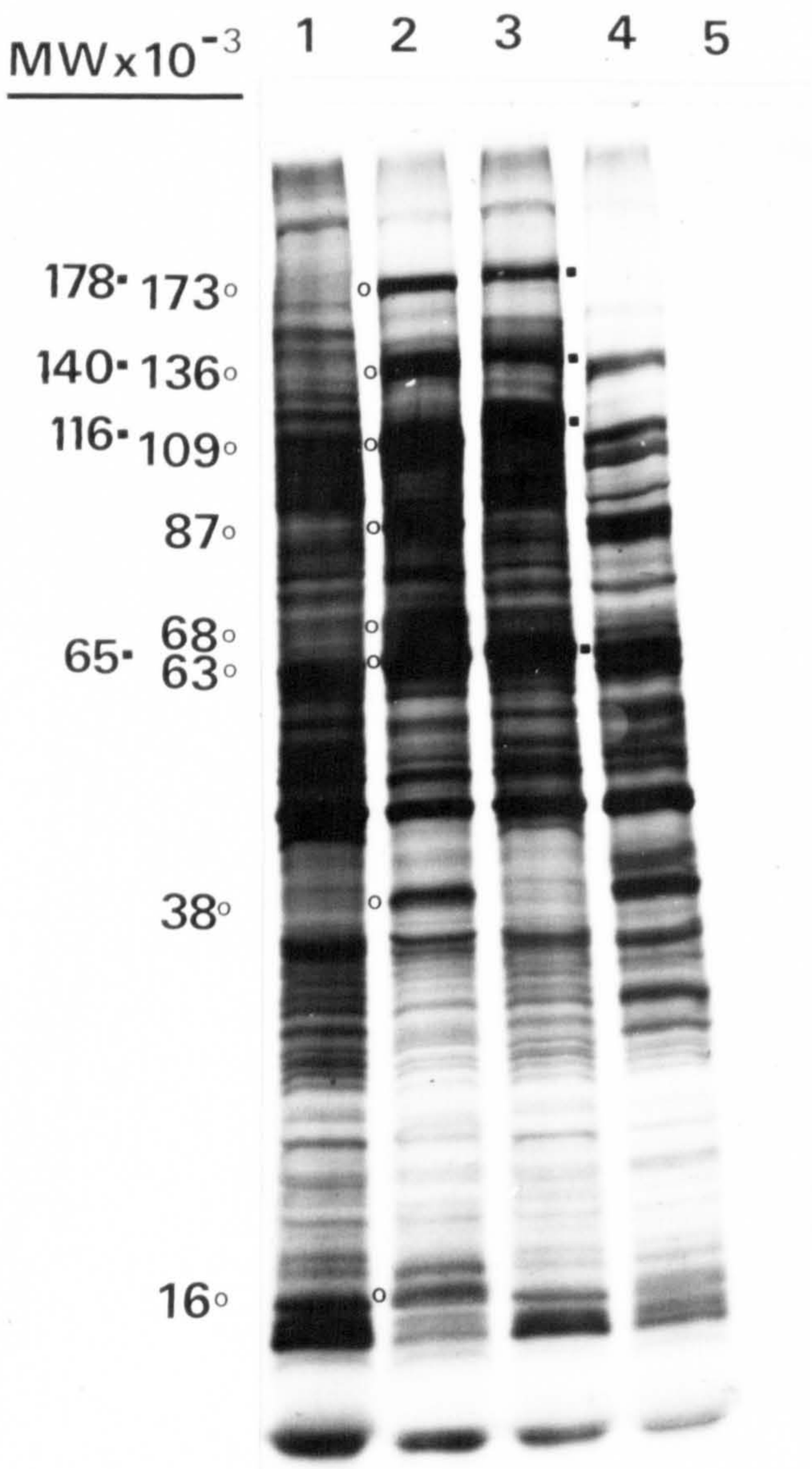


FIGURE 28

PAGE separation of the <u>in vitro</u> translation products of HSV-2 IE mRNAs.

1. Mock-infected cell RNA
2. HSV-1 IE RNA
3. HSV-2 IE RNA
4. HSV-1 late RNA
5. <u>E. coli</u> rRNA

(o) HSV-1 specific IE polypeptides

(■) HSV-2 specific IE polypeptides

with in vivo labelled samples were also detected with in vitro labelled samples; RNA which hybridized to Bam HI hij was located predominantly in the nucleus.

5. Translation Products of HSV-2 IE mRNAs

HSV-2 cytoplasmic IE RNA was translated in vitro using a rabbit reticulocyte lysate, and the translation products were separated by PAGE. Figure 28 shows the translation products of HSV-1 IE RNA, HSV-2 IE RNA and mock-infected cell RNA. An E. coli. rRNA control is also shown to illustrate the endogenous translation products of the reticulocyte system. Sizes of the HSV-1 IE polypeptides were identical to those determined by Watson et al. (1979). The major HSV-2 IE polypeptides translated in vitro had apparent molecular weights of 178 x 10^3, 140 x 10^3, 116 x 10^3 and 65 x 10^3. These values are similar, but not identical to those of the equivalent HSV-2 polypeptides observed in vivo which had molecular weights of 182 x 10^3, 144 x 10^3, 118 x 10^3, 67 x 10^3, 65 x 10^3 and 12.3 x 10^3 (Preston et al., 1978). The slightly lower molecular weights of certain polypeptides translated in vitro can be attributed to the absence of normal post-translational modification, as shown for HSV-1 (Preston, 1977). No in vitro translation product equivalent to the 67 x 10^3 polypeptide observed in vivo was detected, possibly because the unmodified HSV-2 polypeptide comigrated with a host cell polypeptide. It was not expected that the 12.3 x 10^3 polypeptide would be detected, since it would comigrate with globin, an inherent contaminant of the reticulocyte translation system (Preston, 1977).

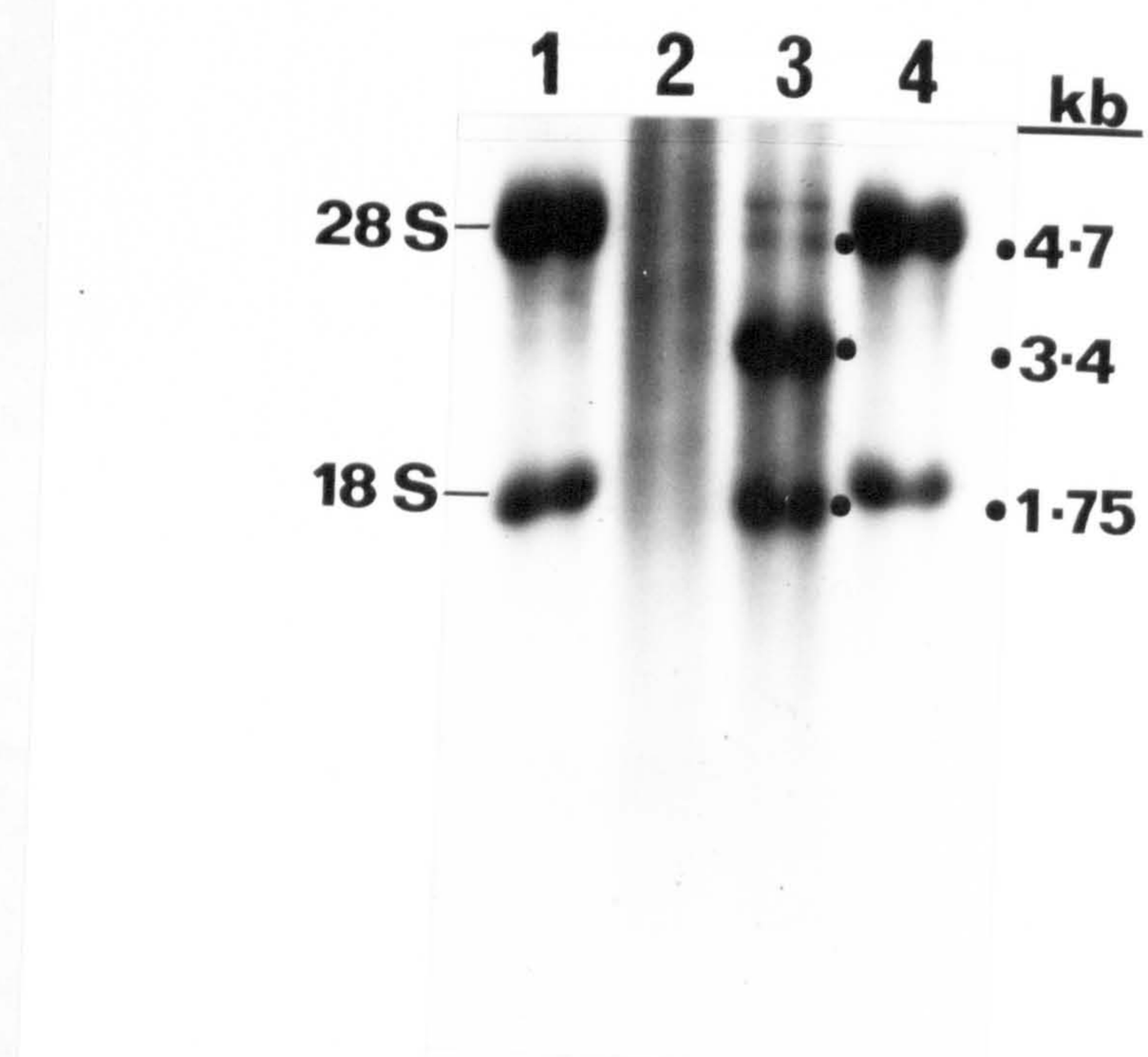


FIGURE 29

Autoradiograph of CH_3HgOH agarose gel of HSV-2 IE and mock-infected mRNAs.

1. BHK rRNA
2. mock-infected RNA
3. HSV-2 IE RNA
4. BHK rRNA

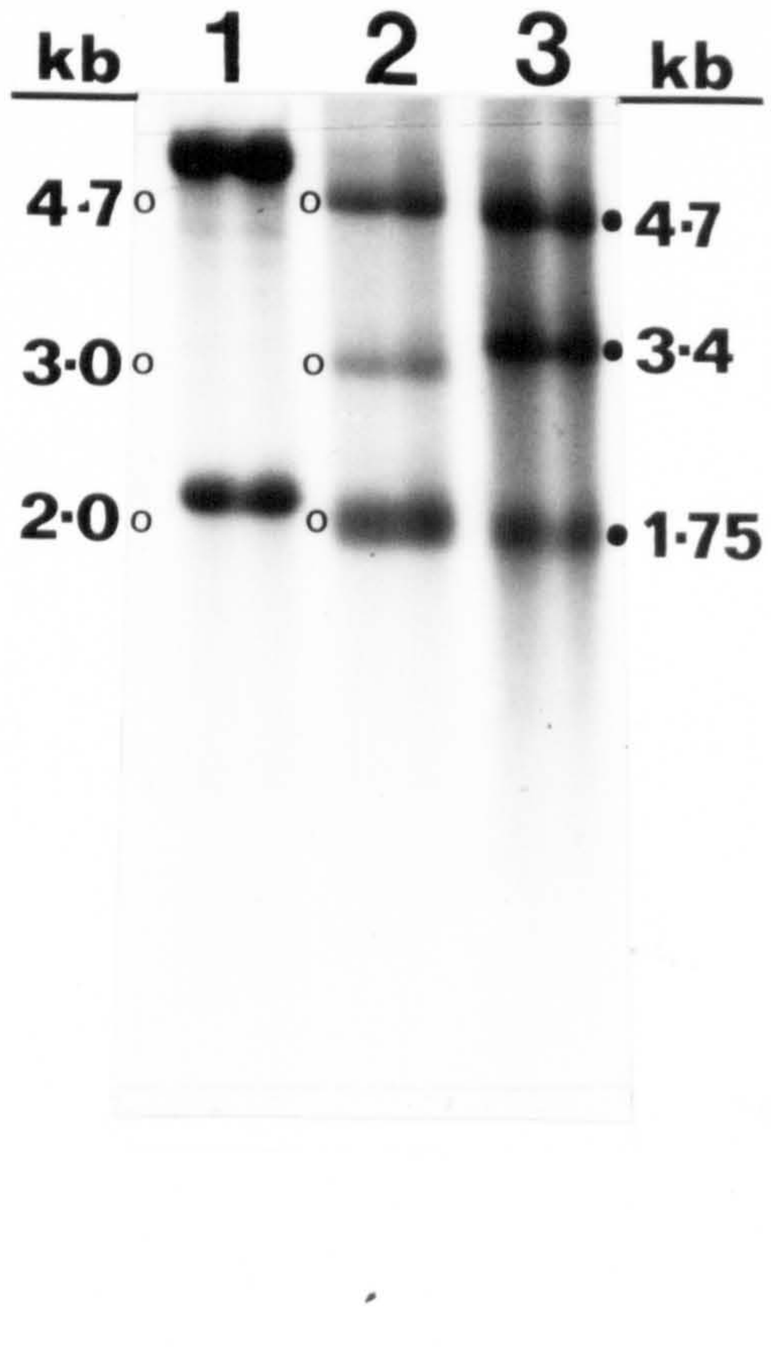


FIGURE 30

Autoradiograph of CH_3HgOH agarose gel of HSV-1 and HSV-2 mRNAs.

1. BHK rRNA
2. HSV-1 IE RNA
3. HSV-2 IE RNA

6. Fractionation of IE mRNAs on Denaturing Agarose Gels

In vivo ^{32}P-labelled cytoplasmic IE RNA was selected on oligo(dT) cellulose and fractionated by electrophoresis through an agarose gel containing 5 mM CH_3HgOH.

a) Size of IE mRNAs

Autoradiography of the gels revealed three bands of 4.7 kb, 3.4 kb and 1.75 kb, which were not present in polyadenylated cytoplasmic RNA isolated from mock-infected cells (Figure 29). Different preparations of IE RNA contained variable small amounts of contaminating 28S rRNA, and the relative proportions of the virus-specific bands varied somewhat.

This was similar to the situation observed with HSV-1 IE RNA in which three virus RNA bands were detected (Watson *et al.*, 1979). However, as shown in Figure 30 there were size differences between the HSV-1 and HSV-2 IE mRNAs. The largest virus-specific bands were of similar size, while the sizes of the other two bands differed between HSV-1 and HSV-2. The HSV-2 IE RNA band of 1.75 kb was smaller than the 2.0 kb HSV-1 band, and the 3.4 kb HSV-2 RNA band was larger than the 3.0 kb HSV-1 equivalent.

b) Genome Locations of IE mRNAs

To determine the genome regions represented by each of the three virus-specific IE RNA bands observed on CH_3HgOH agarose gels, each band was individually excised from a preparative gel, the RNA eluted, and hybridized to blot strips containing HSV-2 DNA fragments. Autoradiographs of the hybridization patterns to Bam HI generated DNA fragments are shown in Figure 31.

The hybridization pattern of total polyadenylated cytoplasmic

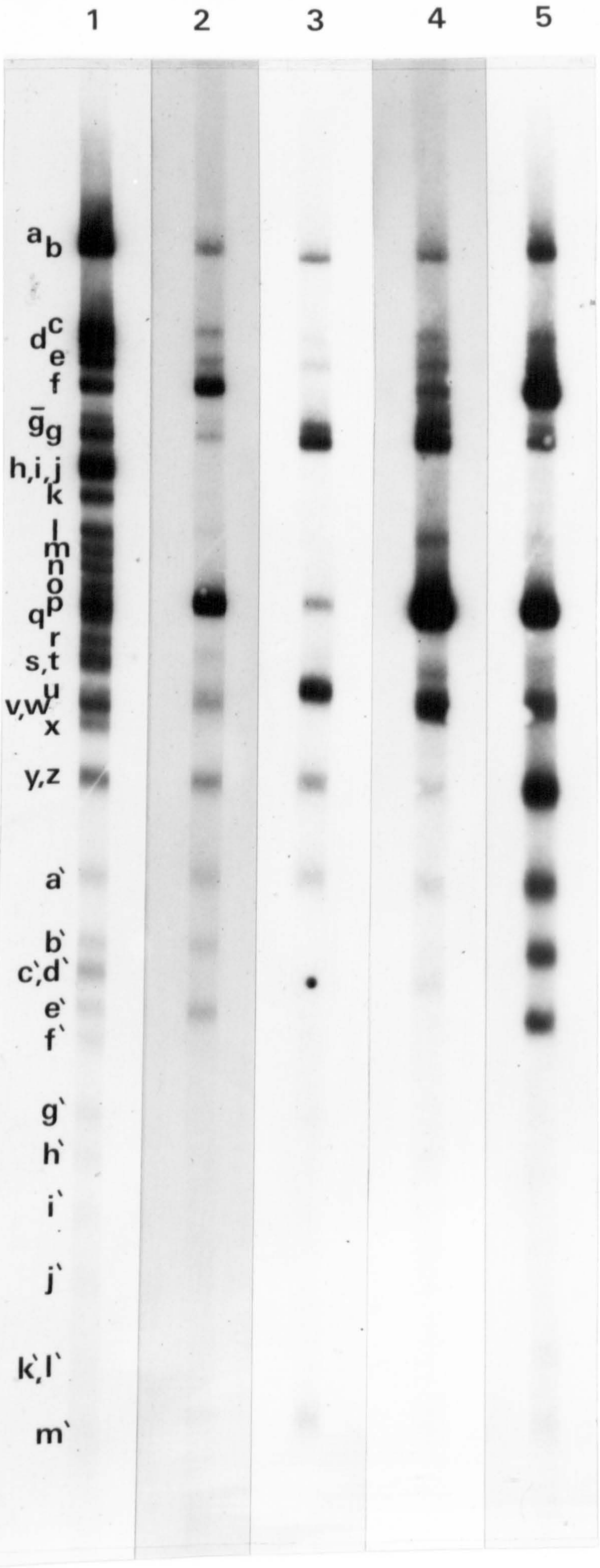


FIGURE 31

Autoradiographs of fractionated HSV-2 IE mRNA bands hybridized to blot strips containing the Bam HI generated fragments of HSV-2 DNA.

1. Nick-translated total HSV-2 DNA
2. Unfractionated cytoplasmic poly(A) + IE RNA
3. 4.7 kb RNA band
4. 3.4 kb RNA band
5. 1.75 kb RNA band

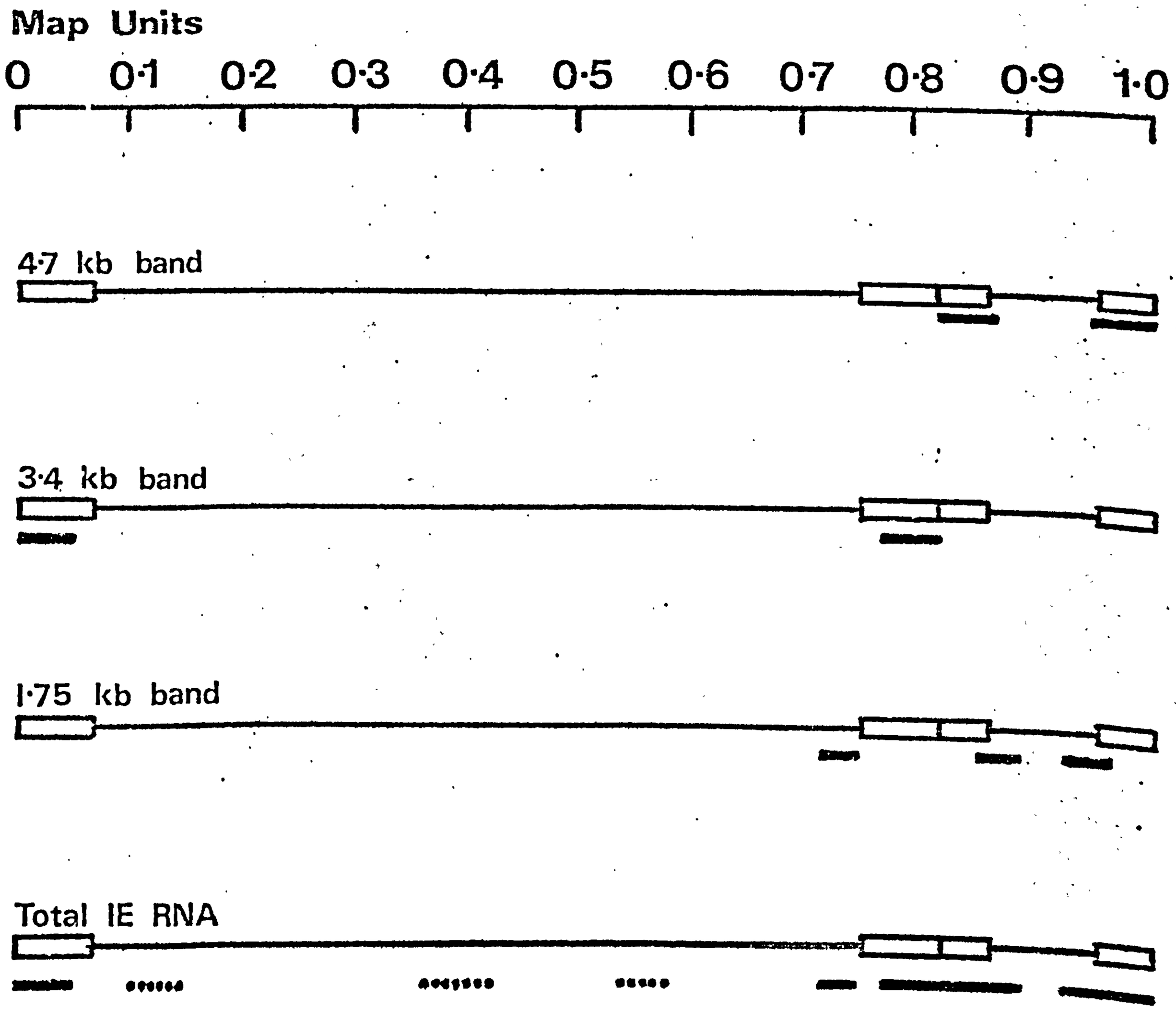


FIGURE 32

Summary of transcript mapping data shown in Figure 31 for fractionated HSV-2 IE mRNAs. The genome map locations of total cytoplasmic IE RNA are shown. Low levels of hybridization are indicated by a broken line.

HSV-2 IE RNA to DNA blot strips is also shown in Figure 31, track 2, and was similar to that of unselected cytoplasmic IE RNA (Figures 25 and 27).

The 4.7 kb RNA band hybridized predominantly to Bam HI g (and $\overline{g}$), u, yz, a' and also to g' and m'. Some hybridization could also be seen to Bam HI b, e and p. The major hybridization indicates that the 4.7 kb mRNA is located entirely within TR_S/IR_S as indicated in Figure 32.

The 3.4 kb RNA band showed strong hybridization to Bam HI g (and $\overline{g}$), p and vw, with faint bybridization to Bam HI c and f, as well as to certain DNA fragments represented in the larger 4.7 kb mRNA which is likely to be due to contamination with degradation products of the larger band. The genome region containing Bam HI w is not represented in IE RNA (Figure 26), and Bam HI p and v are contiguous indicating that the 3.4 kb mRNA is located entirely within TR_L/IR_L shown in Figure 32.

The 1.75 kb RNA band hybridized strongly to Bam HI f, yz, a', b', e' and k'l' and weakly to DNA fragments represented in the 3.4 kb band. The strong hybridization was to fragments located in three distinct regions of the virus genome; at U_L near the junction with IR_L, and at the junctions of U_S with both TR_S and IR_S. This indicated that the 1.75 kb band contained at least three individual IE mRNAs as indicated (Figure 32).

The 4.7 kb band hybridized to Bam HI z and a', as did the two 1.75 kb transcripts which cross the junctions of U_S with TR_S and IR_S. This does not, however, suggest that these RNAs contain overlapping or complementary sequences, but simply that the ends of the two relevant mRNAs lie within the same Bam HI DNA fragment, and are not separated by a Bam HI cleavage site. The equivalent HSV-1 IE mRNAs

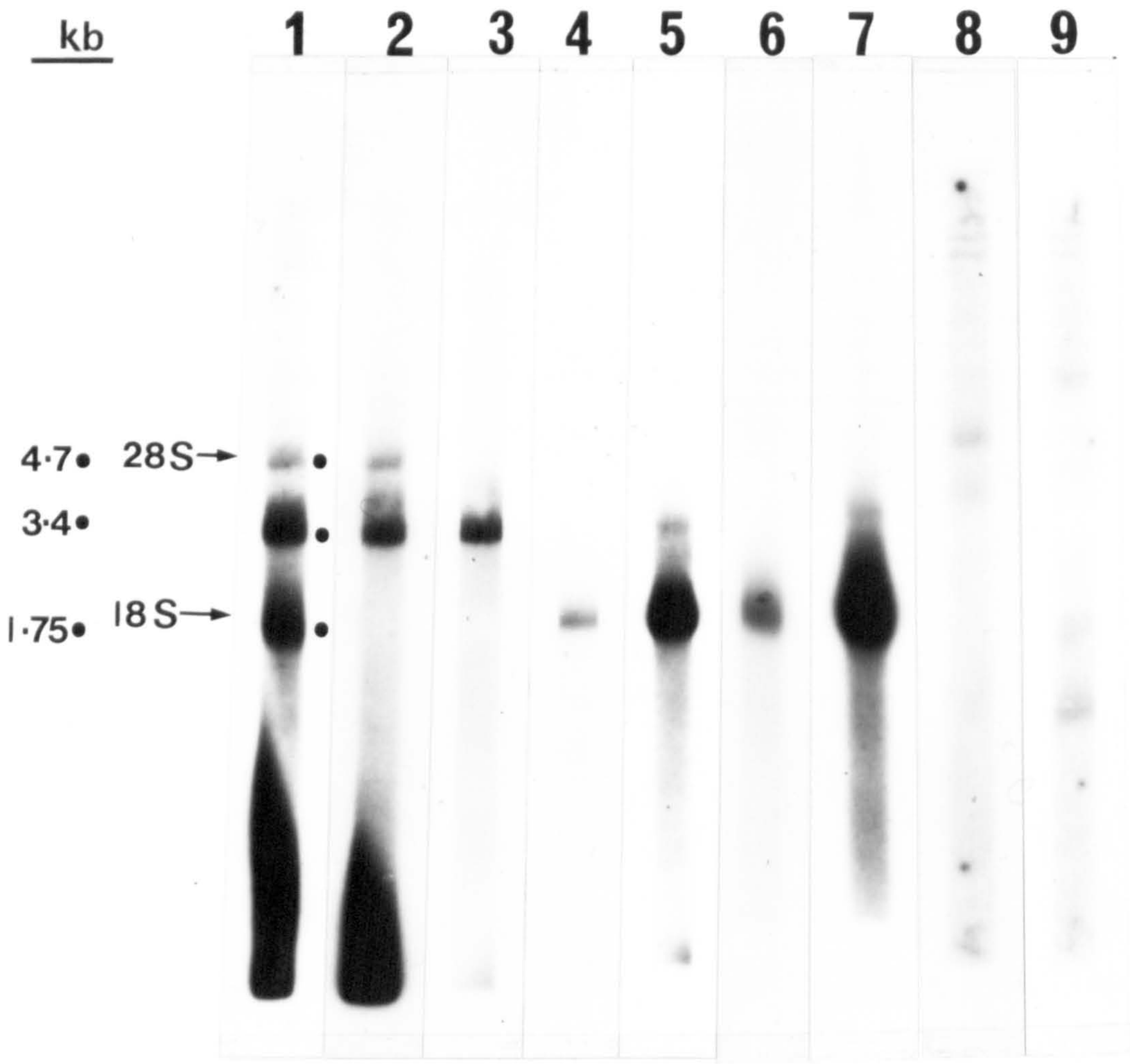


FIGURE 33

Autoradiographs of nick-translated HSV-2 DNA fragments hybridized to poly(A) + IE RNA immobilized on DBM paper.

1. Total HSV-2 DNA
2. Bam HI g
3. Bam HI p
4. Bam HI f
5. Bam HI f
6. Hind III o
7. Hind III o
8. Hind III h
9. Bgl II n

located in these regions of the genome do not overlap (Watson et al., 1979).

7. Genome Locations of IE mRNAs Determined by RNA Blotting

The genome locations of certain IE mRNAs were also determined using the RNA blot procedure of Alwine et al. (1977) in which polyadenylated cytoplasmic HSV-2 IE RNA was transferred to activated DBM paper. Nick-translated total HSV-2 DNA, and cloned DNA fragment probes were hybridized to the immobilized RNA. Autoradiographs of the RNA blot strips are shown in Figure 33.

Nick-translated virus DNA hybridized to three major bands corresponding to sizes of 4.7 kb, 3.4 kb and 1.75 kb. This reflects the situation obtained after size fractionation of in vivo labelled polyadenylated cytoplasmic IE RNA (Figures 29 and 30). Cloned Bam HI $\bar{g}$ hybridized both to the 4.7 kb and 3.4 kb bands (Figure 33, track 2). Bam HI $\bar{g}$ contains sequences from TR_L/IR_L and from TR_S/IR_S. Bam HI p, which contains sequences from TR_L/IR_L directly adjacent to those in Bam HI $\bar{g}$, hybridized solely to the 3.4 kb band (Figure 33, track 3). The 4.7 kb mRNA must therefore be located within TR_S/IR_S, while the 3.4 kb mRNA must be located within TR_L/IR_L. This confirms the previous data.

Bam HI f hybridized predominantly to the 1.75 kb RNA band, as did Hind III o (Figure 33, tracks 4 and 6). This confirms the location of a 1.75 kb mRNA at the junction of U_L and IR_L.

Overexposure of the DBM blot strip hybridized with Bam HI f showed faint hybridization to the 3.4 kb mRNA (Figure 33, track 5) suggesting that the 3.4 kb mRNA extended a small distance into Bam HI f. This location also would explain the faint hybridization

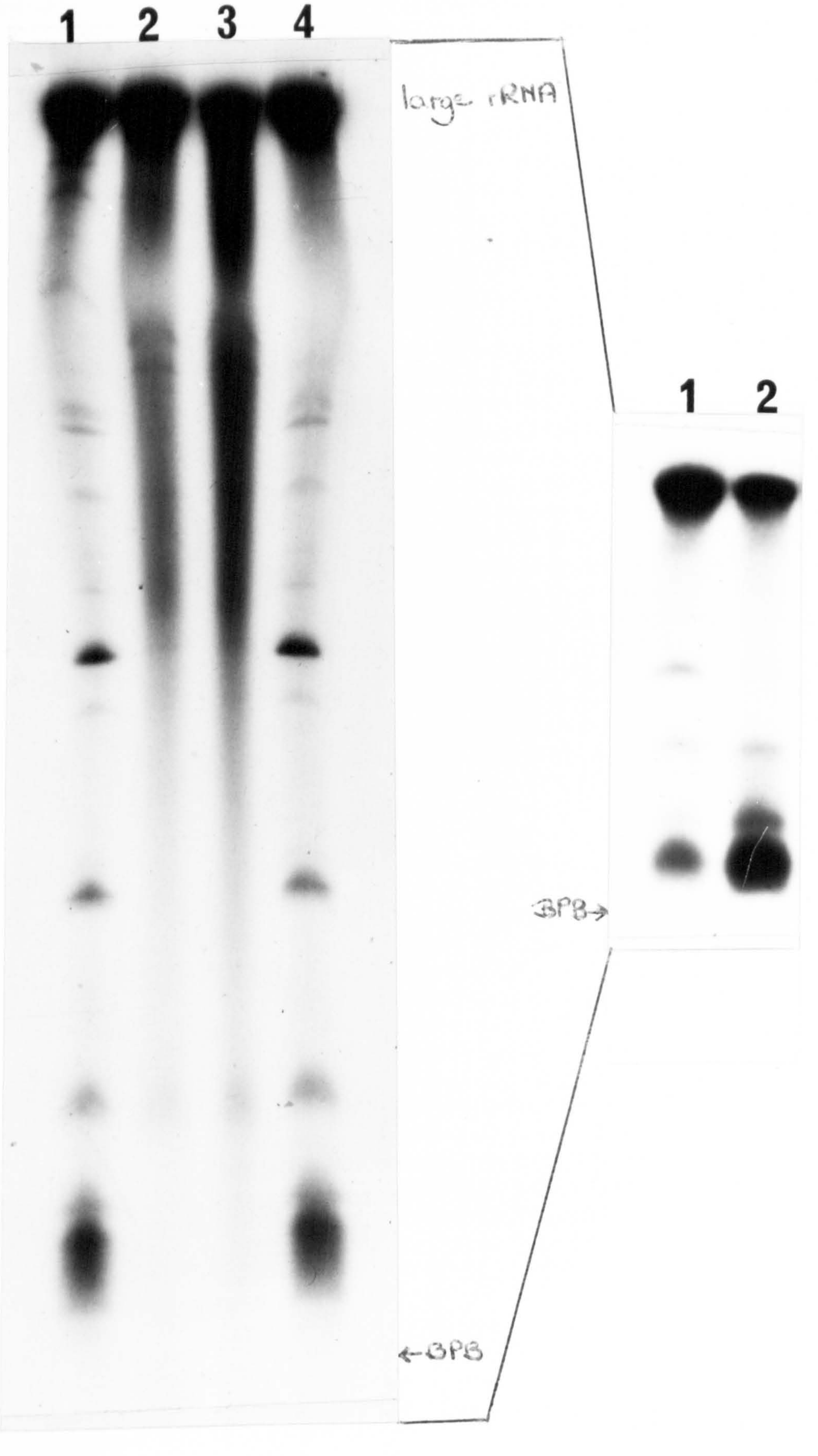


FIGURE 34

Autoradiograph of denaturing acrylamide gel of cytoplasmic poly(A) + HSV-2 IE and mock-infected RNA.

1. BHK uninfected cell cytoplasmic RNA
2. Poly(A) + IE RNA
3. Poly(A) + mock-infected RNA
4. BHK uninfected cell cytoplasmic RNA

FIGURE 35

Autoradiograph of denaturing acrylamide gel of total cytoplasmic IE and mock-infected RNA.

1. Mock-infected RNA
2. HSV-2 IE RNA

of the 3.4 kb band to Bam HI f in Figure 31. The 3.4 kb band also hybridized to Bam HI c, which shares common sequences in TR_L/IR_L with Bam HI f (Figures 20 and 31). However, Hind III o, which contains sequences entirely from U_L, hybridized faintly, on long exposure, to the 3.4 kb band (Figure 33, track 7) suggesting that Hind III o (and therefore Bam HI f which contains all the sequences present in Hind III o) may contain a small region of homology to the 3.4 kb band.

Hind III h (0.28-0.40 map units) hybridized to two RNA bands of 5.0 kb and 4.3 kb approximately (Figure 33, track 8), and faint hybridization of in vivo labelled IE RNA was detected at this genome location (Figure 26). Hybridization to this region was also observed with RNA selected on oligo(dT) cellulose (Figure 31).

Bgl II n (0.57-0.62 map units) hybridized to three distinct RNA bands of 7.0 kb, 1.75 kb and 1.25 kb approximately (Figure 33, track 9). Faint hybridization of IE RNA also was found in this region of the virus genome (Figures 25 and 26).

8. Fractionation of IE mRNAs on Denaturing Polyacrylamide Gels

Analysis of small RNA species was performed by fractionation of in vivo ^{32}P-labelled polyadenylated cytoplasmic IE RNA by electrophoresis through a 12% polyacrylamide gel containing 7 M urea. An autoradiograph of the gel is shown in Figure 34. The IE pattern was identical to the mock-infected cell RNA.

Unselected, total cytoplasmic IE RNA was also fractionated on a denaturing gel, and no virus-induced small RNA bands were detected (Figure 35).

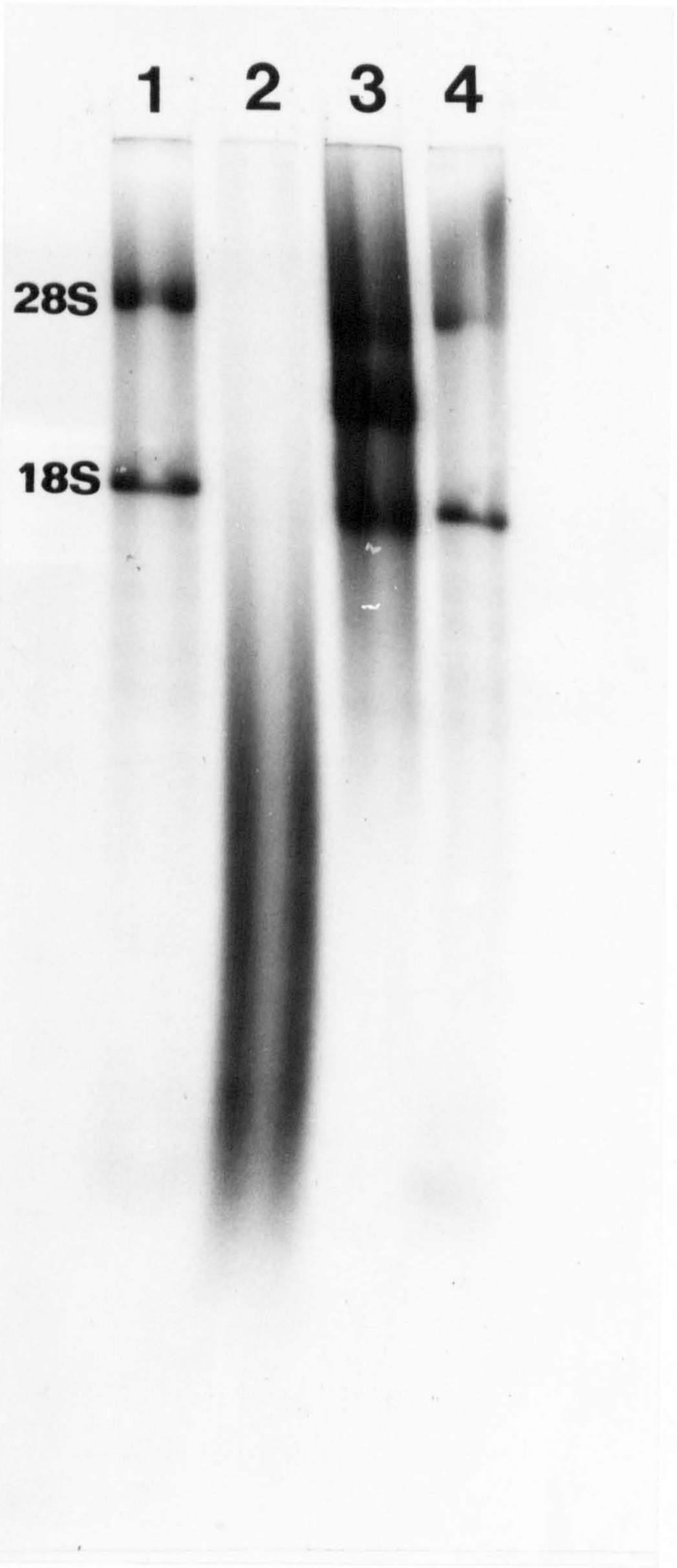


FIGURE 36

Autoradiograph of IE RNA and IE cDNA fractionated on a CH_3HgOH agarose gel.

1. BHK rRNA
2. IE cDNA
3. Poly(A) + IE RNA
4. BHK rRNA

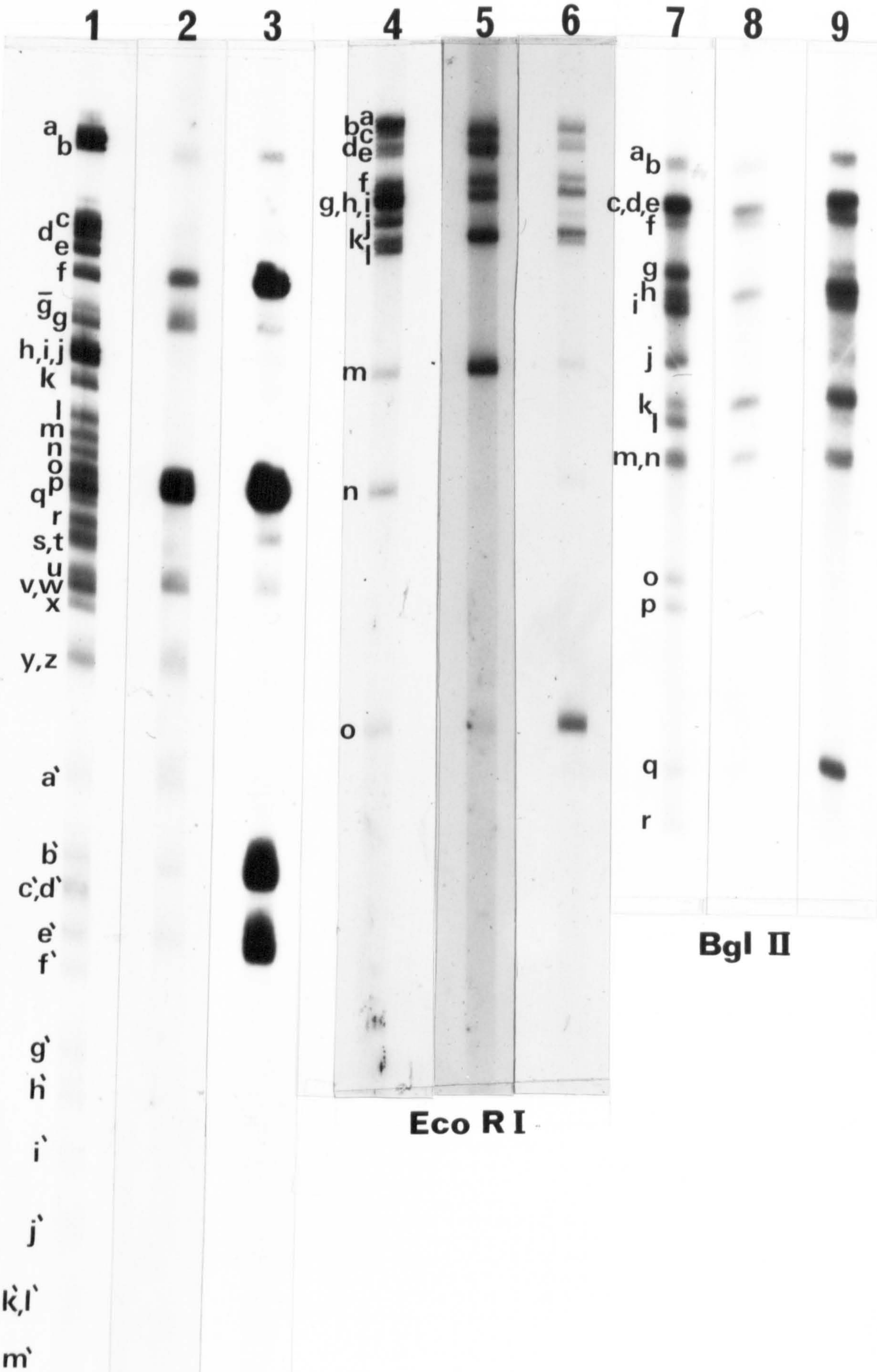


FIGURE 37

Autoradiographs of HSV-2 IE cDNA hybridized to blot strips containing Bam HI (tracks 1-3), Eco RI (tracks 4-6) and Bgl II (tracks 7-9) generated fragments of HSV-2 DNA.

1, 4 and 7 Nick-translated total HSV-2 DNA
2, 5 and 8 Cytoplasmic IE RNA
3, 6 and 9 IE cDNA

9. Orientations of IE mRNAs on the Virus Genome

Almost all eukaryotic mRNAs are polyadenylated at the 3'-end (Molloy and Darnell, 1973), and this can be utilized to determine the orientation of mRNAs on the genome. ^{32}P-labelled cDNA was prepared using IE RNA as a template with oligo$(dT)_{10}$ as primer, and the reaction products were electrophoresed through a CH_3HgOH agarose gel. An autoradiograph of the gel is shown in Figure 36. The cDNA was very small, and by virtue of the method of synthesis, contained sequences complementary to the 3'-termini of the IE mRNAs. Hybridization of the cDNA to nitrocellulose blot strips allows the mRNAs to be oriented on the virus genome. A similar analysis for HSV-1 IE mRNAs was performed by Clements et al. (1979).

The hybridization patterns of IE cDNA and those of in vivo labelled total cytoplasmic IE RNA to Bam HI, Bgl II and Eco RI generated virus DNA fragments are shown in Figure 37.

a) Major IE mRNA Species

Abundant hybridization to Bam HI p was detected, with much less to Bam HI vw (Figure 37 , track 3), indicating that the 3'-end of the 3.4 kb mRNA (IE mRNA I) is located within Bam HI p, and that the direction of transcription in TR_L is rightwards as drawn in Figure 38. Due to the structure of the HSV-2 genome, IE mRNA I synthesized from the IR_L template would be made on the complementary strand to that used in TR_L, and must therefore be transcribed in a leftwards direction (Figure 38).

IE cDNA hybridized to Bam HI u, but not to Bam HI g' or z, which indicates that the direction of transcription of the 4.7 kb mRNA (IE mRNA III) in TR_S is rightwards as shown in Figure 38. IE mRNA III transcribed from IR_S is made from the complementary

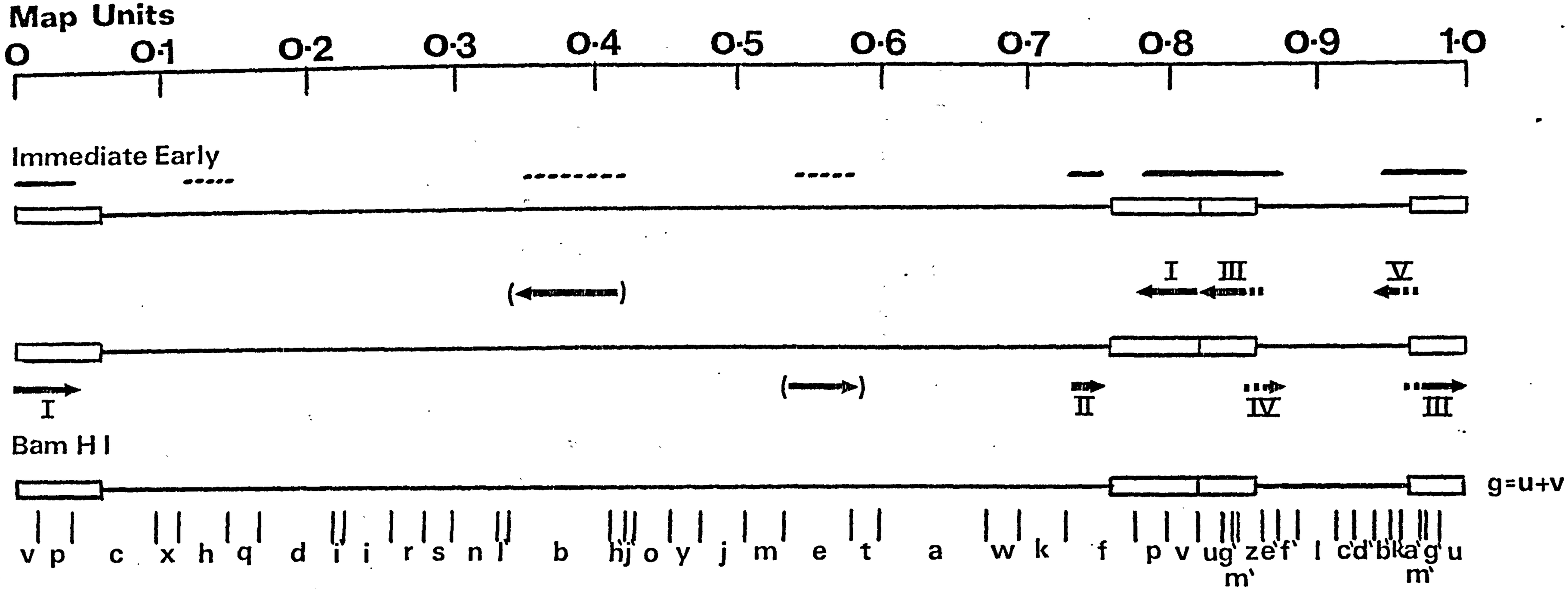


FIGURE 38

Summary of genome locations and orientations of HSV-2 IE mRNAs.

The major IE mRNAs are indicated by numerals and minor species are shown in brackets. A summary of the genome regions represented by total IE RNA and the Bam HI map are also shown.

The dashed areas of IE mRNAs III, IV and V indicate regions where the precise locations of the 5'-ends of the mRNAs are not known.

strand, and therefore in a leftwards direction.

IE cDNA hybridized to Bam HI e', and also very strongly to Bgl II g, but not to Bam HI z. Hence, the transcription of one of the 1.75 kb mRNAs (IE mRNA IV) is initiated within IR_S and continues into U_S (Figure 38). This RNA is made off the complementary DNA strand in IR_S to that used for transcription of IE mRNA III.

Abundant hybridization was observed to Bam HI b', but not to Bam HI k' or a', and there was strong hybridization to Eco RI o with little to Eco RI m (Figure 37, tracks 3 and 6). The transcription of the 1.75 kb mRNA (IE mRNA V) is therefore initiated within TR_S and continues into U_S (Figure 38). Thus, IE mRNA V is made off the complementary strand to that used for transcription of IE mRNA III in TR_S.

b) Minor IE mRNA Species

Nuclear and cytoplasmic IE RNA hybridized to Bam HI b and Bgl II o (Figures 25 and 27). IE cDNA did not hybridize to Bgl II o, however faint hybridization to Bgl II j was detected (Figure 37, track 9). As Bgl II j and o are adjacent, this indicates that a minor RNA in this region is transcribed leftwards as shown in Figure 38.

Total nuclear and cytoplasmic IE RNA hybridized to Bam HI e (Figures 25 and 27), while polyadenylated cytoplasmic RNA hybridized to Bam HI e and also to Bam HI st (Figure 31). IE cDNA did not hybridize to Bam HI e, however hybridization to Bam HI st was detected (Figure 37, track 3). As Bam HI e and t are adjacent, this suggests that a minor RNA in this region is transcribed rightwards as shown in Figure 38.

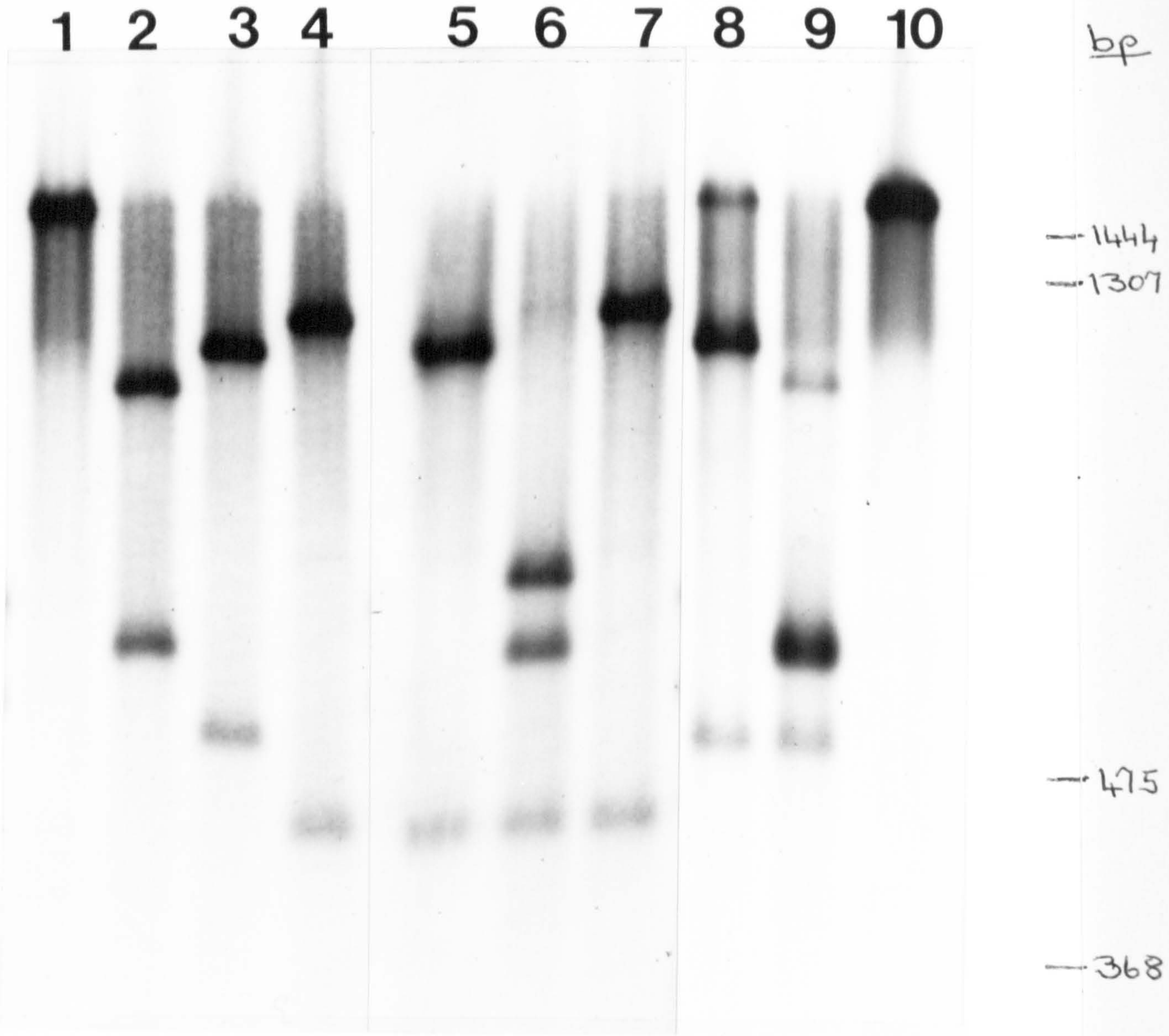


FIGURE 39

Autoradiograph of DNA fragments generated by restriction endonuclease digestion of HSV-2 Hind III o. The enzymes used were:

1. Undigested Hind III o
2. Sma I
3. Sst I
4. Pvu II
5. Sst I + Pvu II
6. Sma I + Pvu II
7. Pvu II
8. Sst I
9. Sma I + Sst I
10. Undigested Hind III o

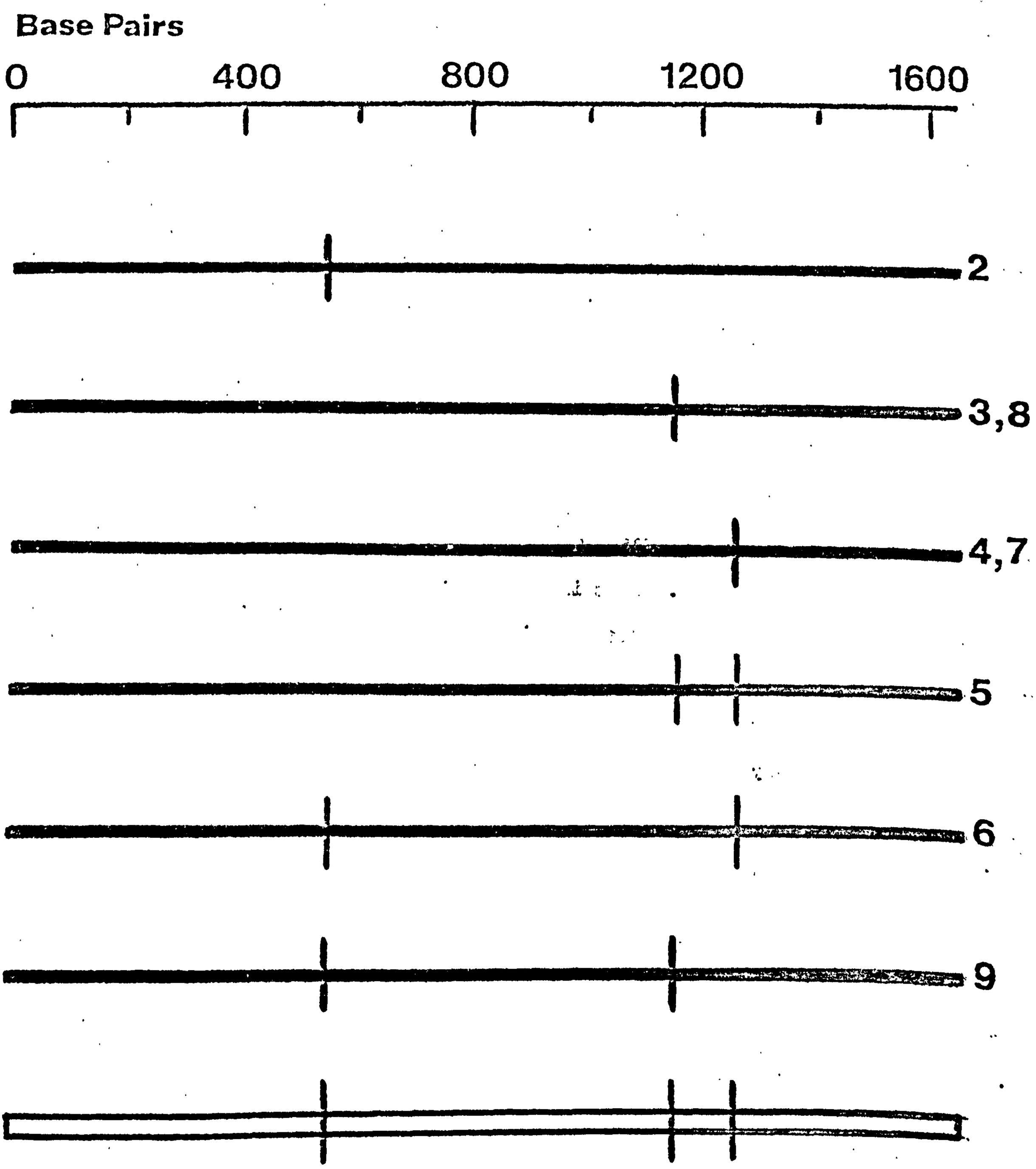


FIGURE 40

Locations of Sma I, Sst I and Pvu II sites in Hind III o.

The numbers on the right refer to the gel tracks shown in Figure 39, with vertical lines indicating the cleavage sites. The complete physical map is shown at the bottom.

IE cDNA hybridized faintly to Bam HI k, Eco RI 1 and Bgl II i (Figure 37). Since no in vivo labelled IE RNAs were mapped to these regions, the hybridization represents the 3'-end of at least one previously undetected IE mRNA, however no orientation could be determined.

As indicated previously, the hybridization of nuclear IE RNA to Bam HI hij was not detected with cytoplasmic IE RNA (Figures 25 and 27). However, IE cDNA prepared using cytoplasmic IE RNA hybridized to Bam HI hij and also to Eco RI j, though not to Bgl II p or r. Eco RI j shares sequences with Bam HI h and with Bgl II p and r (Figure 20). This indicates that the IE RNA is located within Bam HI h (0.12-0.15 map units; Figures 26 and 38), and that these RNA sequences are present within the cytoplasm.

c) Orientation of the 1.75 kb mRNA Mapping in U_L

The HSV-2 physical maps available were insufficiently detailed in the region of U_L containing the 1.75 kb mRNA (IE mRNA II) to allow orientation on the genome. To determine this, it was necessary to further map the Hind III o fragment (0.74-0.75 map units) which was cloned into pAT 153. The Hind III o DNA fragment was isolated, labelled by nick-translation, and the cleavage sites for Sma I, Sst I and Pvu II restriction endonucleases were mapped. An autoradiograph of the gel is shown in Figure 39. The Hind III o DNA fragments were sized by comparison with the fragments produced by cleavage of pBR 322 DNA with Taq I (data not shown); Hind III o had a size of 1650 base pairs.

The mapping rationale is given in figure 40.

Hind III o contains single cleavage sites for Sma I, Sst I and Pvu II, at 550, 1150 and 1260 base pairs respectively. Sst I

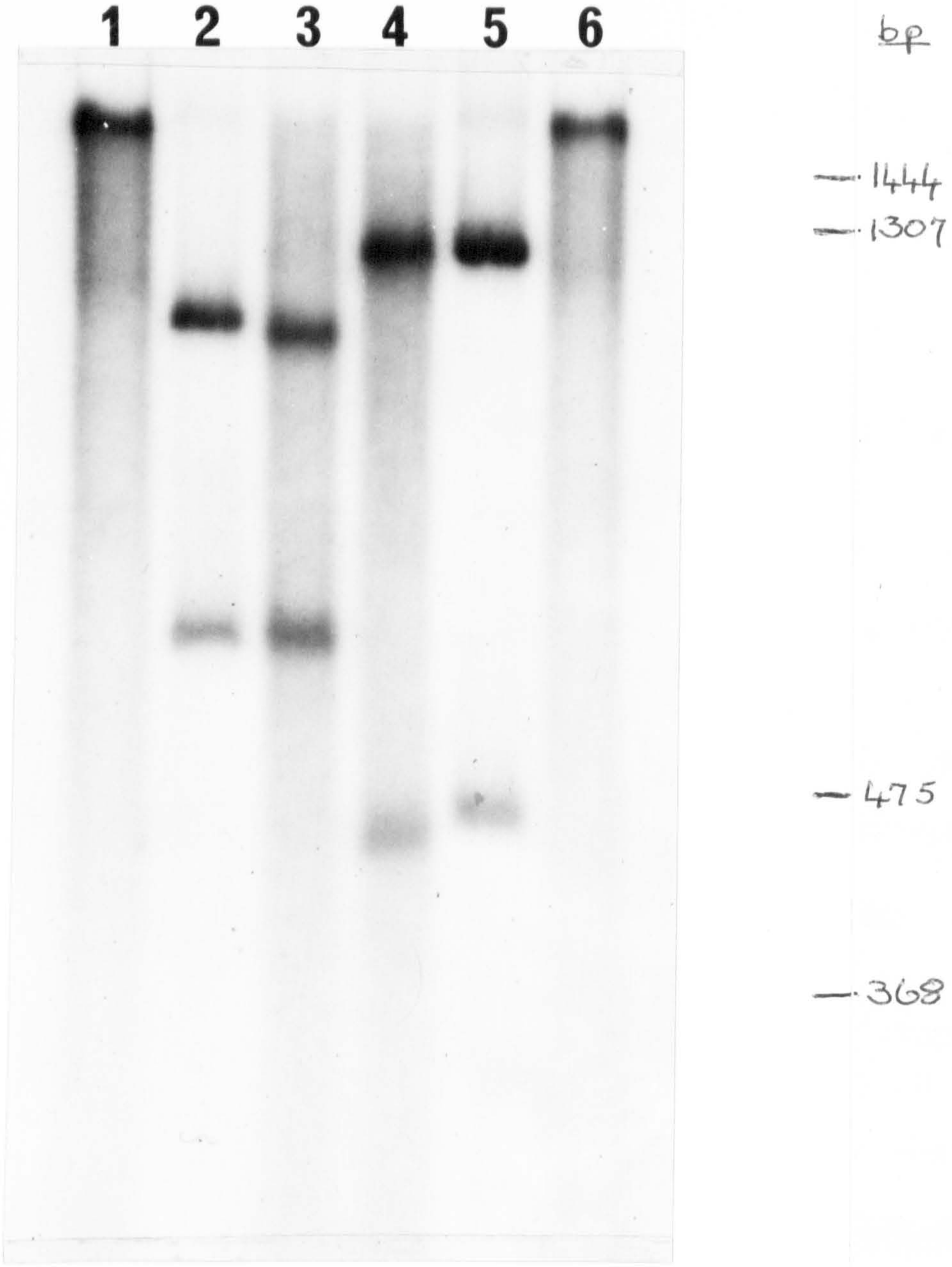


FIGURE 41

Autoradiograph of the restriction endonuclease digestion products of the Hind III o fragment of recombinant RE4 DNA and the HSV-2 Hind III o DNA fragment.

1. HSV-2 Hind III o
2. RE4 DNA + Sma I
3. Hind III o + Sma I
4. Hind III o + Pvu II
5. RE4 DNA + Pvu II
6. HSV-2 Hind III o

digested the largest Pvu II fragment 110 base pairs from one end (the 110 base pair DNA fragment does not appear on these gels), which placed the Sst I and Pvu II cleavage sites 110 base pairs apart, 1150 and 1260 base pairs respectively from one end of Hind III o. Sma I digested the largest Pvu II fragment into two fragments of 550 and 710 base pairs, indicating that the Sma I cleavage site was 550 base pairs from one end of Hind III o and 750 base pairs from the Pvu II site. Sma I digested the largest Sst I fragment into two fragments of 550 and 600 base pairs, which placed the Sst I cleavage site 600 base pairs from the Sma I site.

To orientate the Hind III o fragment within the HSV-2 genome, it was necessary to use an HSV-1/HSV-2 intertypic recombinant RE4 (Wilkie et al., 1979). The genome of this recombinant has a deletion extending from U_L (0.75 map units) to the end of IR_L. This region is substituted with an insertion of a piece of DNA normally found at approximately 0.26 map units in HSV-1. This deletion/insertion extends into the HSV-2 Hind III o fragment, deleting the Hind III site proximal to IR_L. The inserted DNA contains a single Hind III cleavage site, such that digestion of the RE4 DNA generates a Hind III fragment larger than the HSV-2 Hind III o, but which consists almost entirely of sequences found in Hind III o, together with additional sequences at the end proximal to IR_L (A. Davison and N.M. Wilkie, manuscript in preparation).

This Hind III generated fragment of RE4 DNA was isolated, labelled by nick-translation, digested with Pvu II and with Sma I, and the digestion products were separated by gel electrophoresis. An autoradiograph of the gel is shown in Figure 41. The smallest Pvu II, and the largest Sma I generated DNA fragments were larger than the corresponding HSV-2 fragments, indicating that the Pvu II

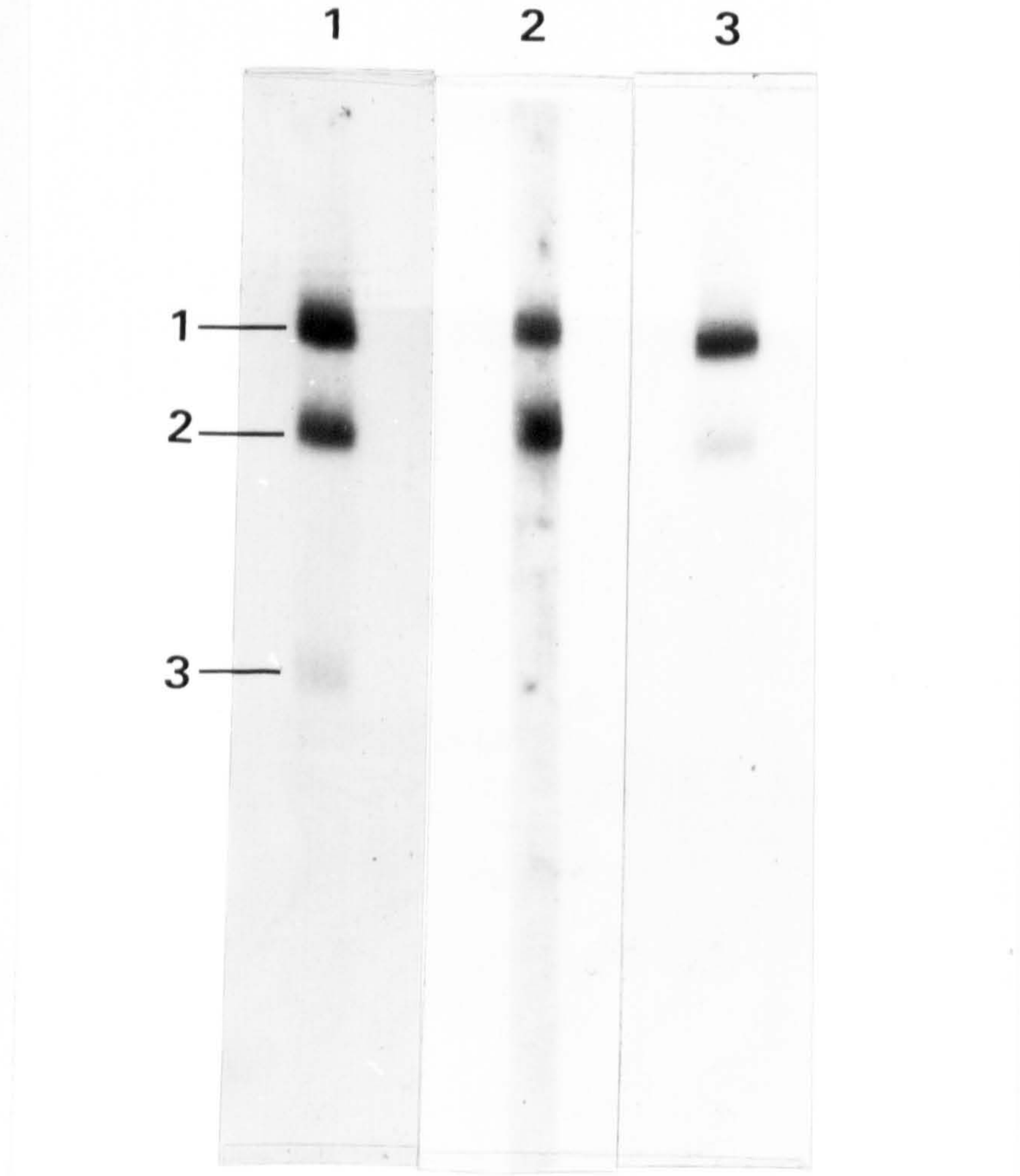


FIGURE 42

Autoradiographs of IE cDNA hybridized to blot strips containing the Pvu II/Sma I generated fragments of the HSV-2 Hind III o DNA fragment.

1. Nick-translated total HSV-2 DNA
2. IE cytoplasmic RNA
3. IE cDNA

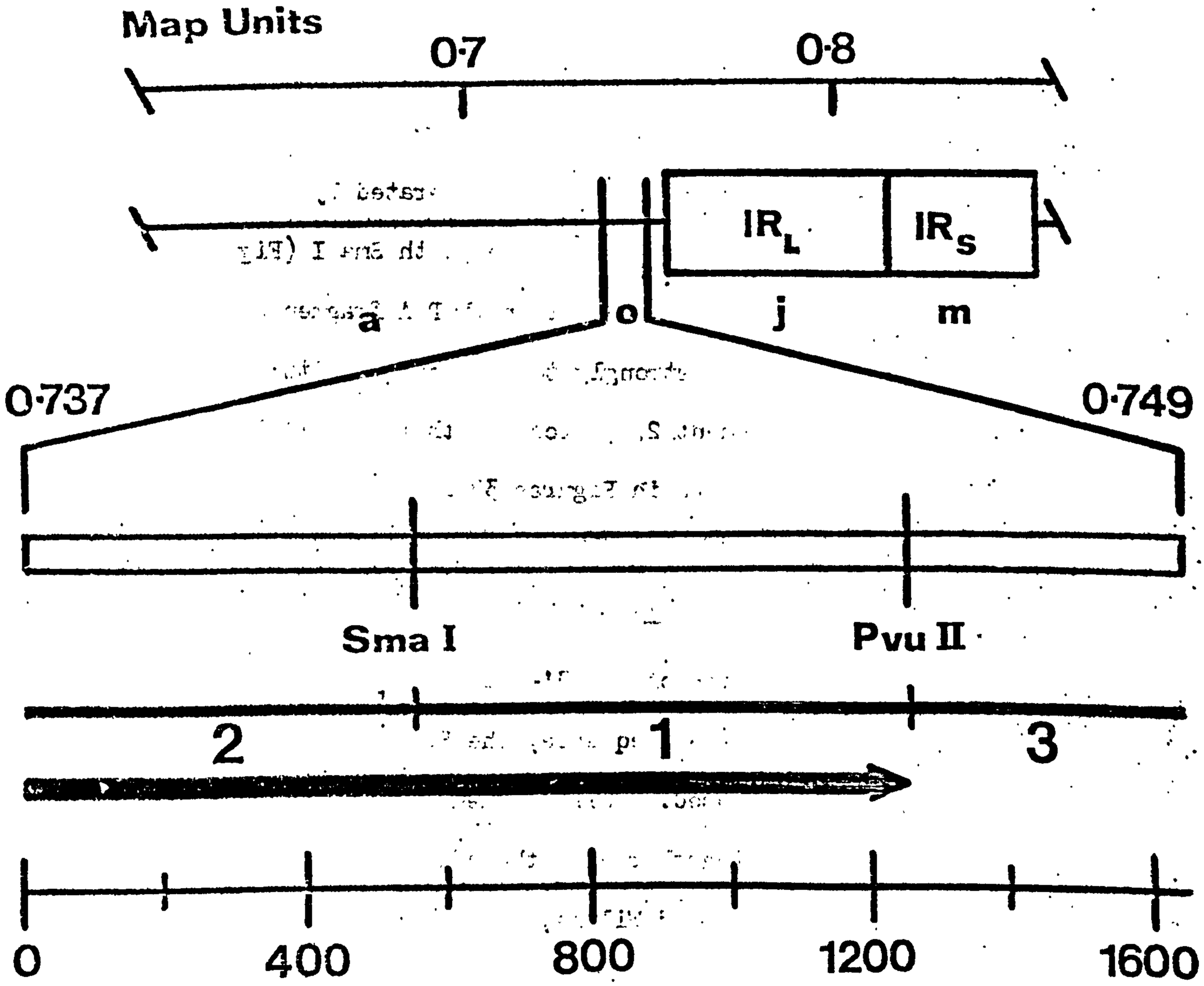


FIGURE 43

The orientation of HSV-2 IE mRNA II.

site in Hind III o is proximal, and the Sma I site is distal to IR_L (Figure 43).

In vivo labelled cytoplasmic IE RNA and IE cDNA were hybridized to blot strips containing the DNA fragments generated by cleavage of cloned Hind III o DNA with Pvu II together with Sma I (Figure 42). Cytoplasmic IE RNA hybridized strongly to both DNA fragments 1 and 2, whereas IE cDNA hybridized strongly to fragment 1, with only faint hybridization to fragment 2, indicating that IE mRNA II is transcribed rightwards as shown in Figures 38 and 43.

10. Mapping the 3'-end of IE mRNA III

To determine the location of the 3'-end of IE mRNA III in TR_S/IR_S with respect to the "a" sequence, the HSV-2 Bam HI joint DNA fragment, Bam HI $\overline{g}$ was used. This fragment contains two copies of the HSV-2 "a" sequence together with the adjacent sequences from TR_S/IR_S and TR_L/IR_L (Davison and Wilkie, manuscript submitted).

In vivo labelled cytoplasmic IE RNA and IE cDNA were hybridized to blot strips containing fragments of Bam HI $\overline{g}$ generated by cleavage with Sst I. Autoradiographs of the blot strips are shown in Figure 44. DNA fragment 5 did not transfer efficiently to these blot strips, and the smaller fragments 6, 7 and 8 were not detected with nick-translated DNA. IE RNA hybridized to fragments 1, 3 and 4, while IE cDNA hybridized mainly to fragment 1, with faint hybridization to fragments 3 and 4. No hybridization was detected to fragment 2. A summary is given in Figure 45.

The 3'-end of IE mRNA III therefore does not extend into the "a" sequence, and the data confirms that the direction of transcription of IE mRNA III is towards the "a" sequence (Figures 38 and 45).

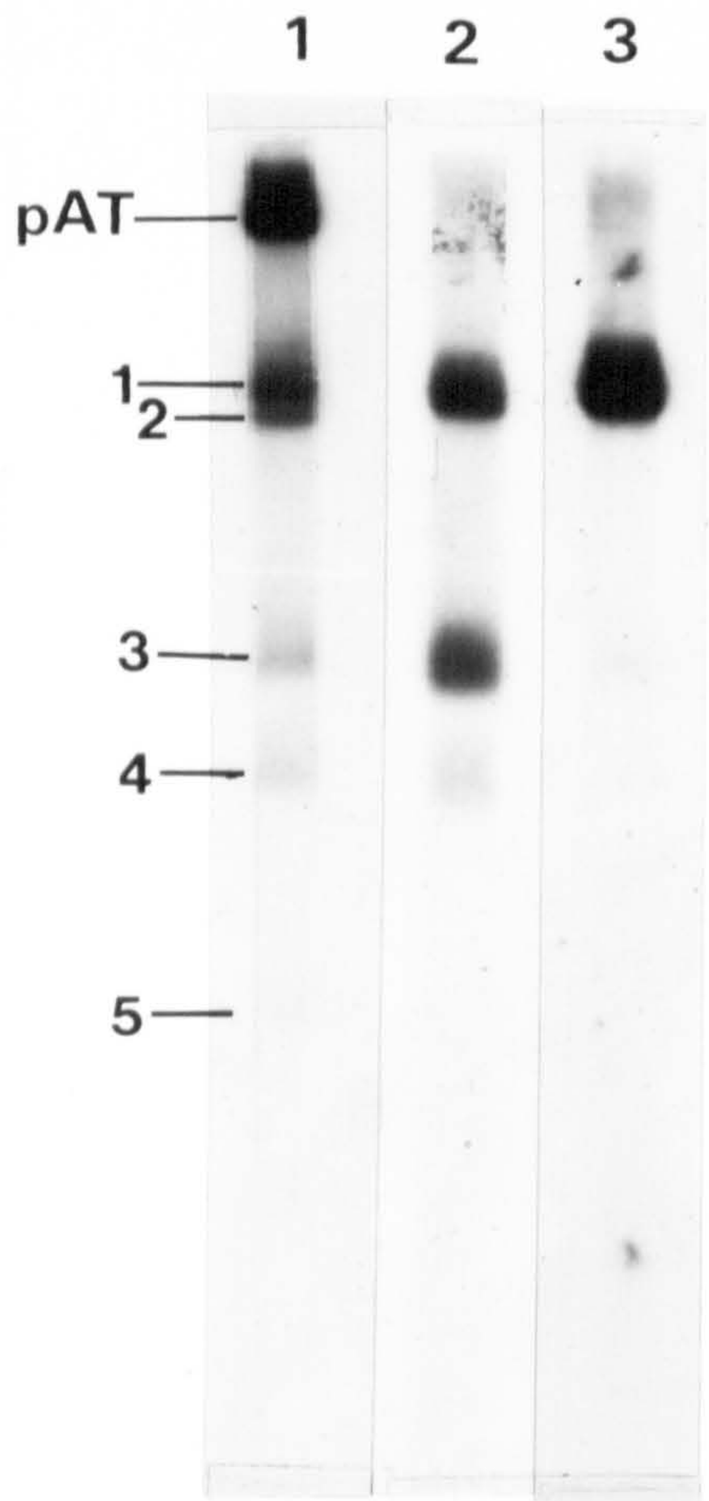


FIGURE 44

Autoradiographs of IE cDNA hybridized to blot strips containing the Sst I generated fragments of HSV-2 Bam HI $\overline{g}$.

1. Nick-translated cloned Bam HI $\overline{g}$ DNA
2. IE cytoplasmic RNA
3. IE cDNA

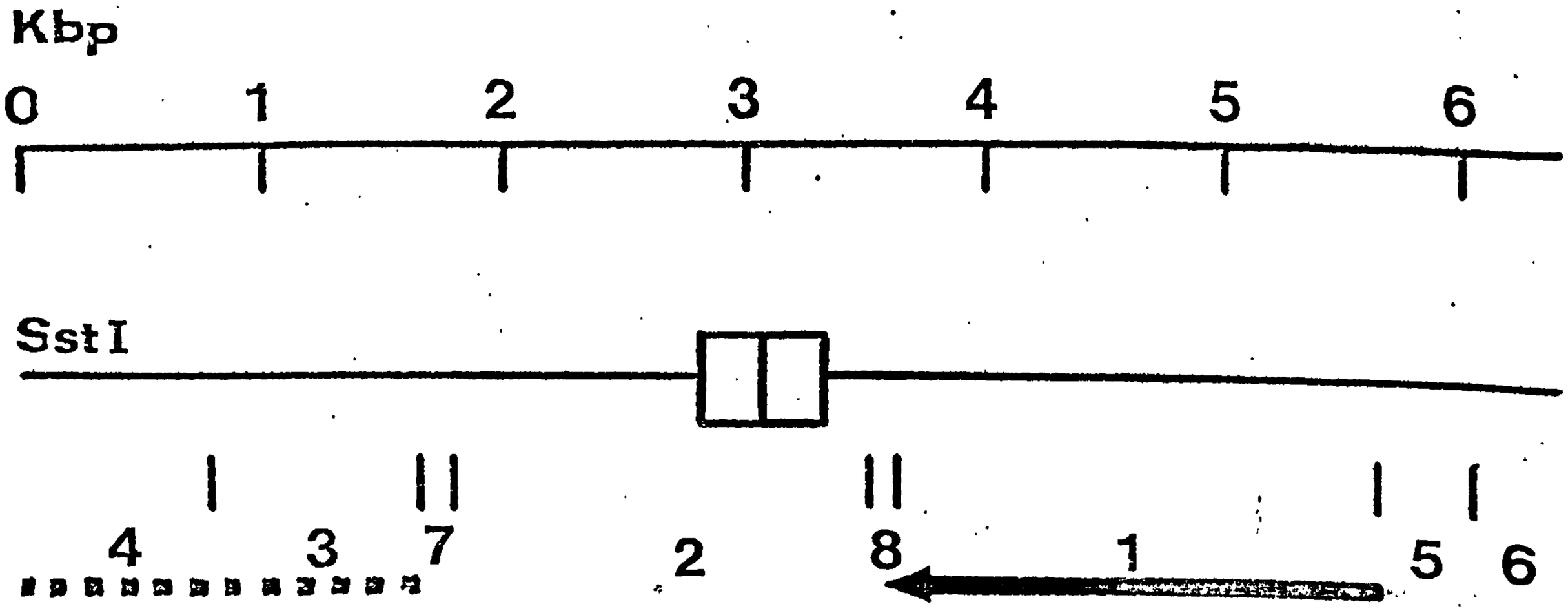


FIGURE 45

Physical map of the HSV-2 Bam HI $\overline{g}$ joint DNA fragment. The "a" sequence is represented by the boxes. Two "a" sequences are present in Bam HI $\overline{g}$.

A summary of the IE cDNA mapping data shown in Figure 44 is also given.

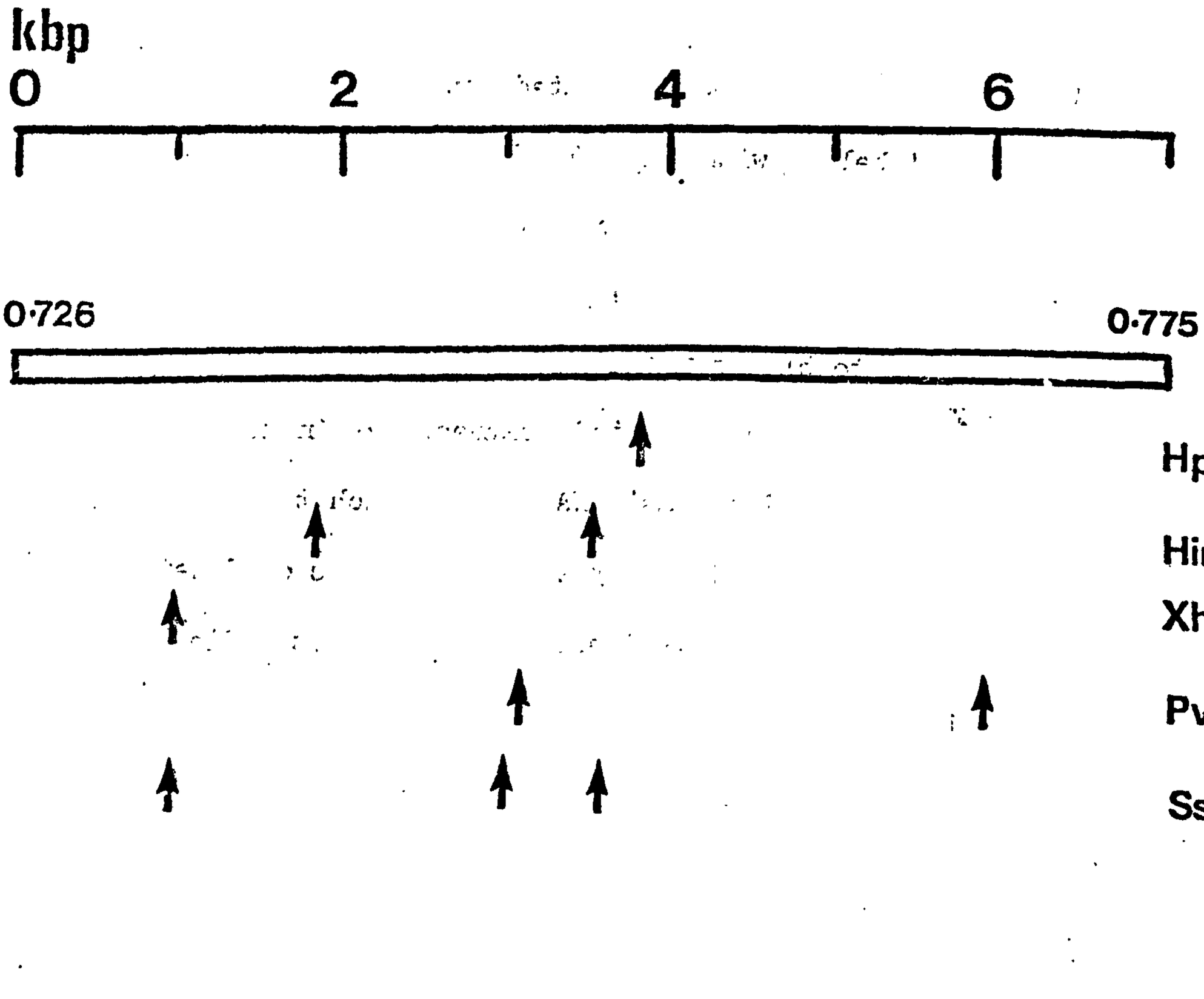


FIGURE 46

Physical maps of the HSV-2 Bam HI f fragment for restriction endonucleases Hpa I, Hind III, Xho I, Pvu II and Sst I.

11. Structure of HSV-2 IE mRNA II

To analyse the structure of IE mRNA II, the Bam HI f fragment (0.73-0.78 map units) was cloned into pAT 153. Bam HI f contains one Hpa I, and two Hind III cleavage sites (Cortini and Wilkie, 1978; Wilkie et al., 1979), and it was further mapped using Pvu II, Xho I and Sst I. The physical maps are shown in Figure 46, and autoradiographs of gels of the Pvu II, Xho I and Sst I fragments are given in a supplement.

a) 3'-end of IE mRNA II

The Hind III o fragment was isolated, and the 3'-ends of both DNA strands were labelled with ^{32}P. The fragment was digested with Pvu II, and the largest fragment isolated. This large fragment previously was shown to contain the 3'-end of IE mRNA II by hybridizing IE cDNA to blot strips (Figures 42 and 43). In separate hybridization reactions, the isolated fragment was incubated with cytoplasmic IE RNA, and with yeast RNA, after which the samples were treated with nuclease S1 followed by electrophoresis on denaturing, non-denaturing and two-dimensional agarose gels. Autoradiographs of the denaturing and non-denaturing gels are shown in Figure 47.

Under the hybridization conditions used, two DNA reassociation products were observed with both the IE RNA and the yeast RNA samples. A third band of 800 nucleotides was present only with the cytoplasmic IE RNA. This band was the same size on both denaturing and non-denaturing gels (Figure 47). On electrophoresis through a two-dimensional agarose gel, the 800 nucleotide band lay on the diagonal i.e. it retained the same relative mobility in both denaturing and non-denaturing dimensions (Figure 48). This confirms the orientation and location of the 3'-end of IE mRNA II,

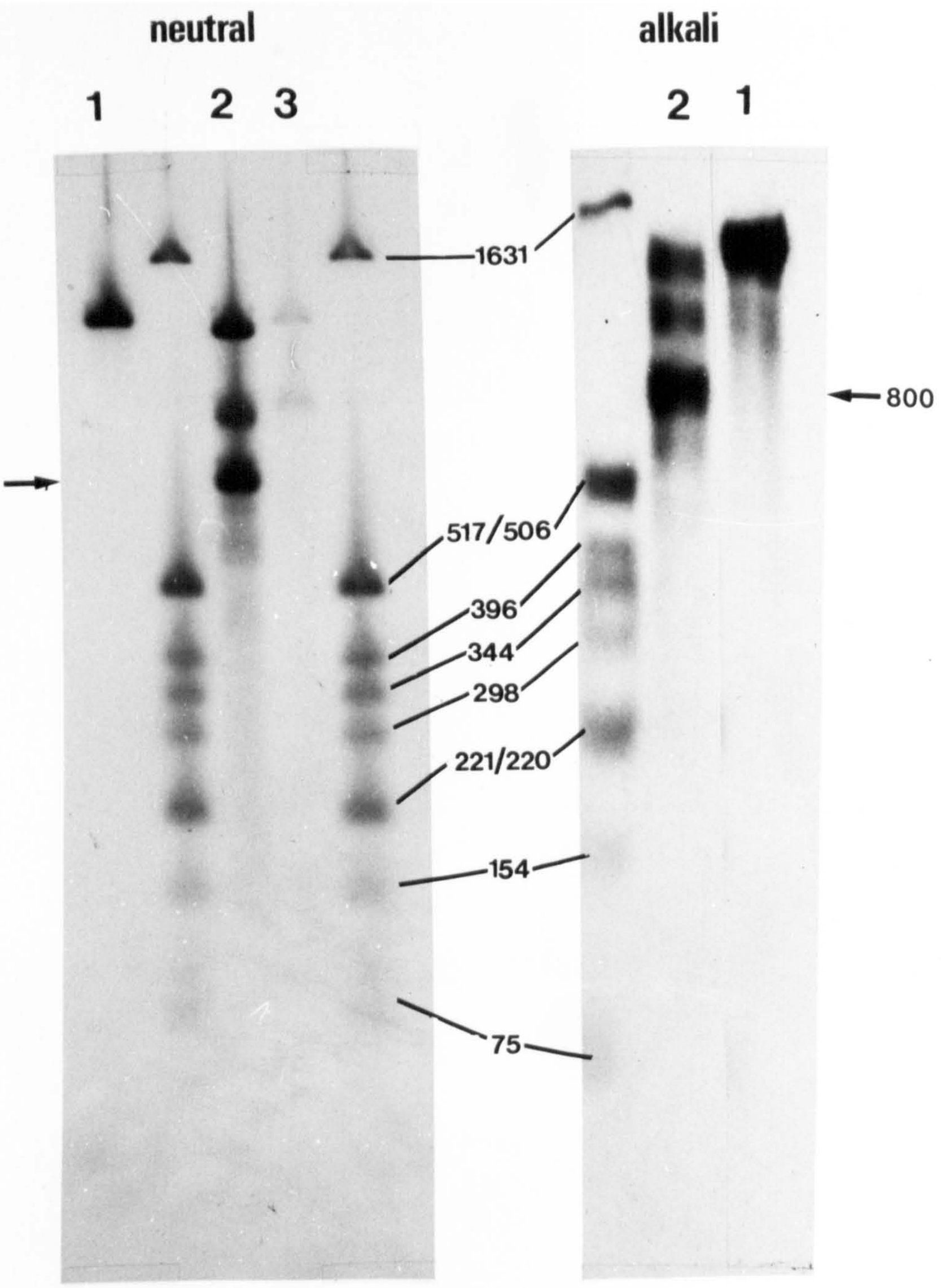


FIGURE 47

Autoradiographs of S1-resistant material prepared by hybridizing HSV-2 IE RNA with 3'-end labelled Hind III o DNA. S1-resistant material was electrophoresed on denaturing and non-denaturing agarose gels. The non-denaturing gel is on the left, the denaturing on the right.

1. 3'-end labelled Hind III o DNA
2. S1-resistant material using cytoplasmic IE RNA
3. S1-resistant material using yeast RNA

DNA size markers are also shown.

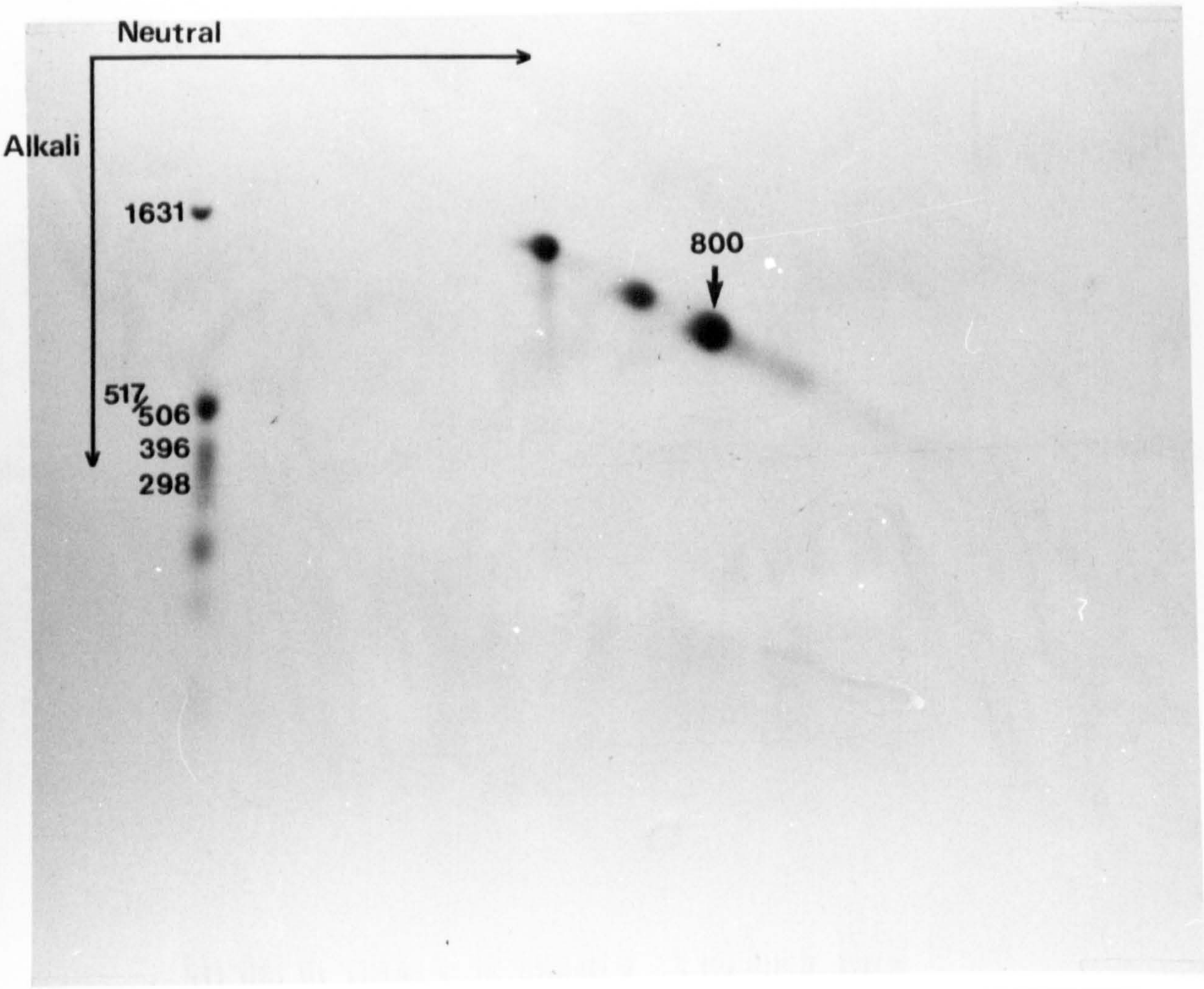


FIGURE 48

Autoradiograph of S1-resistant material prepared by hybridizing IE RNA with 3'-end labelled Hind III o DNA, electrophoresed on a two-dimensional agarose gel. The DNA fragment protected by the virus RNA is indicated with an arrow. DNA size markers were electrophoresed in the denaturing dimension.

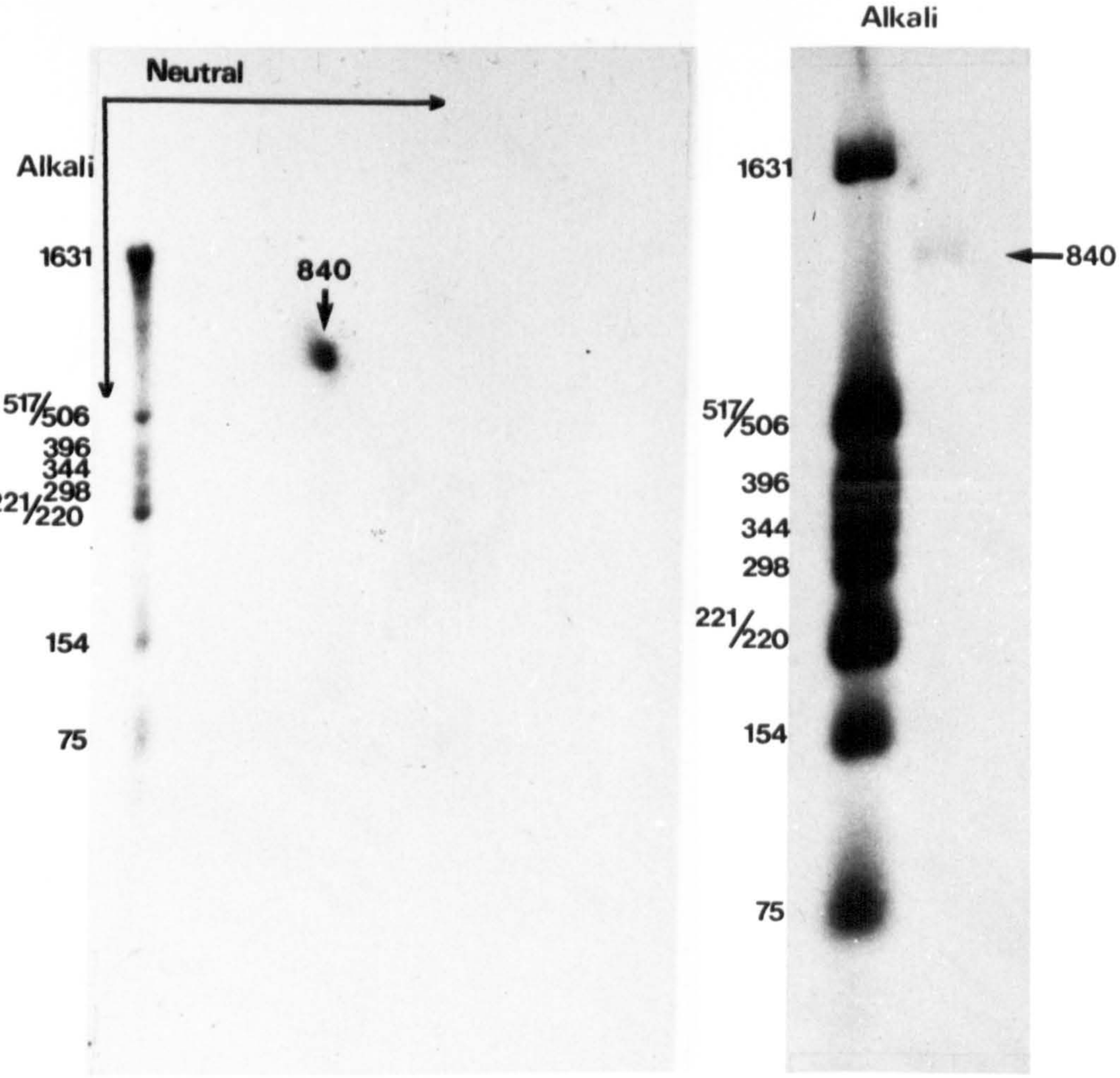


FIGURE 49

Autoradiographs of S1-resistant material prepared by hybridizing IE RNA with 5'-end labelled Hind III digested Bam HI f DNA. S1-resistant material was electrophoresed on denaturing and two-dimensional agarose gels. The DNA fragment protected by IE RNA is indicated with an arrow. DNA size markers are shown.

and indicated that the 3'-end of IE mRNA II was not spliced for 800 nucleotides from the Hind III site shown in Figure 51.

b) 5'-end of IE mRNA II

Cloned HSV-2 Bam HI f DNA was cleaved with Hind III, and the 5'-ends of both strands of all of the resultant DNA fragments were labelled with ^{32}P using polynucleotide kinase. The mixture of labelled fragments was used in the hybridization mixture with IE RNA, followed by nuclease S1 digestion. After electrophoresis, only one nuclease S1-resistant band, of 840 nucleotides, was detected (Figure 49). After two-dimensional agarose gel electrophoresis, the band lay on the diagonal (Figure 49), indicating that IE mRNA II was not spliced at the 5'-end for 840 nucleotides from the labelled Hind III site.

Since both 5'- and 3'-ends of IE mRNA II were analysed by labelling at the same Hind III site, the total size of the mRNA was determined as 1640 nucleotides (Figure 51). An approximate estimate of the size of polyadenylated IE mRNA II on CH_3HgOH gels was 1750 nucleotides.

12. Nucleotide Sequence at the 3'-end of IE mRNA II

Intact plasmid DNA containing the HSV-2 Hind III o fragment was digested with Xma I. This enzyme has the same recognition sequence for digestion as Sma I. Sma I digests;

$$-CCC \downarrow GGG-$$

while Xma I digests DNA leaving a recessed 3'-end;

$$-C \downarrow CCGGG-$$

It is therefore possible to label the 3'-ends of the DNA strands produced by Xma I digestion, using T4 DNA polymerase to fill out

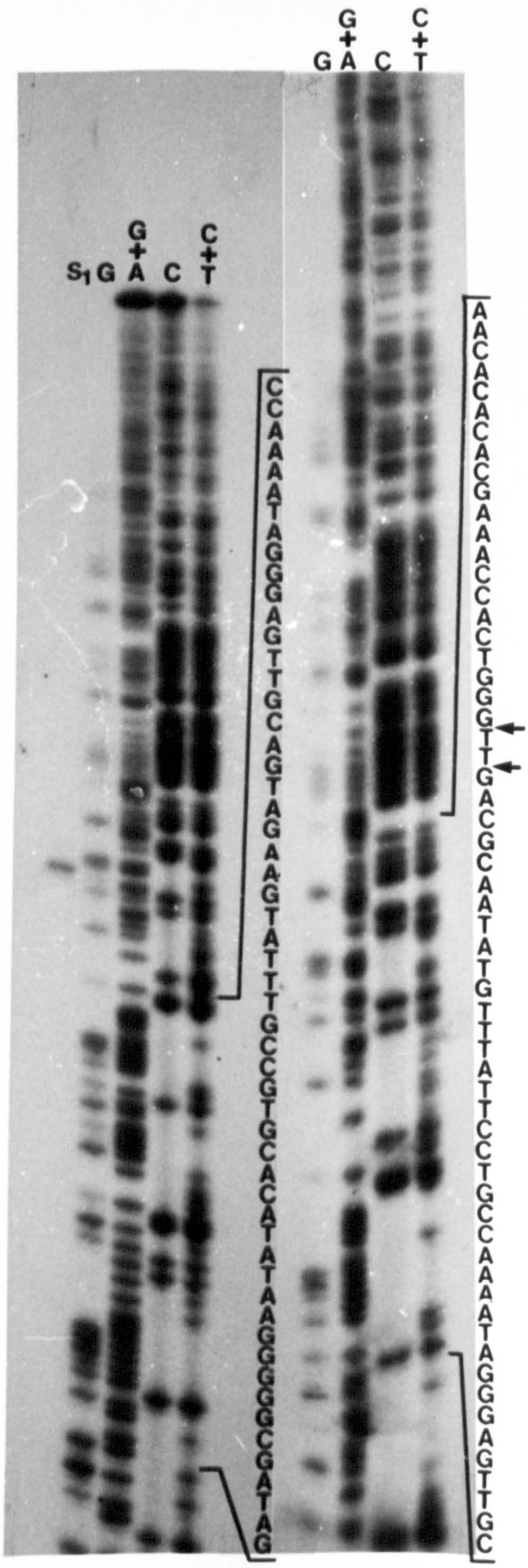


FIGURE 50

Sequencing gels of the DNA sequence at the 3'-end of IE mRNA II. The S1-resistant DNA-RNA hybrid was co-electrophoresed to detect the point of termination of transcription. The region

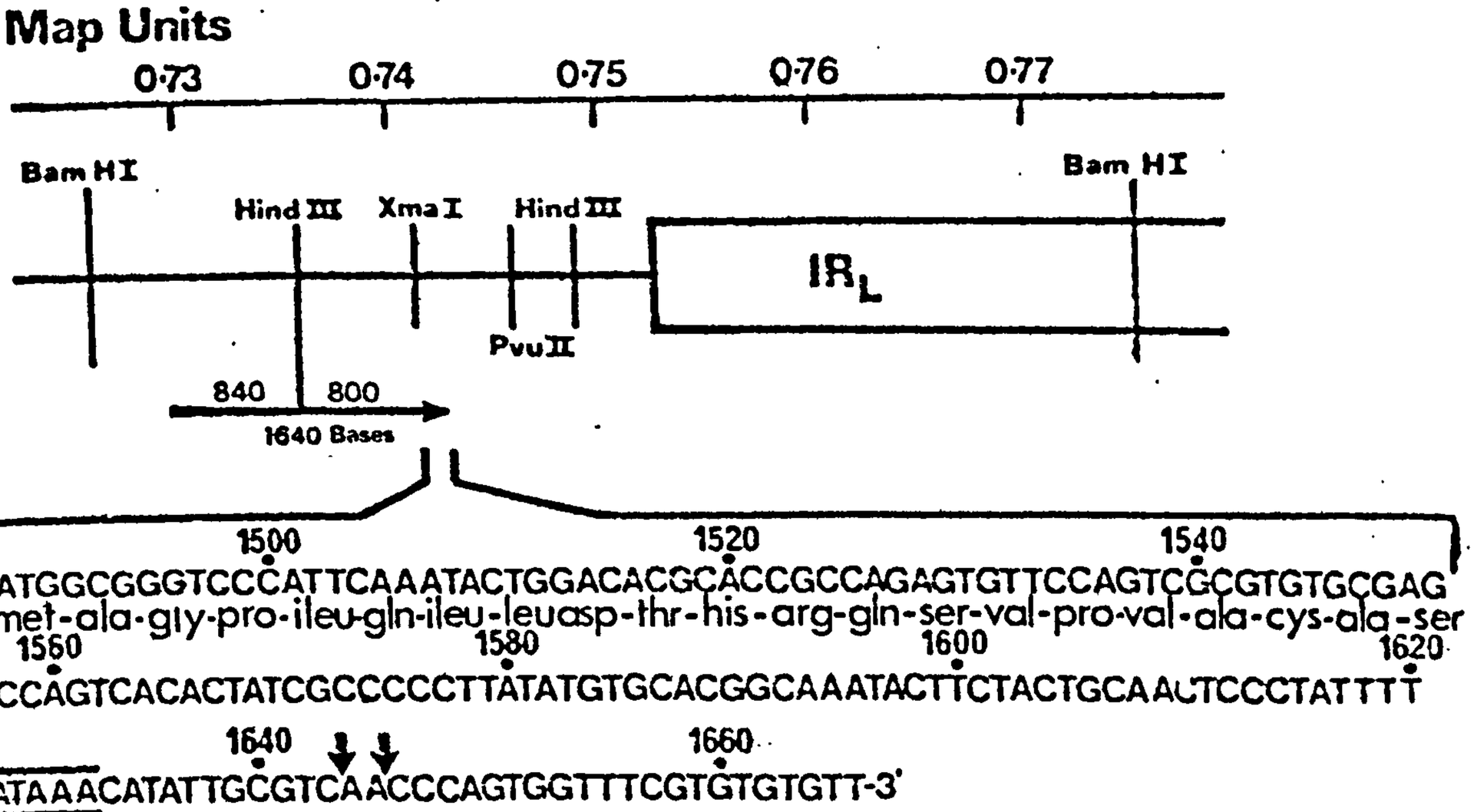


FIGURE 51

Structure and genome location of HSV-2 IE mRNA II. The sequance of the 3' end of the mRNA is also given. The region of termination of transcription is indicated by the arrows. The amino acid sequence of the COOH terminus of a putative polypeptide is given.

the restriction site. Xma I cleaves Hind III o once as previously shown for Sma I (Figures 39 and 43), but does not cleave pAT 153 DNA. The free ends of the linearized DNA were 3'-end labelled using α-^{32}P-dCTP and the DNA was digested with Hind III. The largest labelled DNA fragment was isolated from a polyacrylamide gel, and sequenced. The nuclease S1-resistant DNA-RNA hybrid generated by hybridization of the labelled fragment with IE RNA was co-electrophoresed on the sequencing gels (Figure 50). The S1-resistant band indicated the 3'-end of IE mRNA II on the DNA. The DNA sequence determined (Figure 50) was that of the strand complementary to the RNA, and was read in a 3' to 5' direction. The sequence of the opposite strand, that with the same sequence as the RNA is given in Figure 51. The point of termination of transcription is indicated by the arrows. It was not possible to precisely define the 3'-end of the RNA as the sequence at which the RNA terminated contained two A residues, and it was unclear whether these were transcriptional or post-transcriptional additions.

D I S C U S S I O N

1. Genome Locations of Early and Late HSV-2 RNA

Late RNA samples hybridized to DNA fragments from all regions of the virus genome, and the mapping data presented here is consistent with that obtained using filter-bound DNA fragments (Oakes et al., 1976; Bodemer and Bodemer, 1979), and also with the results of liquid hybridization experiments which indicated that 50% of virus DNA (all of the coding capacity assuming asymmetric transcription) was represented in late RNA (Frenkel et al., 1972; Frenkel et al., 1973). The general pattern of HSV-2 transcription at late times was similar to that observed with HSV-1 using both the blot hybridization and liquid hybridization techniques (Frenkel and Roizman, 1972b; Frenkel et al., 1973; Swanstrom et al., 1975; Oakes et al., 1976; Clements et al., 1977; Jones et al., 1977; Jones and Roizman, 1979).

Several differences in the hybridization patterns of late nuclear and late cytoplasmic RNAs were observed; little cytoplasmic RNA hybridized to both sets of repetitive regions of the virus DNA. A similar situation was observed with HSV-1 (Kozak and Roizman, 1974; Clements et al., 1977; Jones et al., 1977; Jones and Roizman, 1979). In addition, at late times during HSV-1 infection, self-complementary virus RNA was detected in the nucleus, and to a lesser extent, in the cytoplasm (Kozak and Roizman, 1975). These observations could result from either sequestration of specific RNA sequences within the nucleus, or rapid degradation of these sequences in the cytoplasm.

Early HSV-2 RNA when analysed by liquid hybridization was reported to consist of a single abundance class representing only 21% of the genome (Frenkel et al., 1973), in contrast to the

situation with HSV-1 where 44% of the genome was represented by early RNA (Frenkel and Roizman, 1972b). Analysis of HSV-1 early RNA by blot hybridization showed that all regions of the virus genome were represented (Oakes et al., 1976; Clements et al., 1977). This suggested that the transcriptional programmes of HSV-1 and HSV-2 were substantially different.

The HSV-2 blot hybridization data presented here indicates that HSV-2 transcription at early times is similar to that of HSV-1, with early nuclear and early cytoplasmic RNA hybridizing to DNA fragments from all regions of the genome. The hybridization patterns of early RNA were quantitatively different to those observed with late HSV-2 RNA samples. This is consistent with data from studies using isolated virus DNA fragments (Bodemer and Bodemer, 1979). Possible reasons for the disparity between the results obtained using the blot hybridization and liquid hybridization procedures are discussed later.

Although no qualitative differences were observed between the hybridization patterns of HSV-2 early nuclear and early cytoplasmic RNAs, there were differences in the amount of RNA which hybridized to individual DNA fragments. Thus, there was abundant hybridization of early RNA to Bam Hi e and t (0.54-0.60 map units), with little hybridization to Bam HI d and m (Figure 21). A DNA fragment, Bgl II n (0.57-0.62 map units; Figure 20) located within the region of abundant early transcription has been implicated in the morphological transformation of mouse cells and primary rat embryo cells cultured in vitro (Reyes et al., 1979; I.R. Cameron and J.C.M. MacNab, personal communication). Three mRNAs, of 7.0 kb, 1.75 kb and 1.25 kb transcribed from this region were detected in

IE RNA (Figure 33), and the orientation of at least one of these was determined (Figure 38). A 38,000 molecular weight polypeptide has been mapped within Bgl II n (J. Docherty and C.M. Preston, personal communication).

2. Genome Locations of HSV-2 IE RNA

In contrast to the early and late situations, HSV-2 IE RNA hybridized to only certain DNA fragments, located in both sets of repetitive regions, and to a lesser extent, in both unique regions. The mapping data is summarized in Figure 26, and precise map locations of certain IE mRNAs are discussed later.

No major differences in the blot hybridization patterns of IE nuclear and cytoplasmic RNAs were observed using *in vivo* labelled RNA samples, and this is similar to the situation reported for HSV-1 (Clements *et al.*, 1977; Watson and Clements, 1979; Watson and Clements, 1980). The results do not agree with liquid hybridization analysis of HSV-2 RNA which indicated that 45% of the virus genome was represented in total IE RNA (Frenkel *et al.*, 1973). Similarly, for HSV-1, liquid hybridization data suggested that 50% (Kozak and Roizman, 1974) to 30% (Jones and Roizman, 1979) of the genome was represented in IE nuclear RNA, whereas only 13% was represented in the cytoplasm.

The hybridization patterns of *in vitro* labelled nuclear and cytoplasmic HSV-2 IE RNAs were qualitatively similar to those of *in vivo* labelled RNA, with no additional RNA species detected (Figure 27). However, there were quantitative differences between the hybridiation patterns of *in vitro* and *in vivo* labelled samples, and also between the hybridization patterns of *in vitro* labelled

nuclear and cytoplasmic RNA. With *in vitro* labelled IE RNA, sequences of the 4.7 kb IE mRNA III were located predominantly within the nucleus, while sequences of the 1.75 kb IE mRNAs II, IV and V were located mainly within the cytoplasm. The cell fractionation method used was developed for rapid and reproducible preparation of intact mRNA, and does not necessarily rigorously separate nuclei and cytoplasm. The different distribution of the RNA sequences between the nuclear and cytoplasmic fractions may represent a division between sequences strongly associated with the nucleus and those located predominantly within the cytoplasm, or weakly associated with the nucleus, rather than a precise indication of intracellular compartmentalization. The results may also reflect rapid degradation of IE mRNA III within the cytoplasm.

The blot hybridization results presented here indicate that approximately 20% of the HSV-2 genome is represented by major IE mRNA species, together with several minor mRNAs from U_L, while all regions of the genome are represented at early times with both abundantly and scarcely represented regions. This is clearly at variance with the liquid hybridization data of Frenkel *et al.* (1973) which suggested that 45% of the genome was represented in total IE RNA, with only 20% represented by a single abundance class of early RNA. These results make it unlikely that the disparity is due to differences in sensitivities of the two techniques. Liquid hybridization analysis is very dependent on the concentration of virus RNA used to drive the DNA probes into a hybrid, and this may result in inaccurate estimates of the proportion of the genome represented by RNA. That such inaccuracies may arise can best be illustrated by example.

Liquid hybridization of excess RNA to specific virus DNA fragments has been employed to map HSV-1 cytoplasmic IE mRNAs (Jones et al., 1977). These analyses failed to detect scarce IE mRNAs mapping in U_L which were detected by the blot hybridization technique (Clements et al., 1977; Clements et al., 1979). Furthermore, it was reported that 18% of the DNA sequences (36% of the coding capacity assuming asymmetric transcription) of the HSV-1 Hind III (or Hsu I) m fragment was represented by cytoplasmic IE RNA (Jones et al., 1977). Separation of HSV-1 IE mRNAs and nuclease S1 analysis of cytoplasmic IE RNA has shown that Hind III m contains a 4.7 kb IE mRNA, and part of the 5'-end of a spliced, 2.0 kb IE mRNA (Watson et al., 1979; F.J. Rixon, personal communication). Together, these non-overlapping mRNAs comprise 85% of the coding capacity of Hind III m. These data are quite clearly inconsistent.

It has been suggested that the disparity between the liquid hybridization and blot hybridization analyses of HSV-1 may be due to artefacts of the labelling and purification procedure used to prepare RNA for blot hybridization (Jones and Roizman, 1979). However, HSV-2 IE RNA samples used in this analysis for in vitro labelling were prepared without purification on Cs_2SO_4 gradients, and the restricted hybridization profiles observed were qualitatively identical to those obtained using in vivo labelled IE RNA, with no additional sequences detected (Figure 27).

RNA samples used in the analysis here were prepared from virus-infected BHK cells, whereas Frenkel et al. (1973) prepared RNA from HEp-2 cells. It is possible, therefore, that the regulation of HSV transcription is fundamentally different in these two cell types. However, HSV-1 transcription in BSC-1 cells was reported to be identical to that observed in BHK cells (Watson, 1979).

3. Temporal Regulation of HSV Transcription

Regulation of virus mRNA synthesis may occur at two levels. Firstly, the rate of transcription of specific genome regions at various times post-infection may determine levels of mRNA synthesis. Secondly, post-transcriptional RNA processing may regulate levels of mRNA synthesis, determined by the rate of the processing event. Regulation may occur at one or both of these levels, and both have been shown to occur during adenovirus infection.

The data presented here suggest that the transcriptional programme of HSV-2 during the infectious cycle is identical to that of HSV-1, consisting of three distinct phases. IE RNA was transcribed from restricted regions of the genome, and these sequences were present in both the nucleus and cytoplasm. Pulse labelling experiments with HSV-1 have indicated that the IE phase is transient (Watson, 1979), however at least one IE gene product is required continuously throughout infection for transcription of the other genome regions (Watson and Clements, 1980). At early times, all regions of the HSV-2 genome were represented by RNA, with both abundantly and scarcely represented regions. At late times, as at early, all genome regions were represented by RNA, however the degree of hybridization to individual DNA fragments was different to that observed at early times. Also, certain late RNA sequences were preferentially located within the nucleus.

Liquid hybridization analysis of size-fractionated HSV-1 late nuclear RNA and total late cytoplasmic RNA indicated that sequences representing regions transcribed under IE conditions were predominantly contained within large nuclear transcripts, with only relatively small amounts present in the cytoplasm. However, sequences representing regions transcribed at late times were

distributed equally among the RNA classes analysed suggesting that regulation of mRNA synthesis at late times occurs in the nucleus at the level of post-transcriptional cleavage of large precursor transcripts (Jacquemont et al., 1980).

HSV transcription therefore appears to be regulated, at least in part, at the level of transcription, with initiation of transcription of several regions occuring after the switch from the immediate early to early phases. There is also an abundance control, evidenced by differences in the relative abundance of RNAs which hybridize to various regions of the genome, both within, and between the three classes of RNA described. The level at which this abundance control is exerted, whether transcriptional, post-transcriptional or both of these is unclear.

The pattern of transcription is reflected in the production of virus polypeptides during HSV-1 and HSV-2 infection. Following release from a cycloheximide block in the presence of actinomycin D, infected cells synthesize a restricted number of virus-induced polypeptides, and IE RNA directs the in vitro synthesis of a restricted number of similar polypeptides (Figure 28). At early and late times, the rate of synthesis of the IE (or α) polypeptides declines, and the synthesis of at least two further classes of polypeptides, β polypeptides and γ polypeptides is observed (Honess and Roizman, 1974; Powell and Courtney, 1975).

4. Polypeptides Encoded by IE RNA

IE polypeptides are defined as those virus-induced polypeptides synthesized in infected cells released from a cycloheximide block in the presence of actinomycin D. Powell and Courteney (1975) described the synthesis of two groups of HSV-2 IE polypeptides

represented by ICSP 5-8 and ICSP 0, which were labelled on release of a cycloheximide block.

In this laboratory, synthesis of a number of HSV-2 polypeptides is consistently observed. These are Vmw 182, 144, 134, 118, 67, 65, 42.5 and 12.3 (Preston et al., 1976; MacDonald, 1980). Of these polypeptides, only Vmw 182, 144, 118, 65 and 12.3 are made in large amounts in vivo.

Similarly for HSV-1, three IE polypeptides (ICP 4, 0 and 27) have been described, with small amounts of a fourth (ICP 6) being synthesized immediately following release of the cycloheximide block. However, in contrast to the others, ICP 6 was made in increasing amounts as virus infection proceeded (Honess and Roizman, 1974). Other studies from this laboratory have observed the production of several HSV-1 IE polypeptides (Vmw 175, 136, 110, 87, 68, 63 and 12). Polypeptides Vmw 175, 136, 110 and 63 are synthesized in large amounts in vivo (Preston et al., 1978; MacDonald, 1980). Synthesis of additional minor polypeptides is sometimes observed under IE conditions, and two of these (Vmw 38 and 16) can be seen in Figure 28 (track 2). Synthesis of these minor IE polypeptides may be related to the small amounts of IE mRNA which hybridized to several additional regions of the virus genome (Clements et al., 1977; Clements et al., 1979; Watson et al., 1979).

HSV-2 IE RNA directed the in vitro synthesis of four major virus-specific polypeptides with molecular weights of 178,000, 140,000, 116,000 and 65,000. These probably correspond to the in vivo polypeptides Vmw 182, 144, 118 and 64 respectively. The slight differences between the molecular weights of the polypeptides synthesized in vivo and in vitro are likely to be due to the absence

of normal post-translational modification of the polypeptide products in the in vitro translation system. The IE polypeptides are phosphorylated in vivo. No in vitro translation product equivalent to Vmw 67 was detected, possibly because the unmodified polypeptide comigrated with a host polypeptide band. Minor IE polypeptides with molecular weights of 38,000 and 16,000 were also detected. Thus the restricted transcription observed in the IE phase is reflected in the limited number of polypeptides produced both in vivo and in vitro.

5. Sizes, Genome Locations and Orientations of HSV-2 IE mRNAs

Fractionation of HSV-2 IE mRNAs on denaturing CH_3HgOH agarose gels revealed three virus-specific bands with sizes of 4.7, 3.4 and 1.75 kb, and these were mapped on the virus genome. The orientation of several IE mRNAs were determined using cDNA whose synthesis was initiated on polyadenylated IE RNA using oligo$(dT)_{10}$ as primer. This resulted in a product of small size consisting of sequences complementary to the 3'-termini of the mRNAs. Hybridization of this cDNA to blot strips allowed mRNAs to be oriented on the genome.

The 4.7 kb IE mRNA III hybridized to part of the TR_S/IR_S region (0.96-1.00 and 0.82-0.86 map units respectively) and the 3'-end was located towards the terminally redundant "a" sequence (Figure 38). However, the 3'-end of IE mRNA III was not transcribed from the "a" sequence itself.

The 3.4 kb IE mRNA I hybridized to part of TR_L/IR_L (0.0.04 and 0.77-0.82 map units respectively) with the 3'-end located away from the ends of the repetitive regions, towards U_L (Figure 38).

The 1.75 kb RNA band contained three distinct mRNAs which mapped in U_L near the junction with IR_L (IE mRNA II; 0.73-0.74 map

units), and at the junctions of U_S with both IR_S and TR_S (IE mRNAs IV and V; 0.85-0.88 and 0.94-0.97 map units respectively). IE mRNAs IV and V both had their 3'-ends located within U_S and hence these mRNAs share common 5'-end sequences.

The 1.75 kb IE mRNA II mapping entirely within U_L was oriented using the cloned HSV-2 Hind III o fragment, and the 3'-end was located towards IR_L (Figures 38 and 43). This orientation was confirmed by the nuclease S1 data.

The genome locations of the IE mRNAs primarily were defined by the positions of the Bam HI restriction endonuclease cleavage sites. IE mRNAs III and IV both hybridized to Bam HI z, while IE mRNAs III and V hybridized to Bam HI a'. This data does not necessarily imply that these mRNAs overlap, but more likely that the 5'-ends of the two relevant mRNAs lie within the same DNA fragment, and are not separated by a Bam HI cleavage site. The equivalent HSV-1 IE mRNAs which map in this location do not overlap at the 5'-ends (F.J. Rixon, personal communication).

The genome locations of several minor IE mRNAs were obtained. From "northern" blots, three mRNAs of 7.0, 1.75 and 1.25 kb were located within Bgl II n (0.57-0.62 map units), and the orientation of at least one of these was determined. Total cytoplasmic and polyadenylated cytoplasmic IE RNA also hybridized to Bam HI b (0.34-0.41 map units), and to an overlapping fragment, Hind III h (0.28-0.40 map units), which specified two distinct virus IE mRNAs of 5.0 kb and 4.3 kb. The orientation of at least one of these was determined (Figure 38). In addition, the 3'-end of a previously undetected IE mRNA mapping in U_L (0.69-0.73 map units) was observed.

The HSV-2 DNA fragments used in the "northern" blot analysis

are large enough to contain several mRNAs; Hind III h could specify the mRNAs detected, however, Bgl II n is too small to specify all the sequences of the three mRNAs detected, assuming that they are transcribed asymmetrically. It is possible that one or more of these mRNAs is only partially contained within this fragment. Alternatively, it has recently been shown for the region of the HSV-1 genome equivalent to HSV-2 Bgl II n that there are at least two mRNAs of different sizes which have co-terminal 3'-ends (Anderson et al., 1981; J. McLauchlan and J.B. Clements, personal communication). Another HSV-1 mRNA transcribed off the opposite DNA strand of this region also has been located. Since similar orientations of HSV-2 IE mRNAs located in both Hind III h and Bgl II n were determined, this may indicate that the HSV-2 IE mRNAs in these regions exist as families with 3' co-termini.

The transcriptional pattern of HSV-1 ts K at the NPT was more restricted than that of wild-type virus under IE conditions, suggesting that some of these minor IE mRNAs may be present due to incomplete inhibition of early transcription (Watson and Clements, 1978).

The genome locations and orientations of HSV-1 IE mRNAs have been determined (Watson et al., 1979; Clements et al., 1979), and the results are similar to those observed with HSV-2. The sizes of the major HSV-1 IE mRNAs were 4.7, 3.0 and 2.0 kb (Figure 30), and these were mapped equivalent genome regions as their HSV-2 counterparts; the 4.7 kb mRNA in TR_S/IR_S, the 3.0 kb mRNA in TR_L/IR_L, and three 2.0 kb mRNAs to U_L near the junction with IR_L, and at the junctions of U_S with both TR_S and IR_S (Watson et al., 1979; Anderson et al., 1980a).

The difference in size between the 3.4 kb HSV-2 IE mRNA I and the 3.0 kb HSV-1 equivalent may reflect the difference in molecular weight of the two equivalent IE polypeptides shown to map in TR_L/IR_L. These are 118,000 for HSV-2 and 110,000 for HSV-1 (Preston et al., 1978). The polypeptides translated in vitro, and therefore unmodified, have molecular weights of 116,000 and 109,000 respectively (Figure 28; Preston, 1977). By in vitro translation of isolated mRNA fractions, the 3.0 kb HSV-1 mRNA has been shown to encode the 109,000 polypeptide (Watson et al., 1979), and the 3.4 kb HSV-2 IE mRNA I is sufficiently large to encode a 116,000 polypeptide.

The transcriptional pattern of PrV resembles those of HSV-1 and HSV-2, with IE, early and late phases (Feldman et al., 1979). Fractionation of PrV IE mRNAs on denaturing agarose gels revealed two bands of 5.3 and 2.1 kb (F.J. Rixon, personal communication). The 5.3 kb mRNA was mapped to the repetitive DNA regions (Figure 3), and was transcribed towards the ends of those regions, similar to the HSV-1 and HSV-2 4.7 kb mRNAs. Again analogous to the HSV situation, the 2.1 kb RNA band contained three distinct mRNA species mapping in U_L and the junctions of U_S with both repetitive regions (F.J. Rixon, personal communication). The significance of these similarities in the transcription of different herpesviruses is unclear, and further analysis of other virus systems is required to establish whether this pattern is characteristic only of alpha-herpesvirinae.

6. Fine Structures of HSV IE mRNAs

An important aspect of the transcription process is the modification of the primary transcript by splicing. Evidence has been reported suggesting that this step offers a method of regulating

gene expression. The phenomenon of splicing has been described for a number of genes, such as ovalbumin (Breathnach et al., 1978), globin (Jeffreys and Flavell, 1977; Van den Berg et al., 1978) and several immunoglobulins (Honjo et al., 1977; Kataoka et al., 1979; Sukano et al., 1979; Early et al., 1980; Rogers et al., 1980) in addition to the virus genes described in the Introduction.

Both of the 2.0 kb HSV-1 IE mRNAs mapping at the junctions of U_S with TR_S and IR_S contain a single splice located 240 nucleotides from the 5'-end. As with the equivalent HSV-2 IE mRNAs, these HSV-1 mRNAs share common sequences, specified by TR_S and IR_S, at their 5'-ends (Figure 7). The splices, which occur within the repetitive regions, excise 200 nucleotide segments from each mRNA precursor (Watson et al., 1981; F.J. Rixon, personal communication). The HSV-1 mRNAs encode 68,000 and 12,000 molecular weight polypeptides, and the AUG translational start signal for each of these is located within U_S. Hence, the polypeptides share no common tryptic peptides (R.J. Watson, personal communication). The third 2.0 kb HSV-1 IE mRNA which is located entirely within U_L has recently been shown to be unspliced (Figure 4 ; F.J. Rixon, personal communication).

The region of HSV-1 DNA near the junction of U_S with TR_S and IR_S, which specifies the spliced IE mRNAs, is of variable size in different virus isolates (Lonsdale et al., 1979). The sequences which are removed from the mature mRNA by the splicing event contain multiple tandem G + C rich repeats (M.J. Murchie, personal communication). The number of these repeat units is variable.

HSV-2 IE mRNA II has been shown to be 1640 nucleotides in length, and in common with the HSV-1 equivalent mRNA, does not contain a splice. However, there are two caveats to the interpretation of the data concerning the structure of IE mRNA II.

Firstly, nuclease S1 analysis relies on the ability of the gel systems employed to resolve changes in the electrophoretic mobilities of the RNA-DNA hybrid, and a small change due to a very small segment of RNA immediately preceding or following a splice at either the 5'- or 3'-end of the mRNA may not be detected. Similarly, a small RNA segment may be unable to form a stable hybrid with DNA under the conditions used. Secondly, it is possible, though unlikely, that a splice may extend beyond the limits of the DNA fragment used. The Bam HI f fragment extends approximately 600 bp beyond the detected 5'-end of IE mRNA II.

To determine the precise location of the 5'-end of IE mRNA II direct RNA sequencing methods must be used. These involve direct sequencing of capped oligonucleotides, or sequencing cDNA prepared using a known DNA fragment, homologous with sequences adjacent to the 5'-end of the mRNA, as primer. The sequence obtained may then be compared with that of the DNA at the suggested location of the 5'-end. Sequence analysis of the DNA region adjacent to the 5'-end of IE mRNA II is required to show the presence of proposed regulatory promotor sequences.

The structures of the other HSV-1 and HSV-2 IE mRNAs have not yet been accurately determined, however two 3' co-terminal early HSV-1 mRNAs mapping within the Hind III k fragment, and the HSV-1 TK mRNA are unspliced (McKnight, 1980; Anderson et al., 1981; J. McLauchlan and J.B. Clements, personal communication). Thus HSV-1 and probably HSV-2 transcription produce a mixture of both spliced and unspliced mRNAs.

In the DNA virus systems described in the Introduction, splicing generally enhances the coding capacity of a genome region, such as

the SV40 and polyoma late regions, where three polypeptides are encoded as a result of splicing permitting the use of an alternative initiation codon, and also another reading frame (Dhar et al., 1978; Fiers et al., 1978; Reddy et al., 1978a and b; Deininger et al., 1979). Similarily with adenovirus,co-terminal families consisting of several distinct mRNAs, each specifying a different polypeptide may be produced from a single region (Figure 10; Harter and Lewis, 1978; Chow et al., 1979).

The spliced HSV-1 IE mRNAs are both the sole RNA products of the appropriate DNA regions under IE conditions, and each specifies a single polypeptide. In these cases, therefore, splicing in HSV does not increase the genetic diversity of either locus. This resembles the situation observed with several eukaryotic genes rather than those found with other DNA virus systems. HSV does, however, contain co-terminal mRNA families similar to those detected in other virus systems, with each mRNA specifying a unique polypeptide. These co-terminal families may not be the result of differential splicing, and will be discussed later.

Analysis of mutants of SV40 which contain deletions adjacent to the positions at which splices occur in late RNA has shown that while large areas of the intervening and leader sequences are not required for the production of stable mRNAs, the sequences immediately beside the splice junctions must remain intact to ensure efficient processing (Shenk et al., 1976; Hamer et al., 1979; Lai and Khoury, 1979; Subramanian, 1979; Villarreal et al., 1979; Volckaert et al., 1979). Studies on the DNA sequences located at splice junctions have shown a limited number of types of donor and receptor sites. However, it is possible that the specificity of the splicing process, which is particularly important when splices occur within coding

sequences, may reside in higher orders of structure than the sequence of the primary transcript (Breathnach et al., 1978; Zain et al., 1979; Sharp, 1981). The sequences at the splice junctions of HSV-1 conform to the pattern found in several other mRNAs (M.J. Murchie, personal communication).

The precise method by which splicing occurs is unclear, however cleavage of a large primary transcript is the most likely method for virus mRNAs (Evans et al., 1977; Horowitz et al., 1978; Ziff and Evans, 1978; Berget and Sharp, 1979). The primary transcript is capped, and usually, though not always, polyadenylated before splicing (Nevins and Darnell, 1978; Ziff and Evans, 1978; Weber et al., 1980). Small amounts of unspliced HSV-1 mRNAs have been detected (F.J. Rixon, personal communication). In an RNA molecule which contains several splices, the 5'-proximal splice occurs first (Berget and Sharp, 1979).

At least some aspects of the process in virus-infected cells are likely to be of host origin. In undifferentiated mouse teratocarcinoma cells, SV40 early RNA is not processed. However, after the cells have differentiated, normal splicing occurs (Segal and Khoury, 1979). Some aspects, however, are likely to be virus-encoded. Monkey cells are refractory to adenovirus infection, causing the accumulation of unprocessed late RNA. After infection of these cells with Ad-5 ts 125 (which has a mutation in the gene for the 72 K DNA binding protein), late RNA is processed normally, and a lytic infection occurs (Klessig and Grodzicker, 1979). The 72 K polypeptide is probably, therefore, involved in the splicing process.

Several HSV IE polypeptides are DNA binding proteins (Hay, 1979;

Hay and Hay, 1980), and during infection some of these are located predominantly within the nucleus (Preston, 1979). These may therefore be involved in splicing in a way analogous to the adenovirus 72 K polypeptide.

Recently a role for small ribonucleoproteins in splicing has been proposed (Lerner et al., 1980; Roberts, 1980). The RNAs contain in these hybridize with cellular RNAs in the region of the splice junctions, and may serve to align the RNA segments prior to splicing. An equivalent role for the adenovirus VA-RNAs has been suggested (Mathews, 1980). A small RNA, possibly of host origin, has been detected in SV40-infected cells which is homologous with early RNA (Alwine et al., 1980). Similarly, a small virus-induced RNA, possibly of host origin, was detected in HSV-1 infected cells (Talley-Brown and Millette, 1979). No small virus-induced RNA species were detected in HSV-2 IE RNA.

Splicing may, in some cases, be necessary to produce stable mRNA; for example, when all of the intervening sequences of the SV40 late region were removed, without altering the sequences found in the mature mRNA, no polypeptide product was observed (Gruss et al., 1979). However, the RNA polymerase II-transcribed adenovirus polypeptide IX mRNA together with several HSV mRNAs are unspliced (Alestrom et al., 1980; F.J. Rixon, personal communication; J. McLauchlan and J.B. Clements, personal communication; this thesis). Similarly, the mRNAs for the family of related α-interferons, and the single β_1-interferon are unspliced (Nagata et al., 1980; Houghton et al., 1981). Thus splicing is not obligatory for stability of all mRNAs, or for transport into the cytoplasm. However, the small number of unspliced mRNAs analysed has not revealed any common features which may explain the lack of splices, and the significance

of their absence is unclear.

Two unspliced HSV-1 early mRNAs of 5.0 kb and 1.2 kb have 3' co-terminal sequences (Anderson et al., 1981; J. McLauchlan and J.B. Clements, personal communication). These mRNAs specify polypeptides of 136,000 and 38,000 respectively (Anderson et al., 1980). DNA sequence analysis has shown that the genome at the 5'-end of the 1.2 kb mRNA contains sequences similar to the regulatory sequences implicated in initiation of transcription of other eukaryotic genes. Similar sequences were found at the 5'-end of the 5.0 kb mRNA (J. McLauchlan and J.B. Clements, personal communication). The promotor for the 1.2 kb mRNA is therefore located within the DNA sequences which encode the 5.0 kb mRNA. This is unlike the situation usually found with co-terminal families in other virus systems, in which the 3' co-terminal mRNAs generally share a common promotor, and the family of mRNAs is produced by differential splicing. The unspliced adenovirus polypeptide IX mRNA is co-terminal at the 3'-end with the EI b region mRNAs and has a promotor sequence approximately 30 nucleotides upstream from the 5'-end (Figure 10; Alestrom et al., 1980). Initiation of polypeptide IX mRNA transcription is independent of region EI b transcription (Persson et al., 1978; Alestrom et al., 1980). Splicing removes these sequences from the mature EI b mRNAs (Berk and Sharp, 1978b).

7. DNA Sequences at the 5'-Termini of HSV mRNAs

Almost all eukaryotic genes which are transcribed by RNA polymerase II have a very similar short nucleotide sequence approximately 30 nucleotides upstream from the transcription initiation site. This sequence, known as the "Hogness box", is AT rich, and appears to be essential for in vitro transcription of the DNA.

HSV-2 IE mRNA 3

CCCTATTTTGGCAGGAATAAACATATTGCGTCAACCCAGTGGTTTCGT

HSV-1 IE mRNA 4

CCGAAGACGCAATAAACGGCAACAACCTGATTAAGTTTTGCAGTAGCG

SV40 Early

AAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTT

SV40 Late

ACCATTATAAGCTGCAATAAACAAGTTACAACAACAATTGCATTCATT

Ad 2/5 Early Ib

ATAAAAAACCAGACTCTGTTTGGATTT$^{T}_{G}$GATCAAGCAAGTGTCTTGCT

Ad 5 Early Ia

ATGTAAGTTTAATAAAGGGTGAGATAATGTTTAACTTGCATGGCGTGT

chicken ovalbumin

TTCCTTTAATCATAATAAAAACATGTTTAAGCAAACACTTTTCACTTG

mouse β globin major

TCTGACAAATAAAAAGCATTTATCTTCACTGCAATGATGTTTTAAATT

mouse immunoglobulin Y1 chain

TGTATAAATAAAGCACCCAGCACTGCCTTGGGACCCTGCAATAACGTC

HSV-1 TK

CCCGCGCTATGACGGCAATAAAAAGACAGAATAAAACGCACGGGTGTT

FIGURE 52

Comparison of DNA sequences at the 3'-ends of several virus and eukaryote mRNAs. The AAUAAA hexanucleotide is indicated and the position of termination of transcription is shown by an arrow.

A single nucleotide transition in this sequence dramatically reduces the level of *in vitro* transcription of the chicken conalbumin gene (Wasylyk *et al.*, 1980; Wasylyk and Chambon, 1981). The SV40 and polyoma late genes, and the adenovirus early region II do not have a Hogness box upstream of the mRNAs.

Recent evidence suggests that a region of DNA approximately 80 nucleotides upstream of the Hogness box can modulate transcription by RNA polymerase II. Deletion of this region can drastically reduce transcription *in vivo*, with no effect *in vitro* (C. Hentschel, personal communication).

HSV-1 DNA contains both a Hogness box, and sequences related to the upstream regulatory site (CAAT box) at the 5'-end of the TK gene (McKnight, 1980), the 4.7 kb IE mRNA in TR_S/IR_S (M.J. Murchie, personal communication), and the two 3' co-terminal early mRNAs discussed above (J. McLauchlan and J.B. Clements, personal communication). Thus, HSV transcription is likely to be regulated by mechanisms similar to those of other DNA virus and eukaryote genes.

8. DNA Sequences at the 3'-Termini of HSV mRNAs

The DNA sequence of the region containing the 3'-end of HSV-2 IE mRNA II is compared with others of HSV-1 and several other virus and eukaryote genes in Figure 52. The precise location of the point of termination of transcription of IE mRNA II could not be determined due to the presence of two adenine residues in the DNA sequence at this point. It was not possible to determine whether these were present in the RNA as a result of transcriptional or post-transcriptional addition. The presence of at least a single

adenine residue at the polyadenylation site appears to be common for all of the eukaryotic mRNAs analysed.

HSV-2 IE mRNA II contains the hexanucleotide AAUAAA 11 nucleotides from the polyadenylation site. This sequence has been found in almost all eukaryotic and virus mRNAs, adjacent to the polyadenylation site. The mRNAs lacking this sequence are those encoding rat amylase (MacDonald et al., 1980), anglerfish somatostatin (Hobart et al., 1980) and human ∝-interferons C, E and H (Goeddel et al., 1981). These mRNAs contain the related hexanucleotide AUUAAA. Non polyadenylated histone mRNAs do not contain either of these sequences at the 3'-termini.

Although the hexanucleotide AAUAAA, or a related sequence, appears to be required for polyadenylation, it is unlikely to be the only signal for polyadenylation to occur. The major late adenovirus primary transcript spans five possible polyadenylation sites, all of which contain the hexanucleotide. At early times, the first position is preferentially utilized, while at late times, all of the sites are used equally (Nevins and Wilson, 1981). Also, the saccharomyces cerevisiae actin mRNA contains three copies of the hexanucleotide. One of these occurs approximately 1400 nucleotides from the 3'-end of the precursor RNA, and is excised from the mature mRNA, and the other two overlap with each other near the 3'-end of the mRNA (Ng and Abelson, 1980). There are two copies of the hexanucleotide near the 3'-end of HSV-1 TK mRNA, however one of these is not present in the RNA sequences (McKnight, 1980; J. McLauchlan and J.B. Clements, personal communication).

Benoist et al. (1980) described a conserved sequence TTTTCACTGC located after the AATAAA, close to the polyadenylation site of

several, but not all, mRNAs. No related sequence was found in HSV-2 IE mRNA II, however the sequence TTTGCAGTAG is present near the 3'-end of the HSV-1 4.7 kb mRNA (Figure 52). The signal for polyadenylation probably resides at a higher level of structure than the DNA sequences.

The sequence at the 3'-end of HSV-2 IE mRNA II contains two stop codons, UGA at position 1552, and UAA at position 1629. These codons are not in phase with each other, and the UAA forms part of the AAUAAA hexanucleotide sequence. Most mRNAs contain coding sequences which are terminated considerably upstream from the polyadenylation site. The UGA termination codon is therefore the most likely one to be utilized, however the coding sequences within IE mRNA II may be located outside the region sequenced. The sequence of a potential polypeptide is shown in Figure 51. This putative polypeptide is very hydrophobic at the 3'-end. IE mRNA II probably encodes the HSV-2 Vmw 65 polypeptide (Preston et al., 1978).

The control of HSV expression appears to be regulated by mechanisms similar to those observed in other virus and eukaryote systems. Unique problems are also posed due to the structure of the HSV genome, and the presence of genes which map entirely or partly within the repetitive regions. Further study of the structures of primary RNA transcripts and DNA sequence may provide further understanding of the transcription process. This would be assisted by genetic and intertypic recombinant analysis to locate on the genome additional control elements and polypeptide products.

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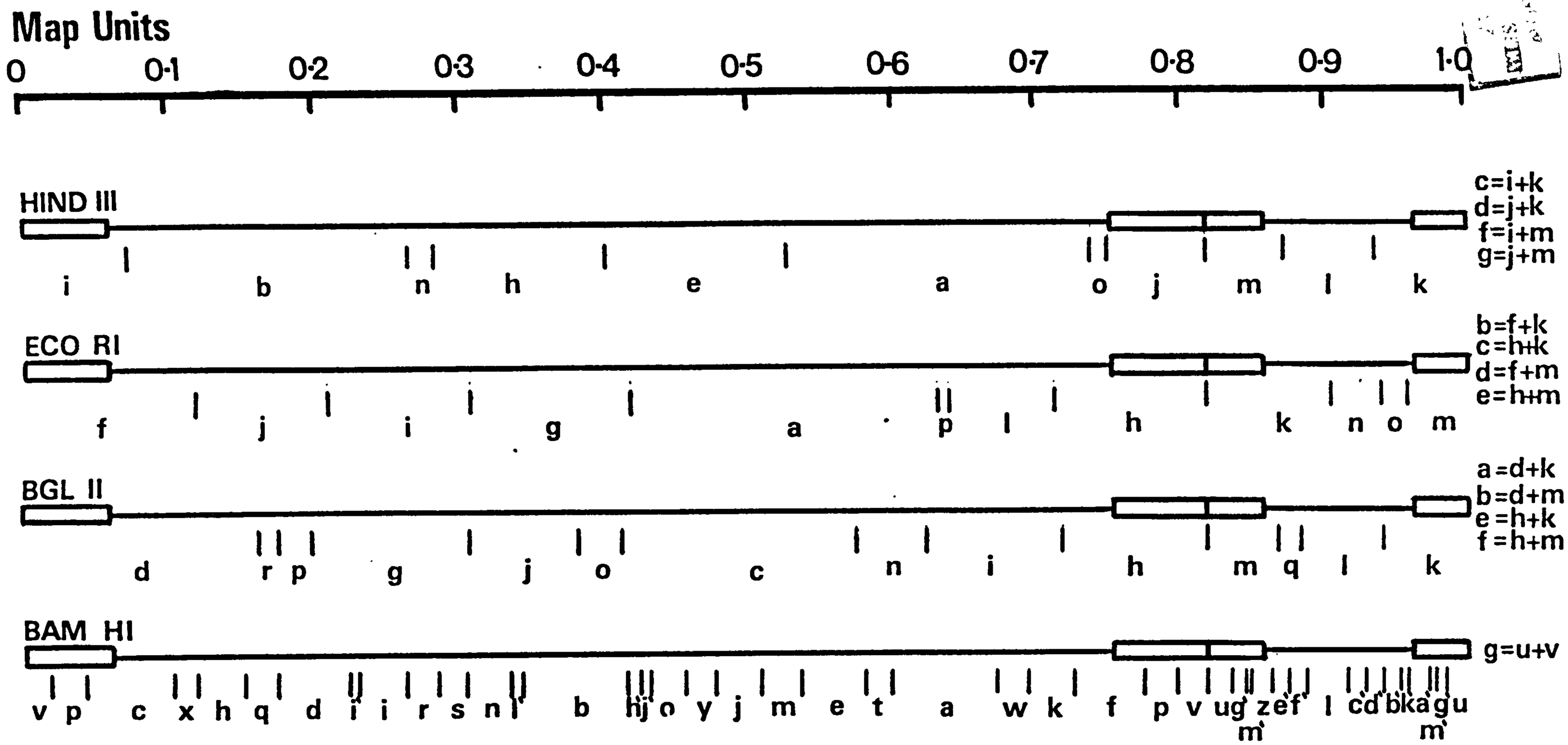

FIGURE 20

Physical maps for the fragments of HSV-2 DNA generated by restriction endonucleases Hind III, Eco RI, Bgl II and Bam HI (Cortini and Wilkie, 1978; Wilkie et al., 1979). These maps are shown for the prototype orientation of both unique regions. Fragments which span the joint region are represented as fusion fragments of the terminal fragments generated on inversion.
A copy of this figure is provided as a supplement at the back of this thesis.

Title: Immediate-early transcription of herpes simplex virus.

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To appear in "Herpesvirus DNA: Recent studies on the Internal Organisation and Replication of the Viral Genome" (Y. Becker, ed.), Martius Nijhoff, B.V., The Hague (1980). in press

THE IMMEDIATE-EARLY REPLICATION PHASE

Infection of permissive cells with HSV in the presence of protein synthesis inhibitors (1, 2) or infection at non-permissive temperature with certain temperature-sensitive mutants (3) results in the accumulation of a restricted set of virus mRNAs which map within certain regions of the virus genome only (1, 4). This restricted set of RNA sequences is transcribed by a pre-existing α-amanitin-sensitive RNA polymerase (5) and has been termed immediate-early (IE) RNA. We have shown for HSV-1 that progression from the IE to the early stage, where much more of the genome is transcribed, (1, 6) requires the continuous function of at least one IE polypeptide (7).

The polypeptides specified by IE mRNA form a sub-set of the virus polypeptides made during productive infection (8, 9). These polypeptides are phosphorylated, preferentially accumulate in the nucleus (10) and have DNA binding properties in vitro.

Hence, study of the IE replication phase and of the switch involved in the transition to the early phase has the potential to provide information on eukaryotic gene transcription by RNA polymerase II, and on how virus-coded polypeptides may act to modify this transcription.

Here we report on the properties of HSV-1 and HSV-2 IE mRNA's. We also describe our experiments with end-labelled DNA probes designed to elucidate the fine structures of several IE mRNA's and present DNA sequence data for the 3'-end of one IE mRNA.

GENOME LOCATIONS, ORIENTATIONS, SIZES AND POLYPEPTIDES ENCODED BY IE mRNA's

Blot hybridizations (1, 11, 12, 13) of *in vivo* and *in vitro* labelled IE RNA to virus DNA fragments generated with various restriction endonucleases, together with a similar analysis of labelled cDNA specific to 3'-ends of mRNA's, has allowed the genome locations and orientations of 6 major IE mRNA's to be determined (Figure 1 and Table 1). IE mRNA orientations have been further confirmed using specific 5'- and 3'-end-labelled DNA probes (see below).

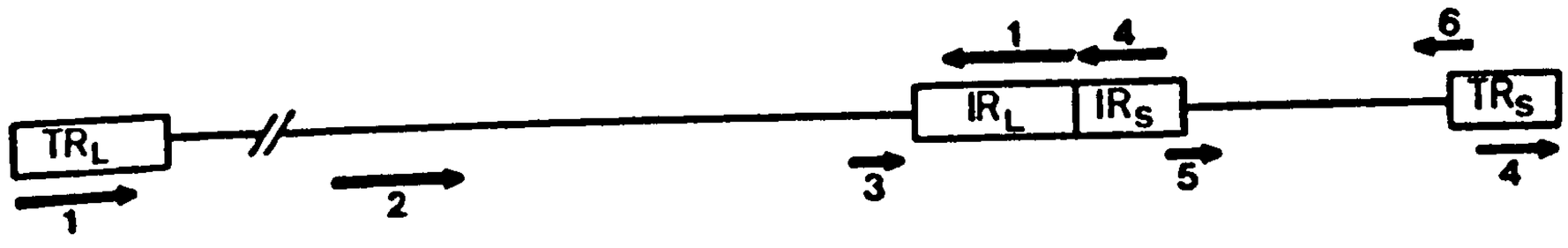


Figure 1. Genome locations and orientations of the major HSV IE mRNAs on the prototype orientation of the virus DNA.

Polyadenylated IE mRNA's were separated by electrophoresis in CH_3HgOH denaturing gels into 3 main virus-specific bands and the genome locations of the different size classes have been determined (13). The polypeptides encoded by IE mRNA's were determined directly by *in vitro* translation of the various size classes (13) and by analyses of polypeptides expressed by intertypic recombinants between HSV-1 and HSV-2 (9).

The major IE mRNA's map primarily in or adjacent to both sets of repetitive DNA regions which flank the two unique DNA regions, and the orientations indicate that there are at least two distinct IE promoters. HSV-1 and HSV-2 IE mRNA's were separated on CH_3HgOH gels to give three bands. The HSV-2 bands were similar but not identical in size to those found with HSV-1 and these size differences may be related to differences in size of the equivalent HSV-1 and HSV-2 IE polypeptides. In contrast to the other IE mRNA's, the IE mRNA 6 is much larger than required for its coding sequences.

Table 1. Genome map locations, sizes and the polypeptides specified by the major HSV-1 and HSV-2 IE mRNA's.

HSV-1 IE mRNA SPECIES	SIZE (kb)	GENOME LOCATIONS	POLYPEPTIDE SPECIFIED ($MW \times 10^{-3}$)
1	3.0	0.00 - 0.04 (TR_L) 0.79 - 0.83 (IR_L)	110
2	5.1	0.54 - 0.59 (U_L)	136
3	2.0	0.74 - 0.76 (U_L)	63
4	4.7	0.83 - 0.87 (IR_S) 0.96 - 1.00 (TR_S)	175
5	2.0	0.87 - 0.90 (IR_S/U_S)	68
6	2.0	0.94 - 0.96 (TR_S/U_S)	12
HSV-2 IE mRNA SPECIES			
1	3.4	0.00 - 0.04 (TR_L) 0.77 - 0.82 (IR_L)	118
2	-	0.54 - 0.60 (U_L)	142
3	1.75	0.73 - 0.74 (U_L)	65
4	4.7	0.82 - 0.86 (IR_S) 0.96 - 1.00 (TR_S)	182
5	1.75	0.85 - 0.88 (IR_S/U_S)	-
6	1.75	0.94 - 0.97 (TR_S/U_S)	12.3

HSV-1 IE mRNA's mapped to genome regions equivalent to those of the respective HSV-1 mRNA's of a similar size, and the data indicates a close similarity in the IE transcription phase between HSV-1 and HSV-2. This is not surprising as at least some HSV-1 and HSV-2 gene products are functionally interchangeable (14) and genetic exchanges have been demonstrated by the production of intertypic recombinants (9, 15, 16, 17). Analysis of intertypic recombinants has demonstrated the colinearity of equivalent genes on the two DNAs (18).

Our studies provide no evidence to support the claim,

based on liquid hybridization studies, by Frenkel et al (19), of a radically different transcriptional programme for HSV-2 as compared to HSV-1. The blot hybridization studies showed no major differences in the hybridization patterns of IE nuclear and cytoplasmic RNA's. This is inconsistent with liquid hybridization data which indicated that 50% (2) to 30% (4) of the HSV-1 genome was represented in IE nuclear RNA whereas only 13% was represented in the cytoplasm (4); 45% of the genome was represented in total HSV-2 IE mRNA (19). Our current knowledge of the genome map locations, sizes and polypeptides specified by both HSV-1 (9, 11, 13) and HSV-2 IE mRNAs (12), together with the molecular weights of those IE polypeptides shown to be unrelated due to breakdown (D. MacDonald and H.S. Marsden, personal communication), indicates that at least 20% of the HSV genome must be represented in the cytoplasm at the immediate-early stage.

No additional IE nuclear sequences were detected with DNAase-treated RNA samples which were not fractionated on Cs_2SO_4 gradients prior to blotting, or with cytoplasmic IE RNA labelled <u>in vitro</u> (12). These controls therefore demonstrate that the restricted hybridization patterns of both nuclear and cytoplasmic IE RNA are not due to artefacts introduced by the methods of RNA isolation or purification.

FINE STRUCTURE ANALYSIS OF HSV IE mRNA's

The rationale of RNA structure analysis using 5'-and 3'-end-labelled DNA probes (20) is shown in Figure 3 for a RNA with a single splice (encoded by one strand of a linear duplex DNA). The procedure uses restriction endonuclease cleaved fragments of HSV-1 and HSV-2 DNA's cloned under UK Category II containment using pAT 153 as the cloning vector and <u>E.coli</u> K12 HB101 as the host bacterium. DNA fragments were 5'-^{32}P-end-labelled using T4 polynucleotide kinase and γ-^{32}P ATP, after dephosphorylation with alkaline phosphatase (20). Fragments containing recessed 3'-ends were 3'-end-labelled using T4 DNA polymerase and one or more α-^{32}P deoxynucleoside triphosphates (21) to fill out the restriction site. The end-labelled probes were normally cleaved with a

single cut restriction enzyme and the two fragments each containing a single labelled end were used in hybridization.

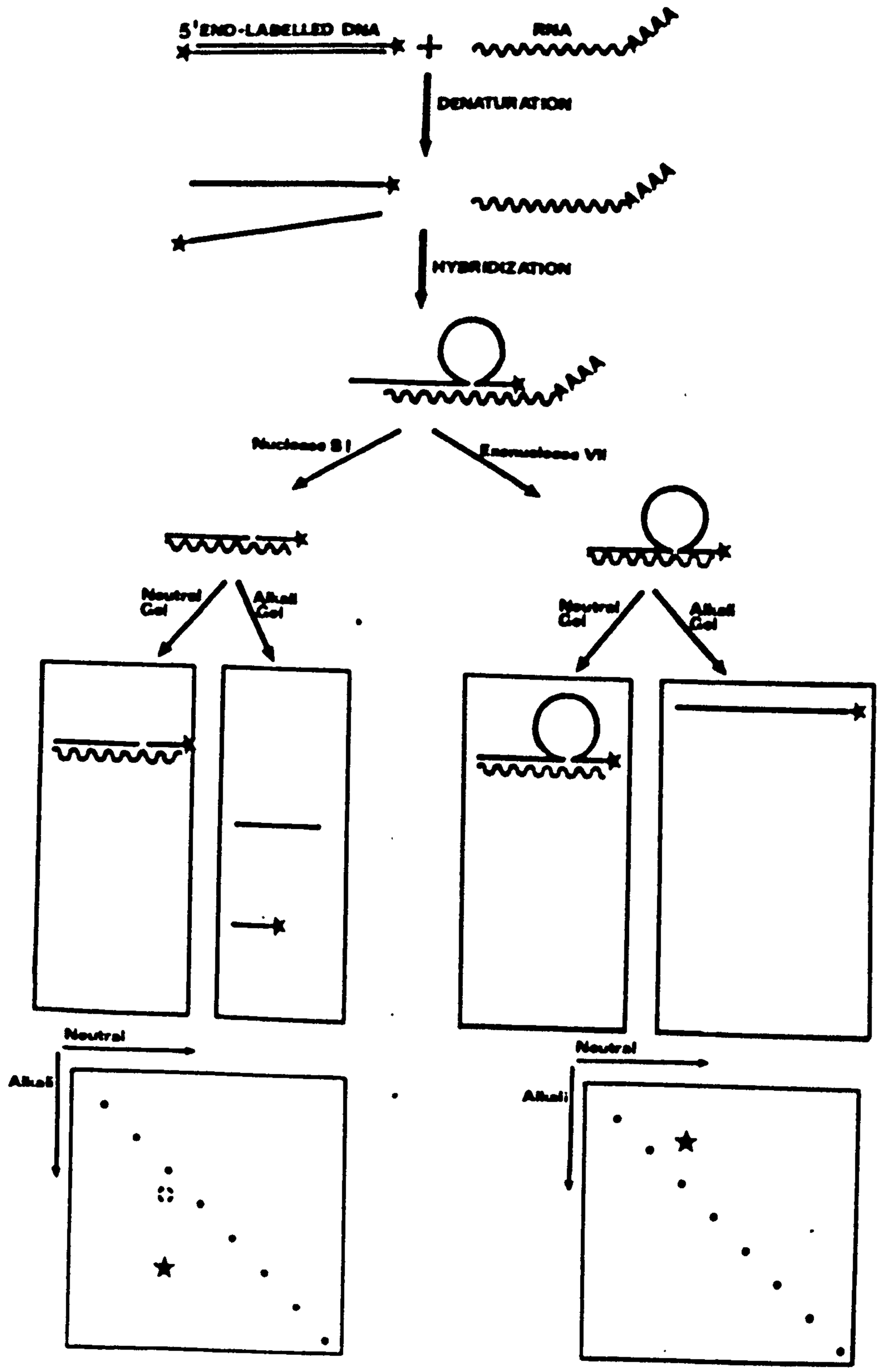


Figure 2. Rationale of RNA structure analysis using a 5'-end-labelled DNA probe to analyse the structure of an mRNA with a single splice.

Excess of end-labelled DNA probes were mixed with infected cell cytoplasmic IE RNA preparations in a solvent containing 90% formamide. After heating to denature the

DNA, hybridizations were performed at temperatures favouring DNA-RNA hybridization and minimising DNA reassociation (22). After hybridization, the non-homologous RNA and the non-hybridized DNA was removed with nuclease S1. The S1-resistant DNA-RNA hybrid consists of a continuous RNA strand with a complementary DNA strand having a specific nick at the site where the loop was excised; in a small proportion of molecules, S1 will also cut through the RNA opposite the nick to form two hybrid molecules, only one of which will be end-labelled.

As a consequence of the polarity of the RNA and DNA strands in a hybrid, the orientation of the RNA with respect to the physical map of the genome can be determined. A 5'-end-labelled DNA probe will only form a S1-resistant labelled hybrid with sequences at the 5'-end of the RNA while a 3'-end-labelled probe will hybridize only with 3'-end sequences of the RNA.

S1-resistant hybrids were electrophoresed in neutral and in alkaline agarose gels. Using end-labelled DNA probes, in both types of gels, only a single band is labelled (Figure 2). If the size of the labelled DNA in alkali gels differs from the value obtained with neutral gels, this indicates that the RNA is spliced. With end-labelled probes on alkaline gels, the size of the labelled DNA fragment determines the distance from the point of labelling to the end of an unspliced RNA, or to the position of the first splice point.

DNA-RNA hybrids were further fractionated using a two-dimensional procedure (23) with electrophoresis in a neutral buffer in the first dimension, and in alkaline buffer in the second dimension. The continuous DNA strands generated by unspliced RNAs will have the same relative mobility in both dimensions and will fall along a diagonal line. A single-spliced RNA will be resolved by the alkaline dimension into two DNA species only one of which will be end-labelled, with both spots vertically aligned below the diagonal. A vertical line through these spots bisects the diagonal at a point equivalent to the sum of the lengths of the component DNA single strands. With the 5'-end-

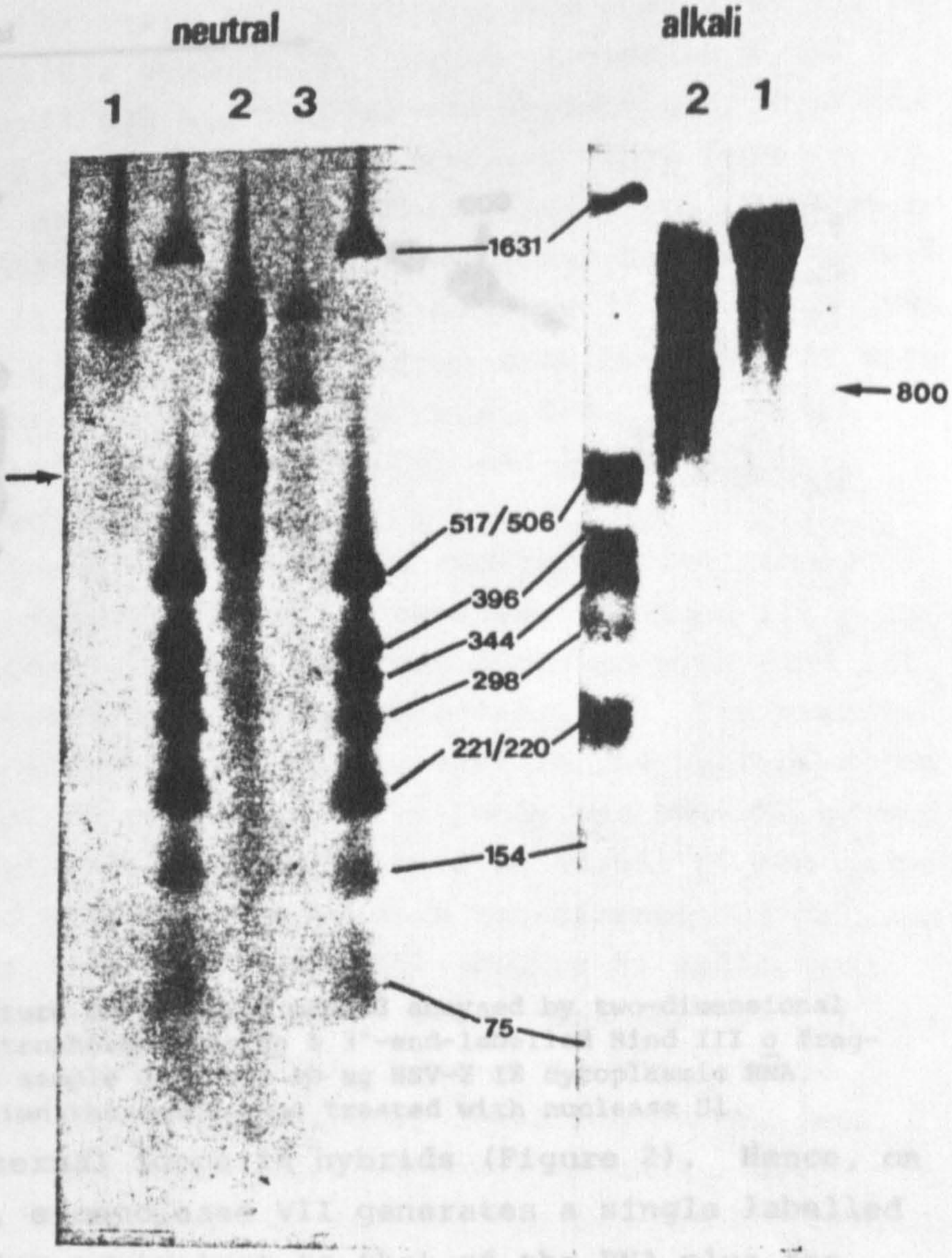


Figure 3. Structure of HSV-2 IE mRNA 3 analysed on neutral and alkaline gels using a 3'-end-labelled Hing III o fragment probe. Sample 1 is the unhybridized fragment probe. Sample 2 is the fragment probe hybridized to 10 µg HSV-2 IE cytoplasmic RNA. Sample 3 is the fragment probe hybridized to 10 µg yeast RNA. Samples were treated with nuclease S1.

labelled DNA probe, the spliced RNA generates a single spot corresponding to the distance from the labelled site to the splice point.

The S1 analyses demonstrate the presence of splices but do not provide direct information on their size. Exonuclease VII (24, 25) can be used instead of S1 to digest hybrids. This enzyme degrades single-stranded DNA but does

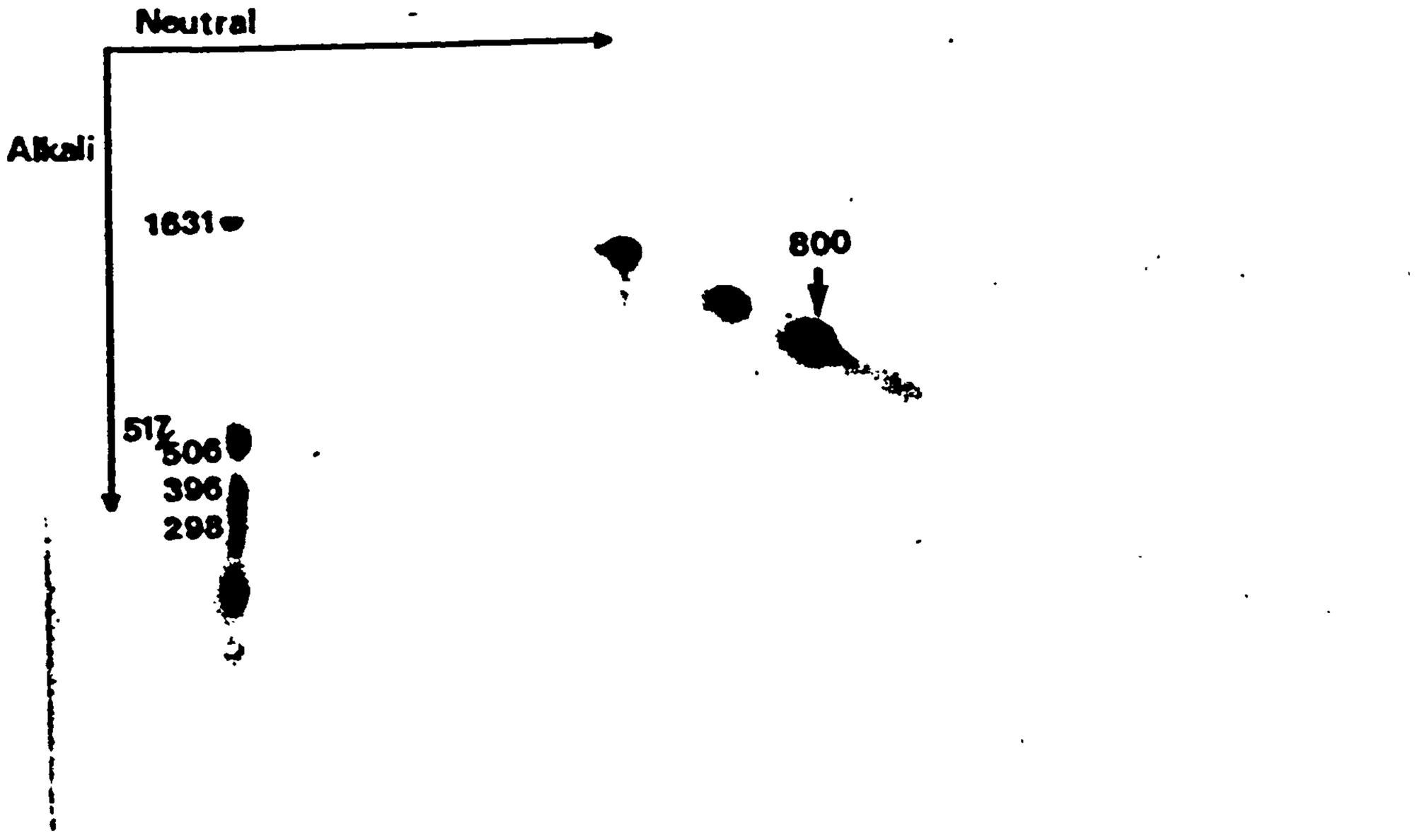


Figure 4. Structure of HSV-2 IE mRNA 3 anaysed by two-dimensional agarose gel electrophoresis using a 3'-end-labelled Hind III o fragment probe. The sample used was 10 µg HSV-2 IE cytoplasmic RNA. After hybridization the sample was treated with nuclease S1.

not cleave internal loops in hybrids (Figure 2). Hence, on alkaline gels, exonuclease VII generates a single labelled band with a size equivalent to that of the RNA plus the spliced out DNA sequences. On two-dimensional gels the end-labelled DNA spot should migrate above the diagonal and the splice length can be estimated.

HSV-2 IE mRNA 3

IE mRNA 3 lies entirely within the U_L region, and has its 3'-end in the Hind III o fragment (12, Figure 7). To analyse the structure of this message, the cloned Hind III o fragment was 3'-end-labelled then cleaved with Pvu II (Figure 7) and the two labelled fragments isolated. These fragments were hybridized individually with HSV-2 IE cytoplasmic RNA and with yeast RNA.

Only the larger fragment directed formation of a DNA-

RNA hybrid, and under the hybridization conditions used two DNA reassociation products were seen with both the IE RNA and yeast RNA control IE (Figure 3, samples 2 and 3). A third band of 800 nucleotides was present only with the infected cell RNA (Figure 3, sample 2). This band was of similar size on neutral and alkaline gels, and furthermore lay on the diagonal after two-dimensional gel electrophoresis (Figure 4). This indicated that the 3'-end of IE mRNA 3 was unspliced for 800 nucleotides from the Hind III site as indicated in Figure 7. This data also confirms the orientation and location of the 3' end of IE mRNA 3 as determined previously (12).

The 5'-end of the RNA was analysed using cloned HSV-2 Bam HI fragment f, which contains the Hind III o fragment (Figure 7). Bam HI f was digested with Hind III, and the resultant ends were 5'-end-labelled. The mixture of 5'-end-labelled fragments was used in the hybridization procedure, and after S1 treatment, only one DNA-RNA hybrid was seen (Figure 5) with a DNA size in alkali of 840 nucleotides. After electrophoresis on a two-dimensional gel, the radioactivity lay on the diagonal (Figure 5) indicating that the RNA was not spliced at the 5'-end for 840 nucleotides from the Hind III site. Since both the 5'- and 3'-ends of this mRNA were studied by labelling at the same Hind III site, the total size of HSV-2 IE mRNA 3 would appear to be the sum of the size of the two DNA-RNA hybrids, 1640 nucleotides (Figure 7). An approximate estimate of the size of polyadenylated IE mRNA 3 on CH_3HgOH gels was 1750 nucleotides.

Nucleotide sequence at the 3'-end of HSV-2 IE mRNA 3

The DNA sequence at the 3'-end of IE mRNA 3 was determined using cloned Hind III o. The intact plasmid containing Hind III o was digested with Xma I which cleaves once within the insert (Figure 7). The two free ends were then 3'-end-labelled using α^{32}P-dCTP. The labelled DNA was cleaved with Hind III and the larger labelled fragment isolated by gel electrophoresis. This DNA fragment was sequenced by the method of Maxam and Gilbert (26), and the S1-resistant

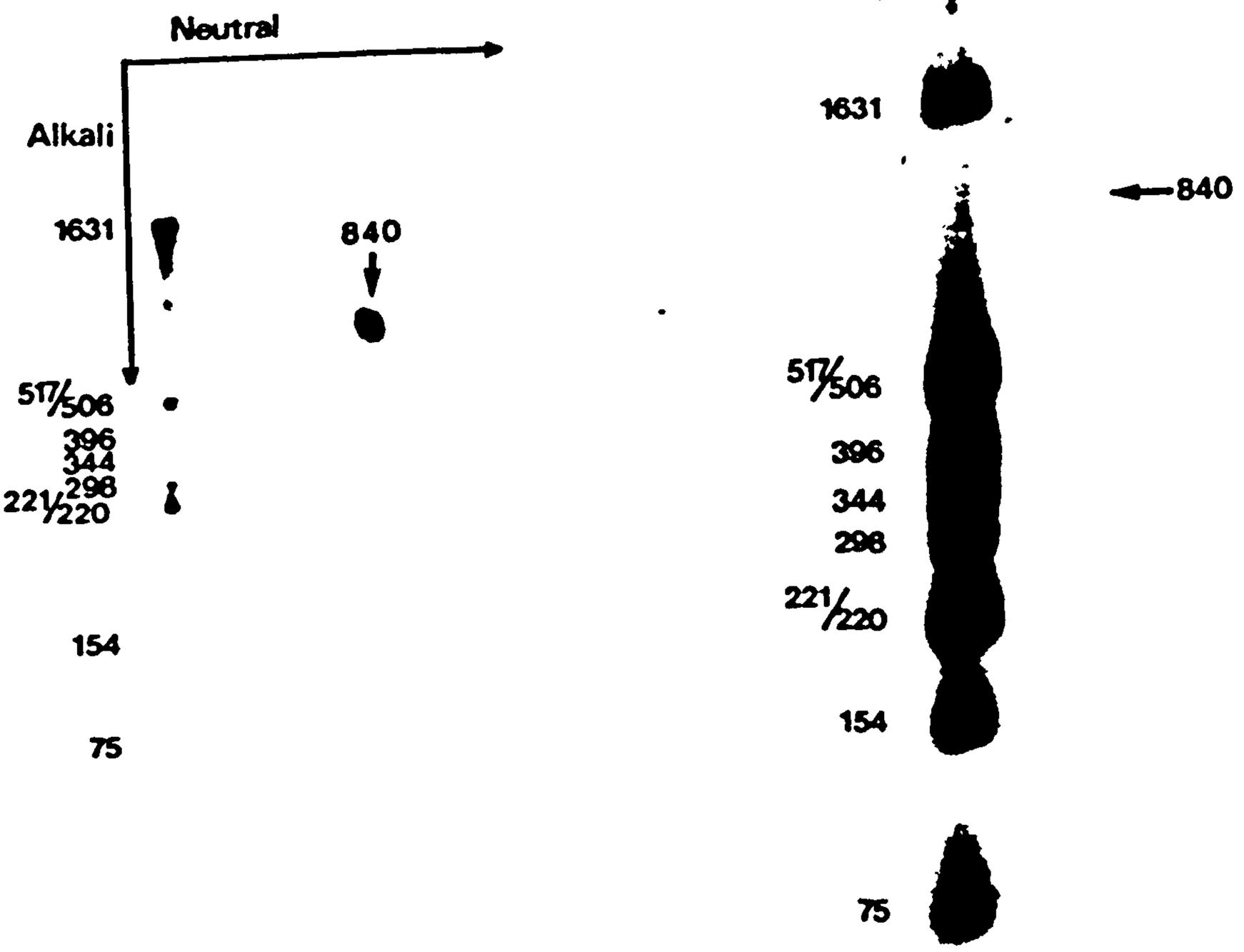


Figure 5. Structure of HSV-2 IE mRNA 3 analysed by two-dimensional and alkaline gel electrophoresis using a 5'-end-labelled Hind III digested Bam HI f fragment probe. The sample was 10µg HSV-2 IE cytoplasmic RNA. Following hybridization the sample was treated with nuclease S1.

DNA-RNA hybrid generated by this fragment was co-electrophoresed on the sequencing gels (Figure 6). This indicated the point at which transcription of IE mRNA 3 terminated on the DNA. The DNA sequence determined was of the strand complementary to the RNA. The sequence given in Figure 7 is that of the DNA strand with the same sequence as the RNA.

The location(s) of the 3'-end shown by the arrows in Figure 7 is not precisely defined as the sequence at which the DNA-RNA hybrid finishes contains two A residues. It is unclear if these are transcriptional or post-transcriptional additions.

The RNA contains the sequence 5'-AAUAAA-3' at 10 to 13 nucleotides upstream from the poly A tail. This sequence

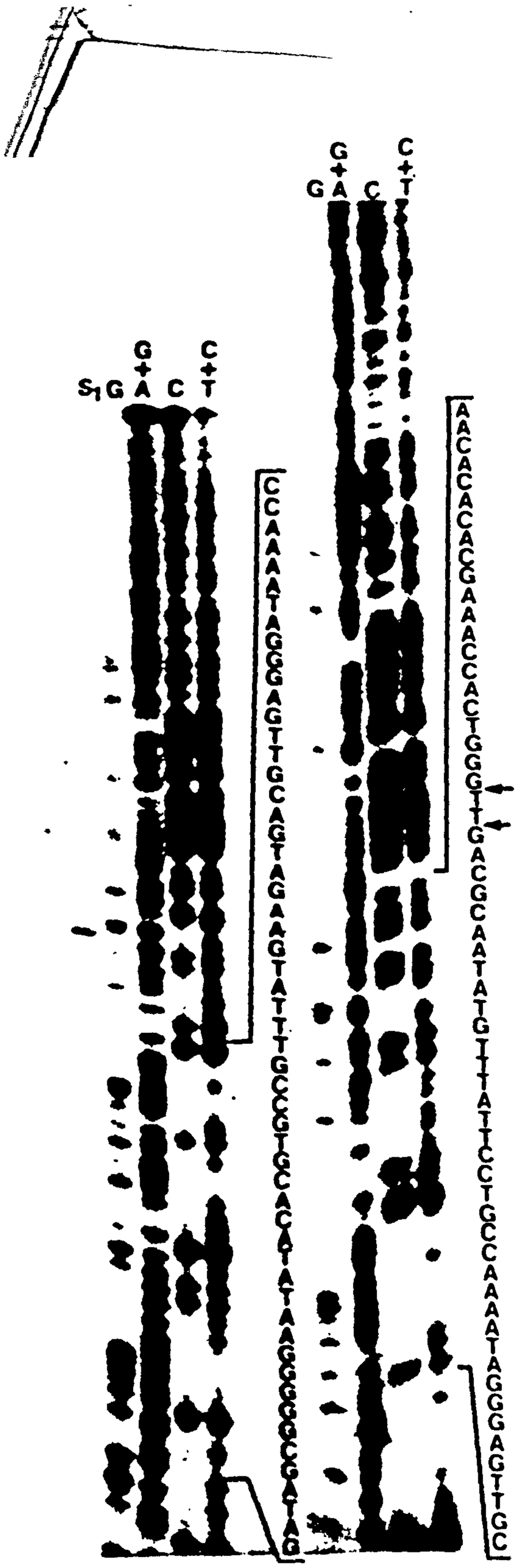
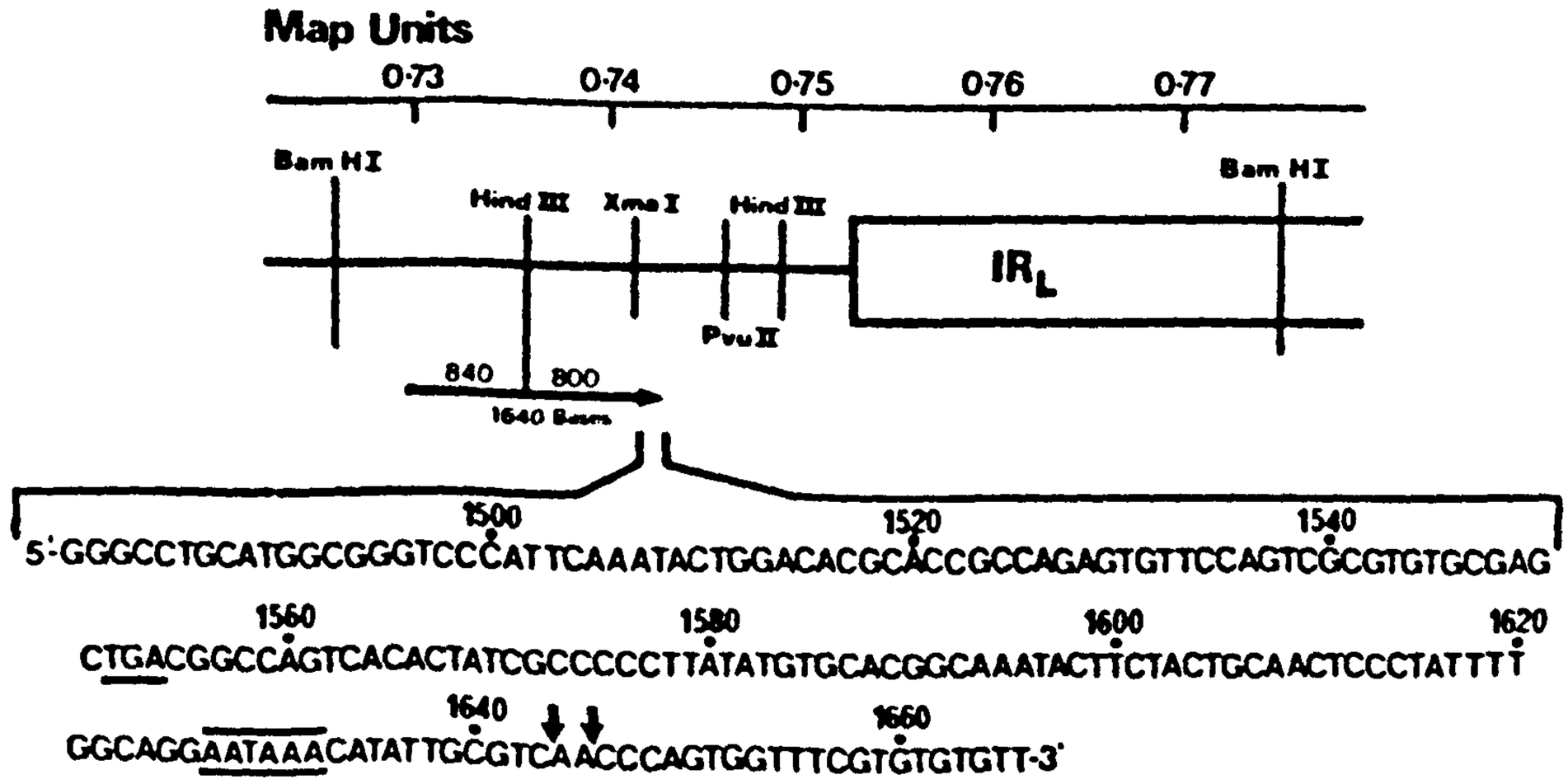


Figure 6. Autoradiogram of sequencing gels showing 3'-end of HSV-2 IE mRNA 3. The lanes are labelled according to the nomenclature of Maxam and Gilbert The 3'-end of the mRNA is determined by co-electrophoresing the nuclease S1-resistant DNA-RNA hybrid produced by hybridizing the 3'-end-labelled fragment probe to be sequenced to 10 µg of HSV-2 IE cytoplasmic RNA. The region in which the RNA terminates is indicated by arrows.

Figure 7. Summary showing the genome map location, size and 3'-end sequence of HSV-2 IE mRNA 3. Arrows indicate the 3'-end of the mRNA.



is present at the 3'-end of all known polyadenylated eukaryotic mRNA's and may serve as a signal for addition of the poly A (27).

Apart from the UAA stop codon present in the AAUAAA sequence, there is only one other stop codon (UGA) in the sequenced region which is not in phase with the UAA stop. The sequences at the 3'-end of IE mRNA 3 therefore appear to conform to those found at the 3'-end of other eukaryotic mRNA's.

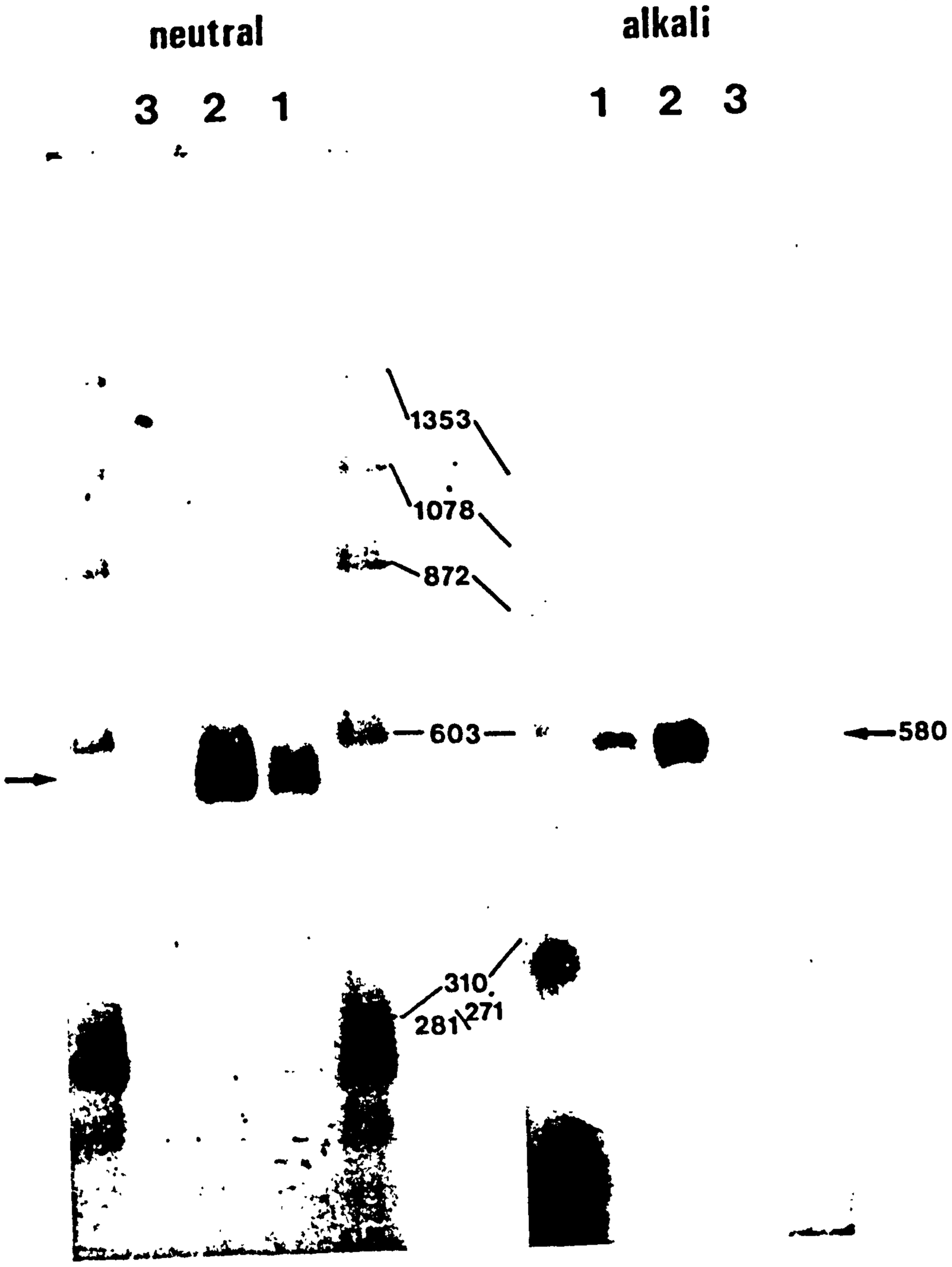


Figure 8. Structure of HSV-1 IE mRNA 6 analysed on neutral and alkaline gels using a 3'-end-labelled fragment probe labelled at Bam HI site a (Figure 12). Sample 1 is the fragment probe hybridized to 5 µg HSV-1 IE cytoplasmic RNA. Sample 2 is the fragment probe hybridized to 15 µg HSV-1 IE cytoplasmic RNA. Sample 3 is the fragment probe hybridized to 15 µg yeast RNA. Following hybridization the samples were treated with nuclease S1.

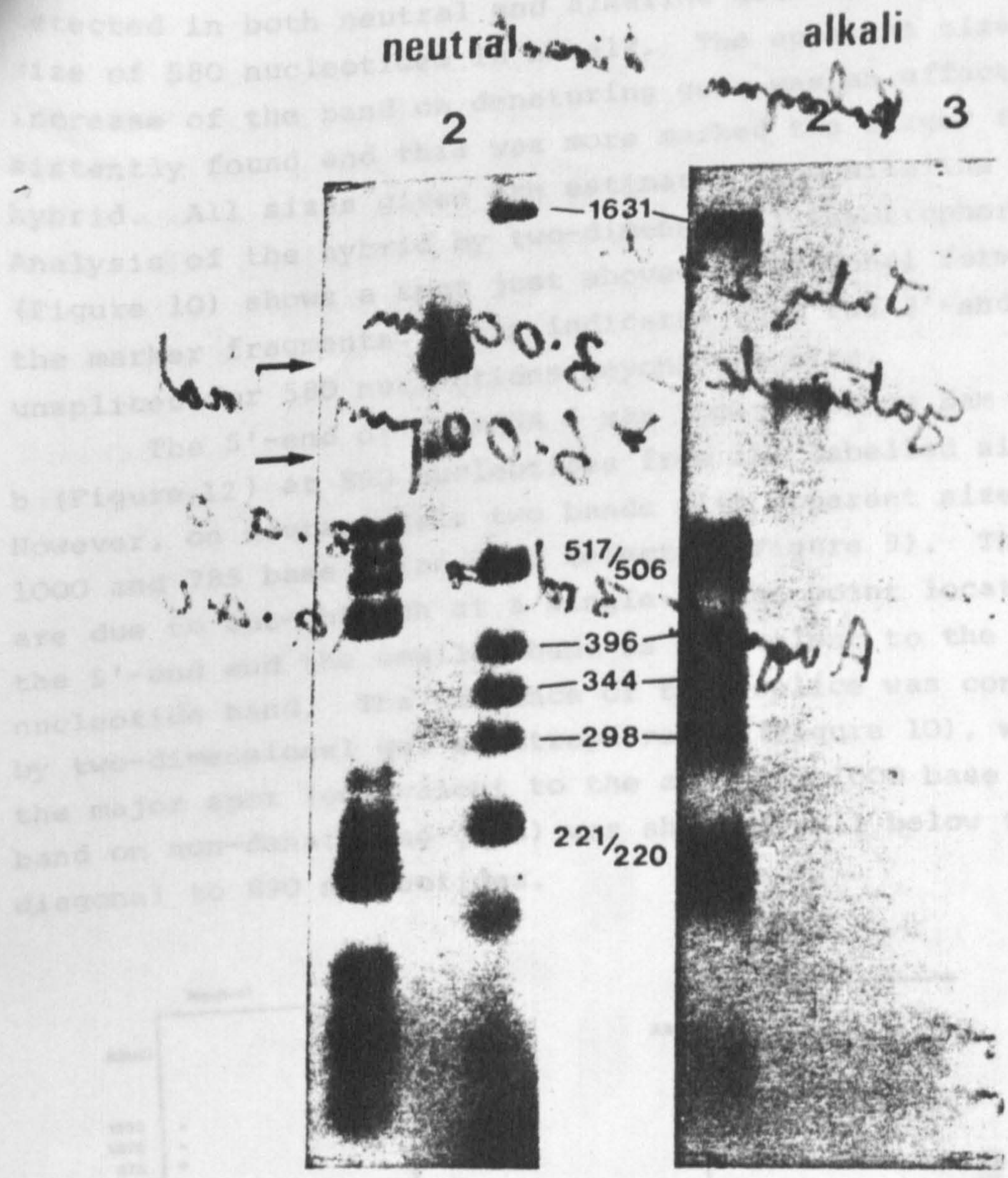


Figure 9. Structure of HSV-1 mRNA 6 analysed on neutral and alkaline gels using a 5'-end-labelled fragment probe labelled at Bam HI site b (Figure 12). Sample 1 is the fragment probe hybridized to 5 µg HSV-1 IE cytoplasmic RNA. Sample 2 is the fragment probe hybridized to 15 µg HSV-1 IE cytoplasmic RNA. Sample 3 is the fragment probe hybridized to 15 µg yeast RNA. Following hybridization, samples were treated with nuclease S1.

HSV-1 IE mRNA's 5 and 6

These mRNA's span the two junctions between IR_S and TR_S with U_S (Figure 1), and 5'-ends of both lie within IR_S and TR_S indicating that these mRNA's have common 5' sequences. Figure 8 shows S1 end-label protection analysis with a 3'-end-label at Bam HI site (Figure 12). A single band was

detected in both neutral and alkaline gels and this had a size of 580 nucleotides in alkali. The apparent size increase of the band on denaturing gels was an effect consistently found and this was more marked the larger the hybrid. All sizes given are estimated from alkaline gels. Analysis of the hybrid by two-dimensional electrophoresis (Figure 10) shows a spot just above the diagonal formed by the marker fragments. This indicates that the 3'-end is unspliced for 580 nucleotides beyond the site.

The 5'-end of IE mRNA 6 was located using Bam HI site b (Figure 12) at 890 nucleotides from the labelled site. However, on neutral gels two bands with apparent sizes of 1000 and 785 base pairs were detected (Figure 9). These are due to cut-through at a single splice point located near the 5'-end and the smaller band is equivalent to the 890 nucleotide band. The presence of this splice was confirmed by two-dimensional gel electrophoresis (Figure 10), where the major spot (equivalent to the apparent 1000 base pair band on non-denaturing gels) was shifted well below the diagonal to 890 nucleotides.

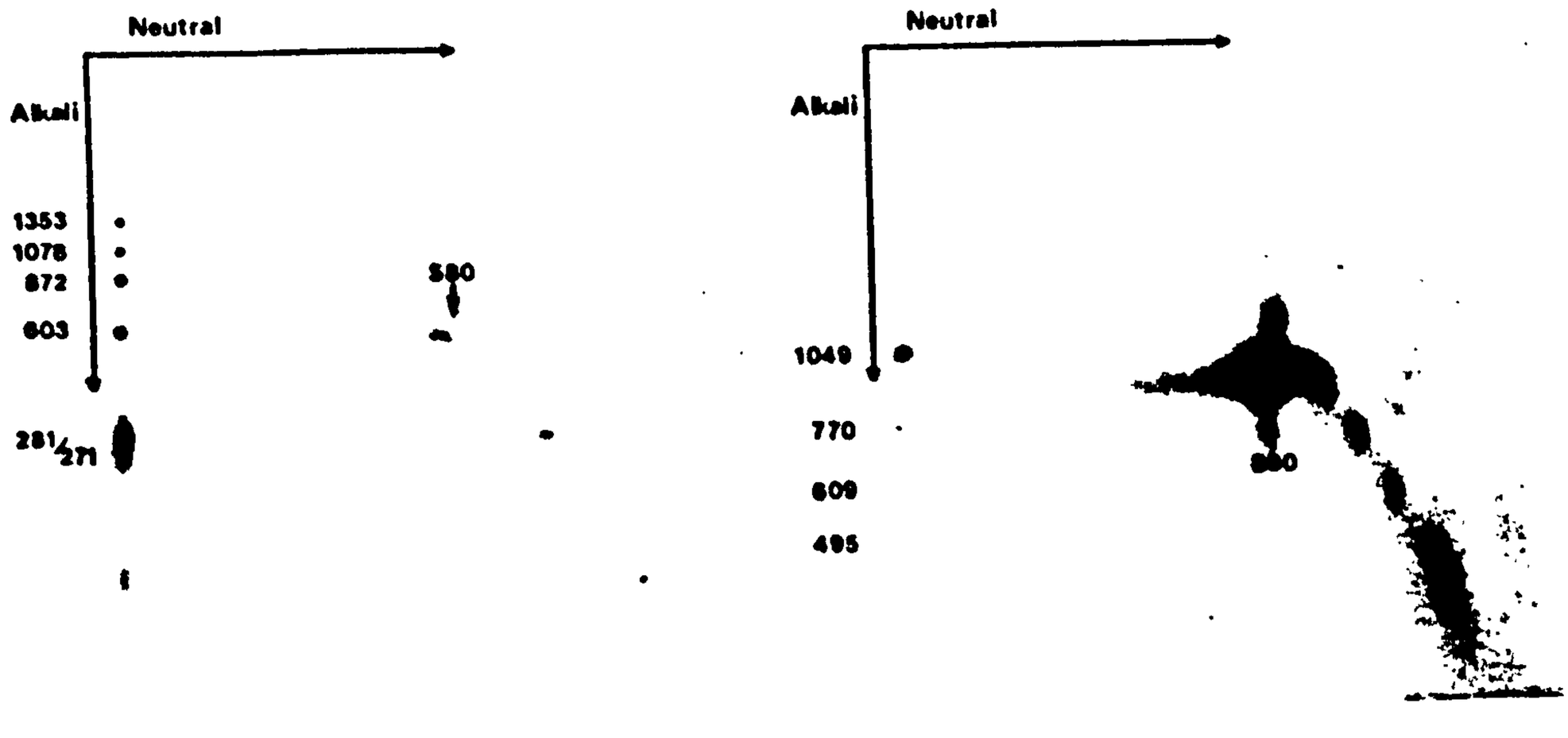


Figure 10. Structure of HSV-1 IE mRNA 6 analysed by two-dimensional gel electrophoresis using 3'-end 5'-end-labelled fragment probes. The fragment probes were 3'-end-labelled at Bam HI site a and 5'-end labelled at Bam HI site b (Figure 12). The fragment probes were hybridized to 15 µg HSV-1 IE cytoplasmic RNA. Following hybridization the samples were treated with nuclease S1.

To determine the size of the splice, the DNA-RNA hybrid formed with a 5'-label at the Bam HI site was treated with exonuclease VII and a labelled band of 1330 nucleotides was detected on alkaline gels as compared to the 890 nucleotide band generated with S1 (Figure 11). The 240 nucleotide stretch from the 5'-end to the splice point was estimated from the cut-through sizes and included a correction for the larger sizes obtained from alkaline gels. Subtraction of 890 plus 240 from 1330 gives a splice of 200 nucleotides.

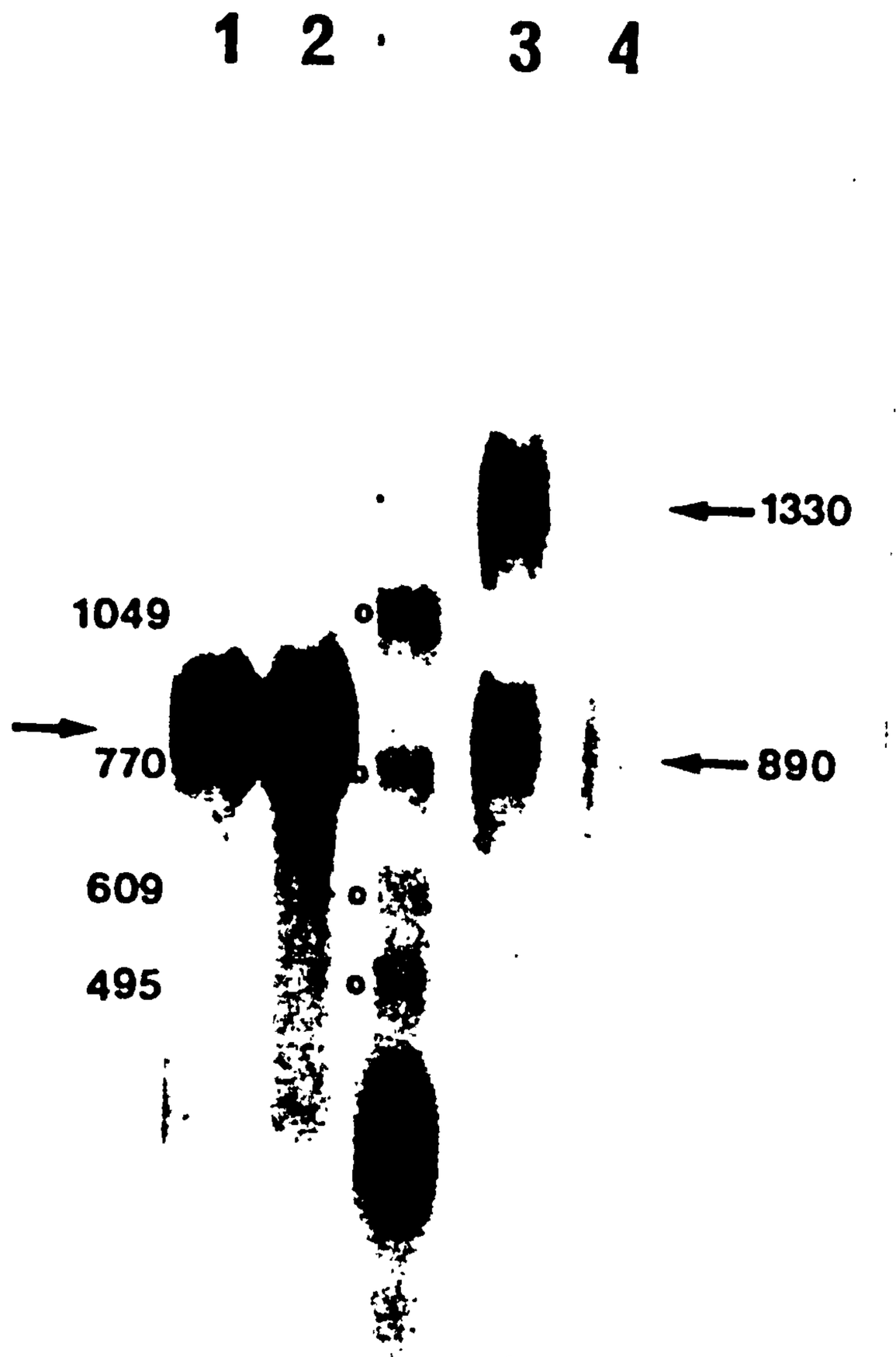


Figure 11. Structure of HSV-1 IE mRNA 6 analysed by electrophoresis on a neutral gel using a 5'-end-labelled fragment probe labelled at Bam HI site b (Figure 12). Sample 1 is the fragment probe hybridized to 5 µg HSV-1 IE cytoplasmic RNA followed by digestion with nuclease S1. Sample 2 is the fragment probe hybridized to 15 µg HSV-1 IE cytoplasmic RNA followed by digestion with nuclease S1. Sample 3 is the fragment probe hybridised to 15 µg HSV-1 IE cytoplasmic RNA followed by digestion with exonuclease VII. Sample 4 is the fragment probe hybridised to 15 µg yeast RNA followed by digestion with exo. VII.

Similarly, the structure of IE mRNA 5 was examined using 5'- and 3'-end-labelled DNA at the Hind III site in Bam HI n. These results are summarized in Figure 12 and show that IE mRNA 5 has a similar splice in IR_S to that of IE mRNA 6 in TR_S. The main body of IE mRNA 5 is approximately 1525 nucleotides, some 100 nucleotides smaller than the body of IE mRNA 6.

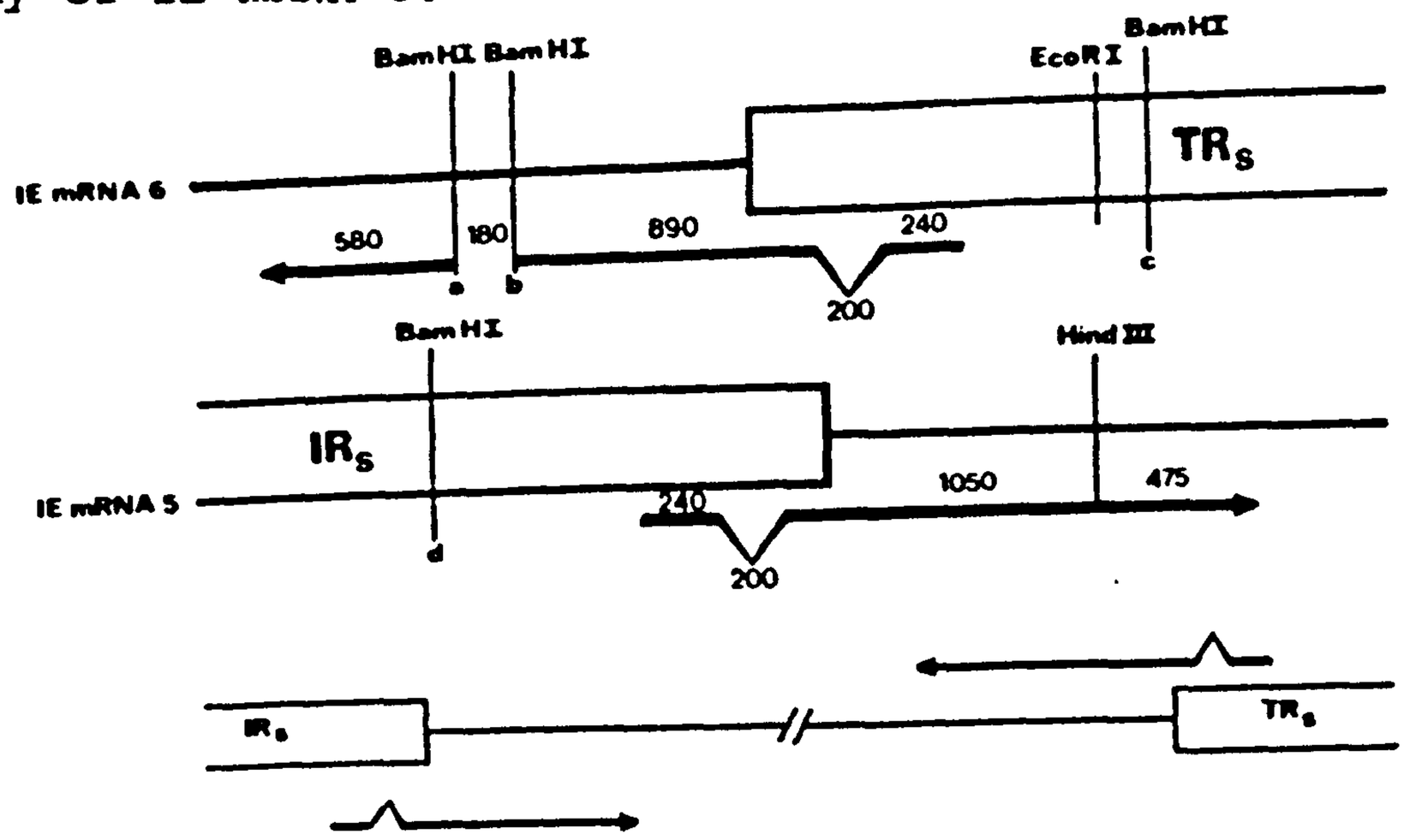


Figure 12. Summary showing genome locations, sizes, and structures of HSV-1 IE mRNAs 5 and 6.

Our data on the structures of these two mRNAs directly confirms similar structures recently described by Watson (28) who showed that both had identical splices at their 5'-ends. This data showed that IE mRNA 6 was continuous through Bam HI sites a and b (Figure 12).

OVERALL CONCLUSIONS

The data presented here for HSV-2, together with other information for HSV-1, suggests that IE mRNA 3 is unspliced. By contrast, HSV-1 IE mRNAs 5 and 6 appear to have a single splice. Adenovirus type 2 specifies an unspliced mRNA (29) as well as the various spliced species (30). The methodology used here may not detect small splices of 50 nucleotides or less which are located at the ends of mRNAs and this is determined by the sensitivities of the gel electrophoretic procedures used.

One would like the precise information on RNA structures that is obtained by comparison of mRNA sequences

with the relevant DNA sequences. Even this information however may not shed much light on the mechanism involved in synthesis of the mRNAs.

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Volume 8 Number 12 1980

Nucleic Acids Research

Temporal regulation of herpes simplex virus type 2 transcription and characterization of virus immediate early mRNA's

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Received 12 May 1980

ABSTRACT

Nuclear and cytoplasmic virus RNAs, synthesized in cells infected with herpes simplex virus type 2 at early and late times post-infection, and in the continuous presence of the protein synthesis inhibitor cycloheximide (immediate early), have been analyzed by blot hybridization to virus DNA fragments generated by Bam HI and Eco RI restriction endonucleases. Polyadenylated immediate early mRNAs were separated on denaturing gels containing CH_3HgOH giving three virus-specific mRNA bands of estimated sizes 4.7, 3.4 and 1.75 kb, and these have been mapped to five discrete regions of the genome. The polypeptides produced by in vitro translation of the HSV-2 immediate early mRNA's have been identified. Orientations of immediate early mRNA's on the virus genome have been determined by mapping cDNAs complementary to the 3'termini of the mRNAs.

INTRODUCTION

Herpes simplex viruses comprise two distinct serotypes (HSV-1 and HSV-2), each with a linear duplex DNA genome of about 150 kbp(1, 2). The HSV-1 and HSV-2 genomes show approximately 50% cross homology (3, 41), and have a similar complex structure (4, 5). The two serotypes can be easily distinguished by the characteristically different DNA fragment patterns generated by cleavage with various restriction endonucleases (6, 7, 42).

At least some of the HSV-1 and HSV-2 gene products are functionally interchangeable, as intertypic complementation has been observed (8). Furthermore, exchange of genetic material has been shown by production of intertypic recombinants containing genomes, parts of which are derived from each parent (9, 10, 11, 12, 13, 23). Analysis of intertypic

recombinants allowed a correlation of the genetic and physical maps of the two serotypes, and demonstrated the colinearity of equivalent genes on the two DNAs (42).

Both HSV-1 and HSV-2 have been shown to be capable of inducing the biochemical and morphological transformation of cells cultured in vitro (24, 25, 26, 27, 28, 40, I. Cameron and J.C.M. Macnab, personal communication). HSV-2 also has been implicated in the aetiology of human cervical carcinoma (14, 29, 30).

Hence the evidence available indicates close similarities between HSV-1 and HSV-2 in terms of the molecular organization, regulation and expression of their genomes.

Previous studies on HSV transcription have been concerned primarily with HSV-1, and have shown that transcription is subject to temporal regulation (31, 33, 34). Inhibitors of virus protein synthesis, or infection of cells at the non-permissive temperature with certain HSV-1 temperature-sensitive mutants, resulted in accumulation of immediate early (IE) RNA which hybridized only to certain restricted regions of the genome (33, 35, 36). By contrast, at both early and late times (before and after the onset of viral DNA replication) the virus transcripts mapped throughout the genome (33). At least one virus gene product is involved in effecting the switch from the restricted IE stage of transcription (37).

On the basis of liquid hybridization studies, Frenkel et al (15) have proposed a radically different transcriptional programme for HSV-2 as compared to HSV-1. The present communication investigates the transcriptional programme of HSV-2 using the Southern blot procedure (16, 33), and also characterises the HSV-2 IE mRNAs.

MATERIALS AND METHODS

Cells and virus

Baby hamster kidney cells (BHK 21-C13) were grown to confluent monolayers in 2 litre roller bottles, or on 90 mm plastic tissue culture plates as described previously (17, 33), and infected with HSV-2 (strain HG52) at a multiplicity of 50 pfu/cell.

For purification of radioactively labelled RNA, confluent monolayers were incubated for 16 hr prior to infection in phosphate-free Eagles medium containing 2% calf serum. After 1 hr virus absorption, the cells were labelled with 0.25 - 0.75 mCi (^{32}P)-orthophosphate/ml of medium at 37°C. For preparation of IE RNA, monolayers were pre-treated with cycloheximide (200 µg/ml) for 30 min at 37°C prior to infection. Infection and subsequent maintenance of monolayers was carried out in the continous presence of 200 µg/ml cycloheximide. RNA was isolated 6 hr after the virus absorption period.

Cell fractionation and isolation of RNA

Nuclear and cytoplasmic cell fractions were prepared and RNA was isolated essentially as described by Kumar and Lindberg (18). In general, RNA preparations to be hybridized to blot strips were further purified by Cs_2SO_4 equilibrium density centrifugation (33). Nuclear IE RNA was also prepared without Cs_2SO_4 banding, by two cycles of DNAase treatment (20 µg/ml) for 1 hr at 37°C followed each time by Sephadex G-50 filtration. Polyadenylated RNA was selected as described previously (36).

In vitro labelling procedures

Cytoplasmic IE RNA was labelled with (γ-^{32}P)-ATP (3000 Ci/mmol; Radiochemical Centre, Amersham) using polynucleotide kinase (17). HSV-2 DNA was labelled to high specific activity with (α-^{32}P) deoxyribonucleoside triphosphates (300 Ci/mmol) by the method of "nick translation" (19). ^{32}P-labelled HSV-2 complementary DNA (cDNA) was prepared as previously described (17), using an oligo $(dT)_{10}$ primer.

Gel electrophoresis

RNA samples were fractionated on denaturing 1.5% agarose gels containing 5 mM CH_3HgOH as described previously (36). RNA bands were excised from preparative gels, and the gel slices were homogenised in 2 ml formamide. Gel homogenates were adjusted to 50% formamide, 3 x SSC, 1 x Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 100 µg/ml yeast RNA, and were incubated overnight at 45°C with shaking prior to hybridization.

In vitro translation

Total cytoplasmic RNA samples were translated *in vitro*

as described by Preston (20).

Cloning procedures

Restriction endonuclease fragments of HSV-2 DNA (7) were cloned under Category II containment within the Institute of Virology as specified by the U.K. Genetic Manipulation Advisory Group. The cloning vector was pAT 153, and the host bacterium, E.coli K12 HB101 (39).

Blot hybridization procedures

Nitrocellulose blot strips containing HSV-2 DNA fragments generated with various restriction endonucleases were prepared as previously described (33). The strips were pre-incubated overnight at 45°C in 50% formamide, 3 x SSC, 1 x Denhardt's solution containing 100 µg/ml yeast RNA. Hybridizations were performed under the same conditions at 45°C for 48 hr. The blot strips were washed and autoradiographed as described previously (33).

RNA was transferred onto DBM paper according to the method of Alwine et al., (44). The DBM strips were then hybridized and washed as described for the nitrocellulose strips with the addition of 1% glycine in the pre-incubation mixture.

RESULTS

The HSV DNA molecule consists of long unique (U_L) and short unique (U_S) regions, each flanked by inverted repeated sequences (TR_L/IR_L, TR_S/IR_S) joined at IR_L - IR_S (4, 5, 6, 7). The genome also contains a true terminal redundancy, designated the "a" sequence, which is present at the junction of the internal repetitions in one or more copies in different DNA molecules (43). Fragment Bam HI $\bar{g}$ (Fig. 1, tracks 1, 8 and 10) consists of the joint fragment, derived from those DNA molecules in the population which contain two internal "a" sequences. Four genome arrangements, resulting from all of the possible combinations of inversions of the two unique regions, are present in DNA preparations in approximately equal amounts (4, 6). A further consequence of this structure is that a number of fragments produced by digestion with a restriction endonuclease may contain sequences in common (7, 42).

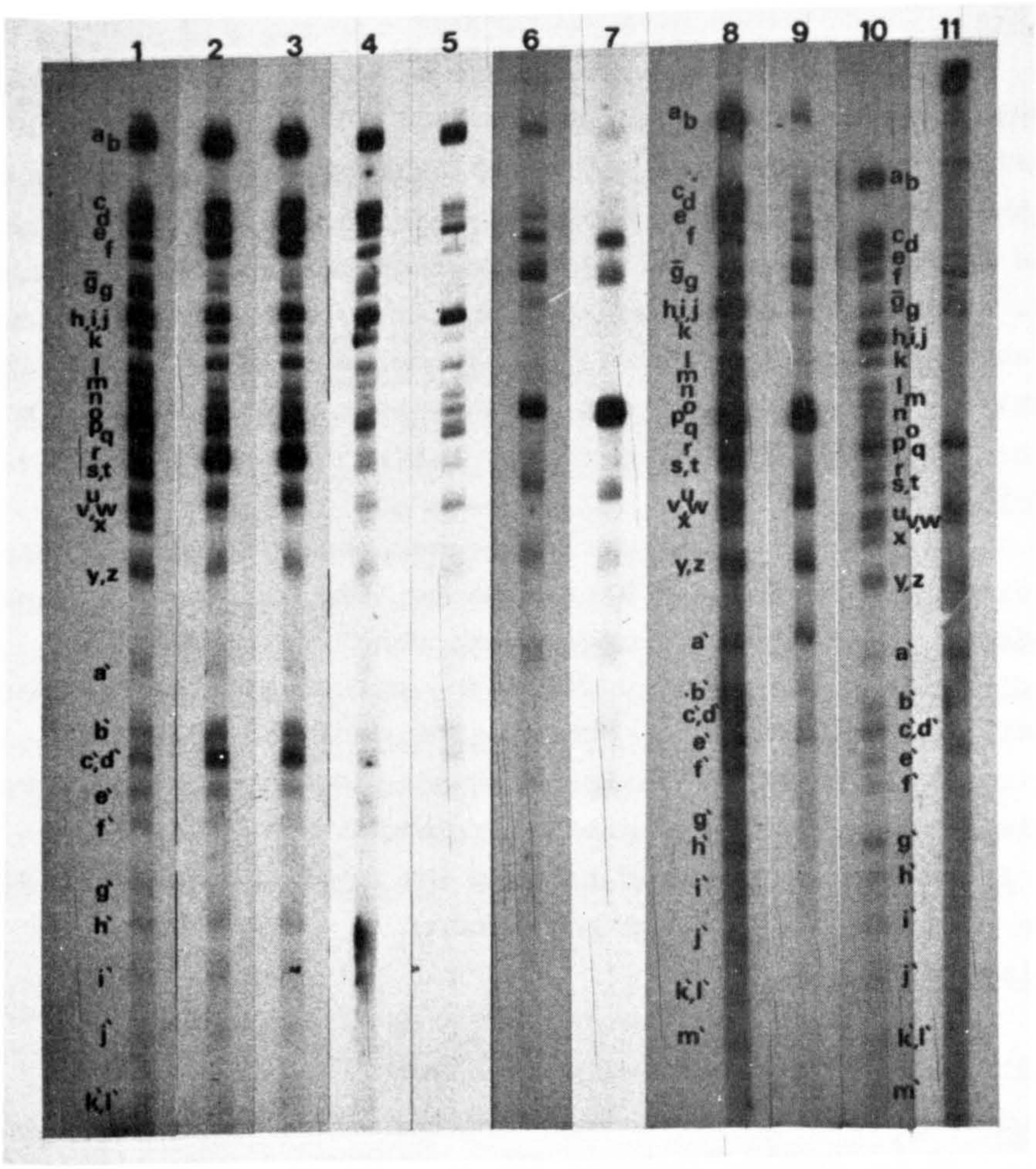


Figure 1: Fluorographs of IE, early and late RNA samples hybridized to the Bam HI fragments of HSV-2 DNA. 1. Nick-translated total HSV-2 DNA the control strip for tracks 2 to 7. 2. Early nuclear RNA. 3. Early cytoplasmic RNA. 4. Late nuclear RNA. 5. Late cytoplasmic RNA. 6. IE nuclear RNA. 7. IE cytoplasmic RNA. 8. Nick-translated total HSV-2 DNA the control stip for track 9. 9. In vitro labelled IE cytoplasmic RNA. 10. Nick-translated DNA the control strip for track 11. 11. Unbanded IE nuclear RNA.

Mapping of HSV-2 transcripts

Nuclear and cytoplasmic RNA samples were isolated from infected cells labelled post-absorption with (^{32}P)-orthophosphate from 0-2 hr (early RNA), 0-10 hr (late RNA), or 0-6 hr in the continuous presence of cycloheximide (IE RNA).

Late RNA

Both nuclear and cytoplasmic late RNA hybridized to all the DNA fragments generated by Bam HI (Fig. 1, tracks 4 and 5). Comparison of the nuclear and cytoplasmic hybridization patterns from this and other experiments revealed consistent differences in the relative amounts of hybridization to several DNA fragments. Late nuclear RNA showed greater hybridization to Bam HI g, f and c, as compared with late cytoplasmic RNA. A similar situation, to a lesser extent, can be seen with Bam HI k, m and x.

Early RNA

As with late RNA, early nuclear and cytoplasmic RNAs hybridized to all the Bam HI fragments (Fig. 1, tracks 2 and 3). No major consistent differences were observed between the hybridization patterns of nuclear and cytoplasmic early RNAs. However, the hybridization patterns of early RNA differed quantitatively from the patterns obtained with late RNA. For example, early RNA showed greater relative hybridization to Bam HI e, f and s t as compared to late RNA, and reduced hybridization could be seen to Bam HI d and m.

Immediate early RNA

As compared to late and early RNA, the pattern of hybridization of nuclear and cytoplasmic IE RNA is severely restricted (Fig. 1, tracks 6, 7 and 9). Some small differences appeared to be present between the nuclear and cytoplasmic RNA samples; there was no apparent hybridization of cytoplasmic RNA to Bam HI e, h i j or k. However, these sequences must be present in cytoplasmic RNA preparations, as they can be detected in RNA selected on oligo-dT cellulose (Fig. 5, track 2), and as minor species in IE cDNA (Fig. 7, track 6).

In order to ensure that the restricted hybridization of IE RNA was not due to preferential labelling in vivo of certain RNA species or to selective fractionation of RNAs, IE cytoplasmic RNA was labelled in vitro with polynucleotide kinase and then hybridized (Fig. 1, track 9). In addition, in vivo ^{32}P-labelled IE nuclear RNA was hybridized without fractionation on Cs_2SO_4 (Fig. 1, track 11). Both hybridization profiles were qualitatively similar to those of IE RNA selected in the normal way

(Figs. 1, tracks 9 and 11).

Summary of RNA mapping data

The RNA mapping data are summarized in Fig. 2. Significant hybridization to a fragment is indicated by a single continuous line, while low levels of hybridization are indicated by a broken line.

Characterization of IE mRNAs

Translation products of HSV-2 IE mRNAs

The HSV-2 IE polypeptides translated *in vitro* have apparent molecular weights of 178 x 10^3, 140 x 10^3, 116 x 10^3 and 65 x 10^3 (Fig. 3). These values are similar, but not identical to those of the equivalent polypeptides found *in vivo*. The *in vivo* labelled HSV-2 polypeptides have molecular weights of 182 x 10^3, 142 x 10^3, 118 x 10^3, 67 x 10^3, 65 x 10^3 and 12.3 x 10^3 (13). The slightly lower molecular weights of some of the polypeptides when translated *in vitro* can be attributed to their absence of normal post-translational modification, as found previously for HSV-1 (20). No *in vitro* translation product equivalent to the 67 x 10^3 polypeptide found *in vivo* was detected, possibly

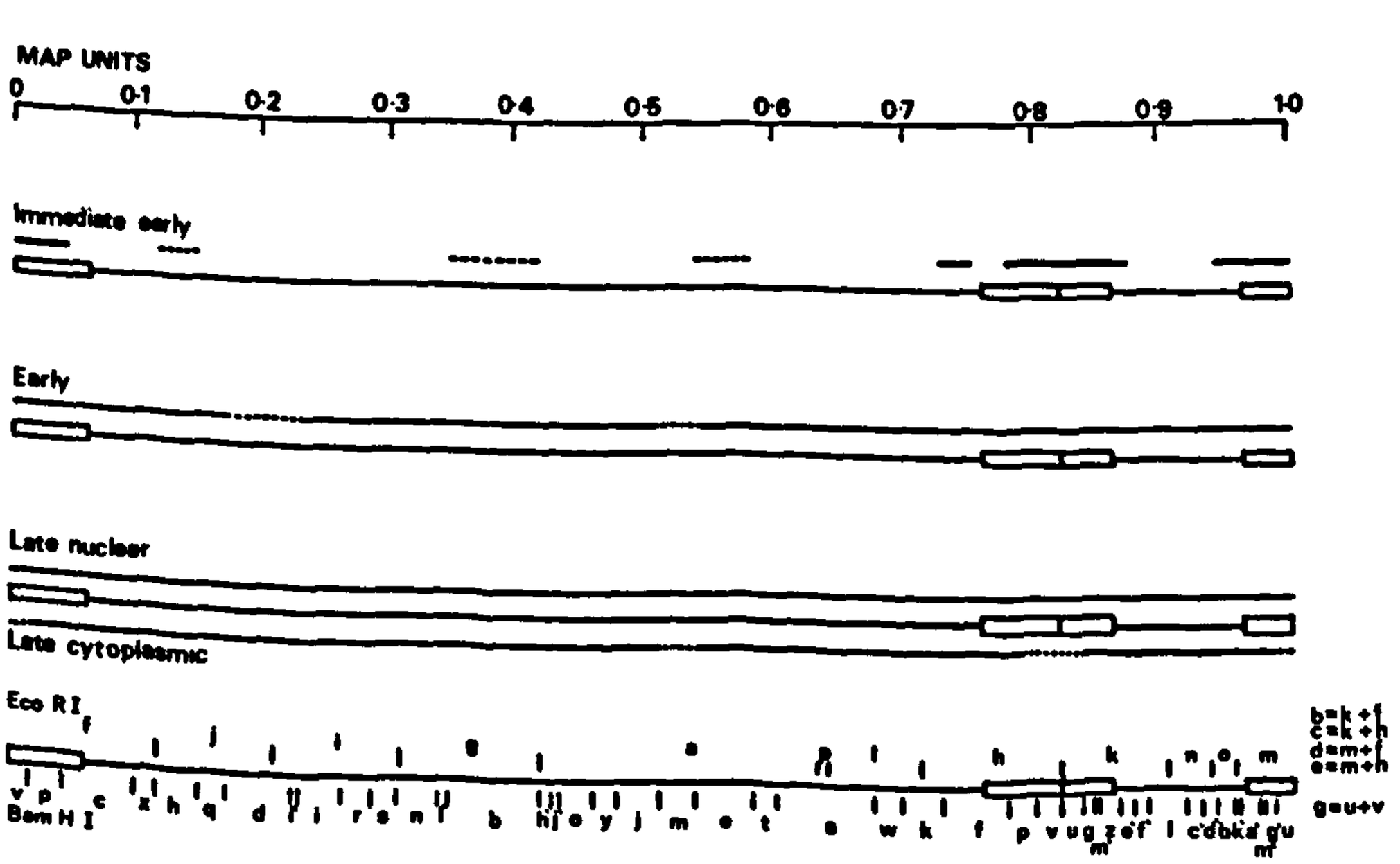


Figure 2: Summary of the RNA mapping data and physical maps for HSV-2 DNA fragments generated by restriction endonucleases Bam HI and Eco RI. The maps are shown for the standard orientation of U_L and U_S (7, 42).

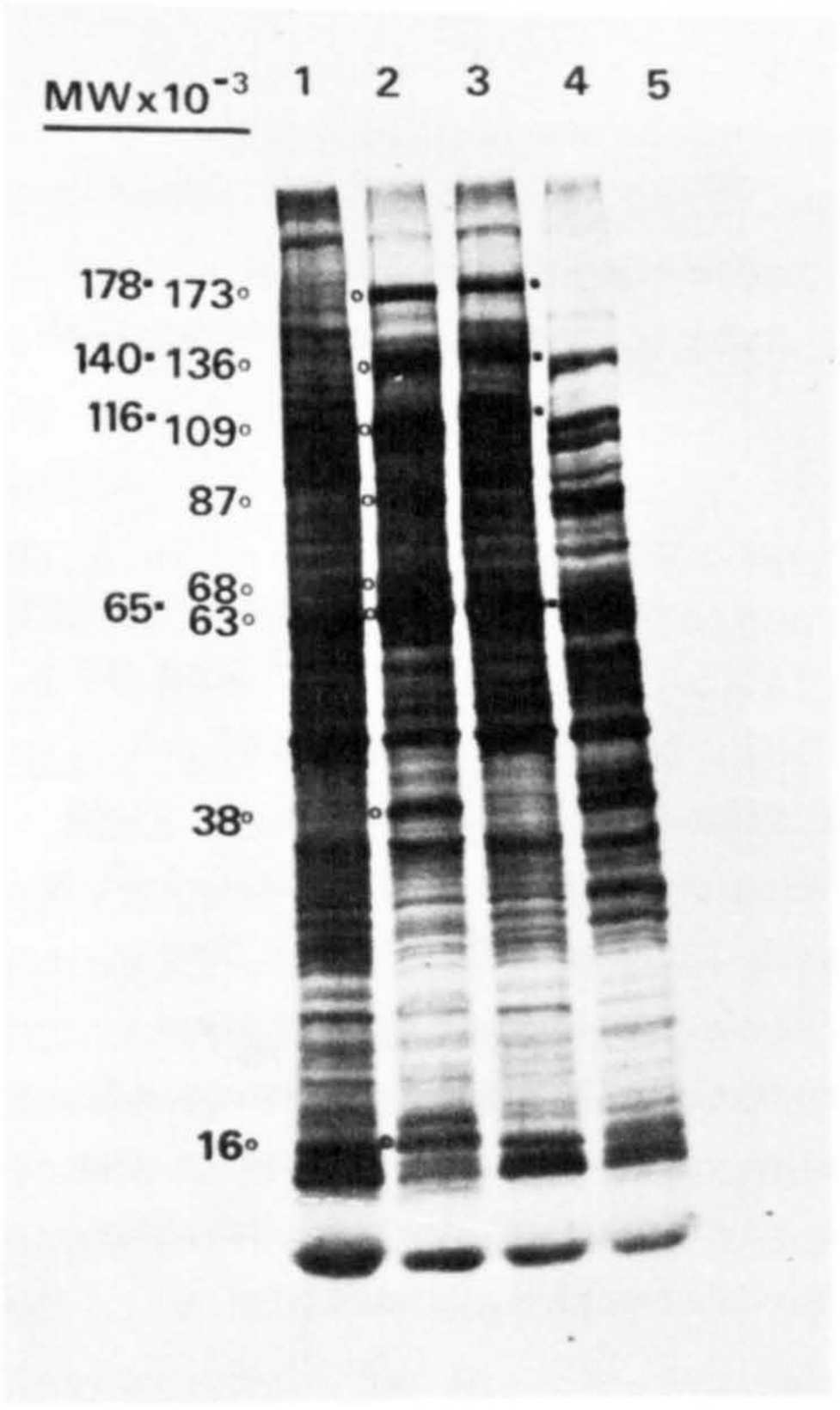


Figure 3: PAGE separation of the *in vitro* translation products of HSV-2 IE mRNAs.
1. Mock-infected RNA. 2. HSV-1 IE RNA. 3. HSV-2 IE mRNA.
4. HSV-1 late RNA. 5. *E.coli* rRNA. HSV-1 specific IE polypeptides (o). HSV-2 specific IE polypeptides (■).

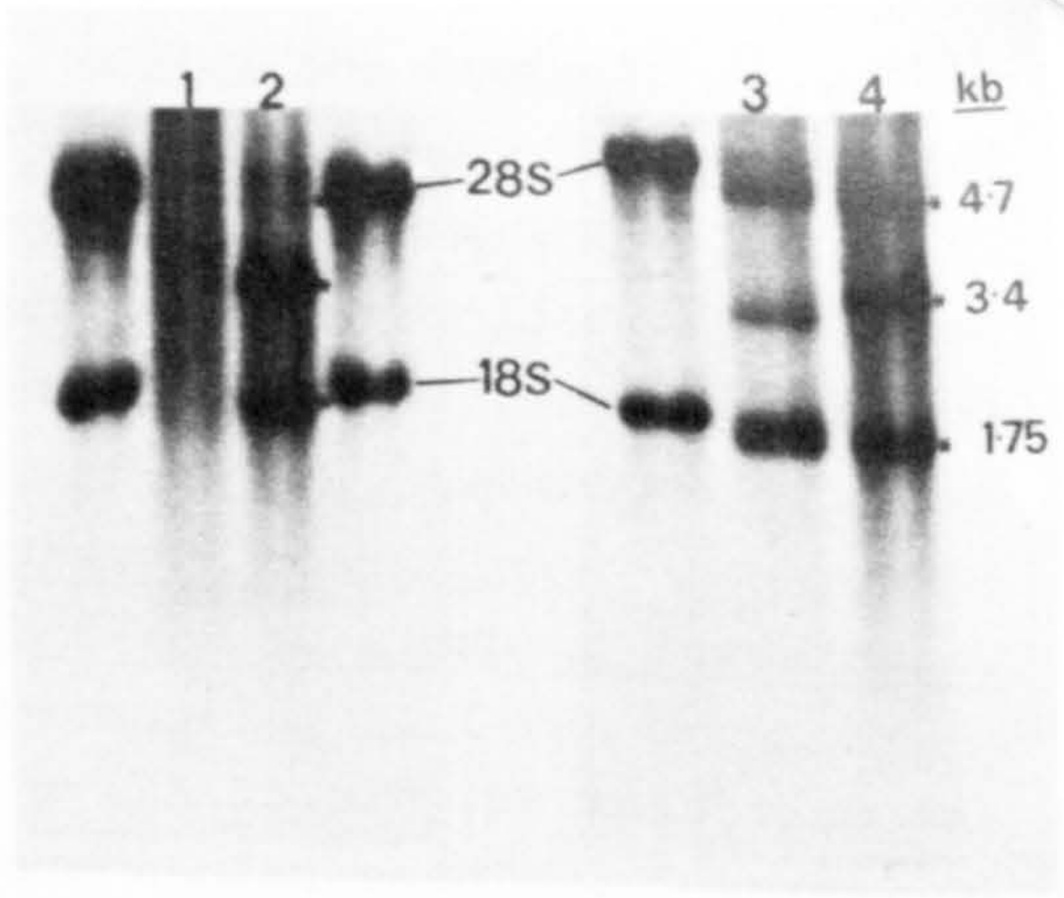


Figure 4: Fluorographs of HSV-1 and HSV-2 mRNAs separated by electrophoresis through CH_3HgOH agarose gels. 1. Mock-infected RNA. 2. HSV-2 IE RNA. 3. HSV-1 IE RNA. 4. HSV-2 IE RNA.

because the unmodified polypeptide comigrates with a host polypeptide band. It is not expected to observe the 12.3 x 10^3 polypeptide, as it would migrate with globin, an inherrent contaminant of the reticulocyte translation system (20).

Sizes of IE mRNAs

Fractionation of ^{32}P-labelled cytoplasmic polyadenylated IE mRNA on denaturing CH_3HgOH agarose gels revealed three bands which were not present in polyadenylated cytoplasmic RNA from mock-infected cells (Fig. 4). Different preparations of poly (A) +RNA contained variable small amounts of 28S ribosomal RNA, and

the relative proportions of the three virus RNA bands varied somewhat (Fig. 4, tracks 2 and 4). These bands had sizes of 4.7 kb, 3.4 kb, and 1.75 kb, consistent with the HSV-1 situation (36), though clear size differences can be seen between the HSV-1 and HSV-2 IE mRNAs (Fig. 4, tracks 3 and 4).

Genome map locations of HSV-2 IE mRNAs

The hybridization pattern of total polyadenylated cytoplasmic IE RNA (Fig. 5, track 2) was similar to that of unselected cytoplasmic IE RNA (Fig. 1, track 6). Individual polyadenylated RNA bands excised from a preparative CH_3HgOH gel were hybridized to blot strips containing the Bam HI fragments (Fig. 5, tracks 3, 4 and 5).

The 4.7 kb RNA band hybridized predominantly to Bam HI g (and $\bar{g}$), u, y z, a', and also to g', and m' (Fig. 5, track 3); some hybridization could also be seen to Bam HI b, e and p. The major hybridization indicates the 4.7 kb mRNA (designated IE mRNA IV) maps within TR_S/IR_S as indicated in Fig. 9.

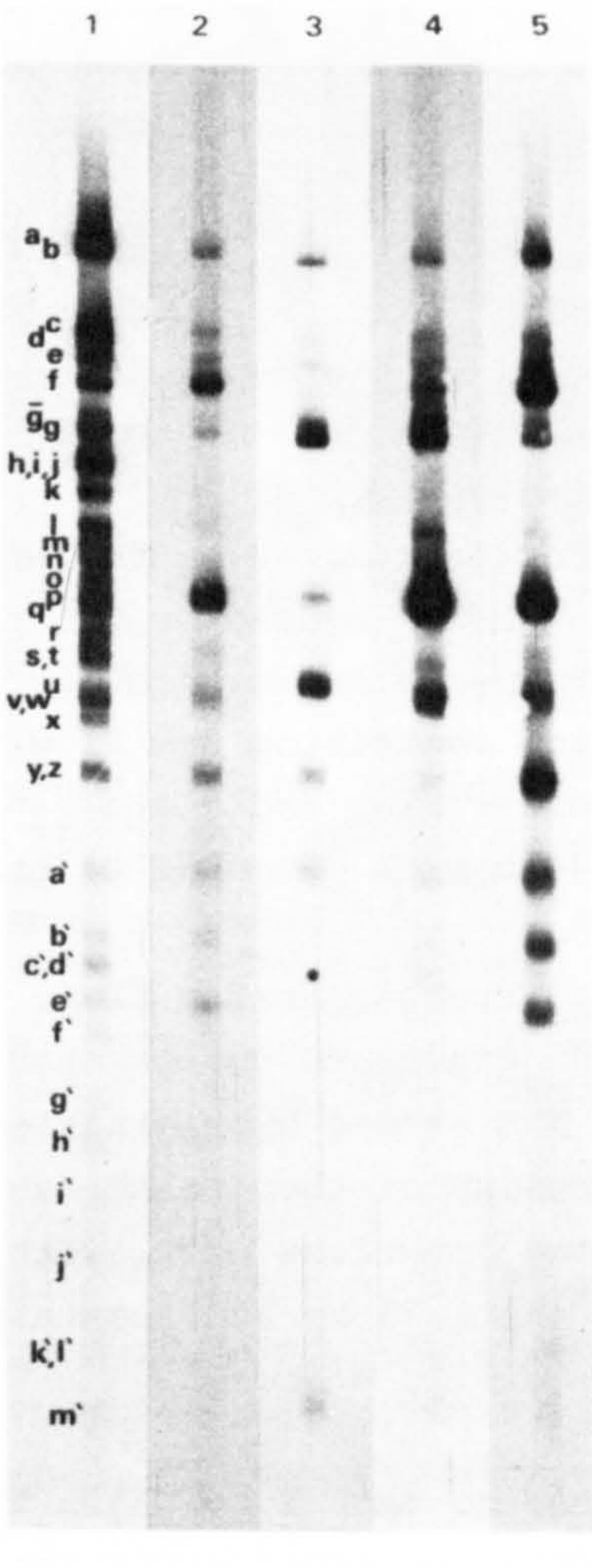
The 3.4 kb RNA band showed strong hybridization to Bam HI g, p, and v w (Fig. 5, track 4), with faint hybridization to Bam HI c and f as well as to certain fragments represented in the larger, 4.7 kb mRNA. Since the region containing Bam HI w is not represented in IE RNA (Fig. 2), and Bam HI p and v are contiguous, this indicates that the 3.4 kb mRNA (IE mRNA I) maps entirely within TR_L/IR_L (Fig. 9).

The 1.75 kb RNA band hybridized strongly to Bam HI f, y z, a', b', e' and k' l', and also to DNA fragments represented in the 3.4 kb mRNA (Fig. 5, track 5). The strong hybridization was to fragments from three distinct regions of the genome; at U_L near the junction with IR_L, and at the junctions of U_S with both IR_S and TR_S. This indicated that the 1.75 kb band contained at least three individual mRNAs (IE mRNAs III, V and VI respectively; Fig. 9).

IE mRNAs IV and V both hybridized to Bam HI z, while IV and VI hybridized to Bam HI a'. This does not necessarily imply that these RNAs overlap, but more likely that the ends of the two relevant mRNAs lie within the Bam HI fragment, and are not separated by a Bam HI cleavage site. The equivalent HSV-1 IE mRNAs which map in these locations do not overlap (36) and these

regions of uncertainty are represented by a discontinuous line in Fig. 9.

The map positions of the major IE mRNA species were confirmed using RNA transferred to DBM paper. Total nick-translated HSV-2 DNA and cloned DNA probes were hybridized to the immobilized RNA. Bam HI g hybridized to both the 4.7 and 3.4 kb RNA bands (Fig. 6, track 2), while Bam HI p hybridized



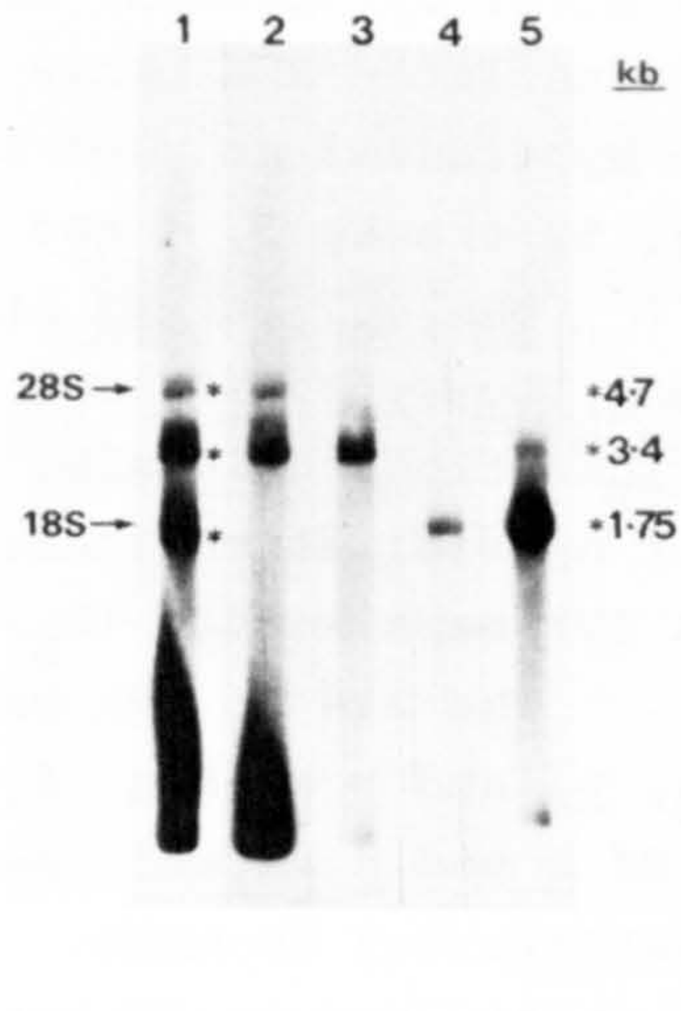


Figure 6: Fluorographs of nick-translated HSV-2 DNA fragments hybridized to IE poly (A)+RNA immobilized on DBM paper. 1. Total HSV-2 DNA. 2. Bam HI g. 3. Bam HI p. 4. Bam HI f. 5. Bam HI f.

Figure 5: Fluorographs of fractionated HSV-2 IE RNA bands hybridized to the Bam HI fragments of HSV-2 DNA. 1. Nick-translated total HSV-2 DNA. 2. Unfractionated cytoplasmic poly (A)+IE RNA. 3. 4.7 kb IE RNA band. 4. 3.4 kb IE RNA band. 5. 1.75 kb IE RNA band.

only to the 3.4 kb mRNA (Fig. 6, track 3). These results, in conjunction with those obtained by hybridizing isolated RNA bands to strips (Fig. 5) confirm the location of the 4.7 kb mRNA in TR_S/IR_S and the 3.4 kb mRNA in TR_L/IR_L. The hybridization observed using the Bam HI f probe confirms the location of a 1.75 kb mRNA at the junction of U_L and IR_L (Fig. 6, track 4). Overexposure of the DBM blot strip hybridized to Bam HI f, showed a small amount of hybridization to the 3.4 kb mRNA (Fig. 6, track 5). This indicates that this mRNA extends a small extent into Bam HI f, and explains the faint hybridization of the 3.4 kb mRNA band to Bam HI f (Fig. 5, track 4). The 3.4 kb mRNA also hybridizes to Bam HI c, as f and c share sequences from TR_L/IR_L (Fig. 2).

Orientation of IE mRNAs

cDNA synthesis initiated on polyadenylated HSV IE RNA using oligo $(dT)_{10}$ as primer results in a product of small size, which consists of sequences complementary to the 3'-termini of mRNAs. Therefore hybridization of this cDNA to blot strips allows these mRNAs to be oriented on the genome (17).

IE cDNA made from polyadenylated cytoplasmic RNA was hybridized to the Bam HI and EcoRI generated DNA fragments. The hybridization patterns of IE cDNA and of in vivo labelled total IE cytoplasmic RNA are shown in Fig. 7.

IE mRNA I There was abundant hybridization to Bam HI p, but much less to Bam HI v w (Fig. 7, track 6). This indicates that the direction of transcription of the 3.4 kb mRNA I in TR_L is rightwards as drawn in Fig. 9. Due to the structure of the HSV-2 genome, IE mRNA I synthesized from the IR_L template would be made on the complementary strand to that used in TR_L, and must therefore be transcribed in a leftwards direction (Fig. 9).

IE mRNA II In total nuclear (Fig. 1, track 6) and polyadenylated cytoplasmic IE RNA (Fig. 5, track 2), hybridization to Bam HI e and s t was observed, and this hybridization is also observed on overexposure with cytoplasmic IE RNA (data not shown) IE cDNA did not hybridize to Bam HI e, but hybridization was detected to Bam HI s t (Fig. 7, track 6). As Bam HI e and t are adjacent on the genome, this indicates that the minor II is transcribed rightwards (Fig. 9).

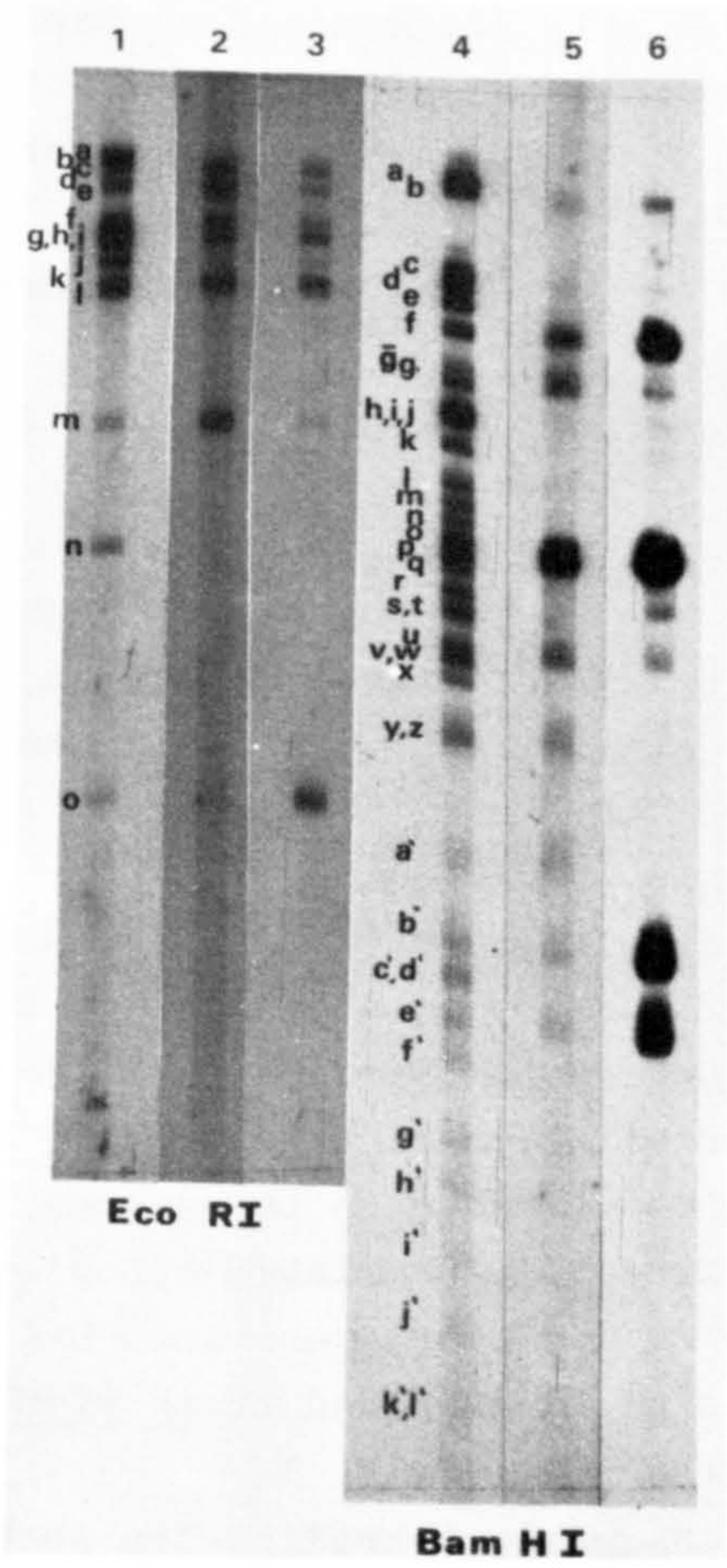


Figure 7: Fluorographs of HSV-2 IE cDNA hybridized to the Bam HI and Eco RI fragments of HSV-2 DNA. 1. Nick-translated total HSV-2 DNA. 2. IE cytoplasmic RNA. 3. IE cDNA. 4. Nick-translated total HSV-2 DNA. 5. IE cytoplasmic RNA. 6. IE cDNA.

IE mRNA III The HSV-2 physical maps available were insufficiently detailed in this region to allow unambiguous orientation of this mRNA. Hence, the Hind III o fragment (0.737 -0.749 map units, Fig. 8) which contains this mRNA was inserted into pAT 153, and the recombinant plasmid was propagated in E.coli HB101. Fine maps of Hind III o were prepared using Pvu II and Sma I restriction endonucleases, and these are shown in Fig. 8. IE cDNA when hybridized to a blot strip containing the DNA fragments generated by a Pvu II/Sma I double-digest showed abundant

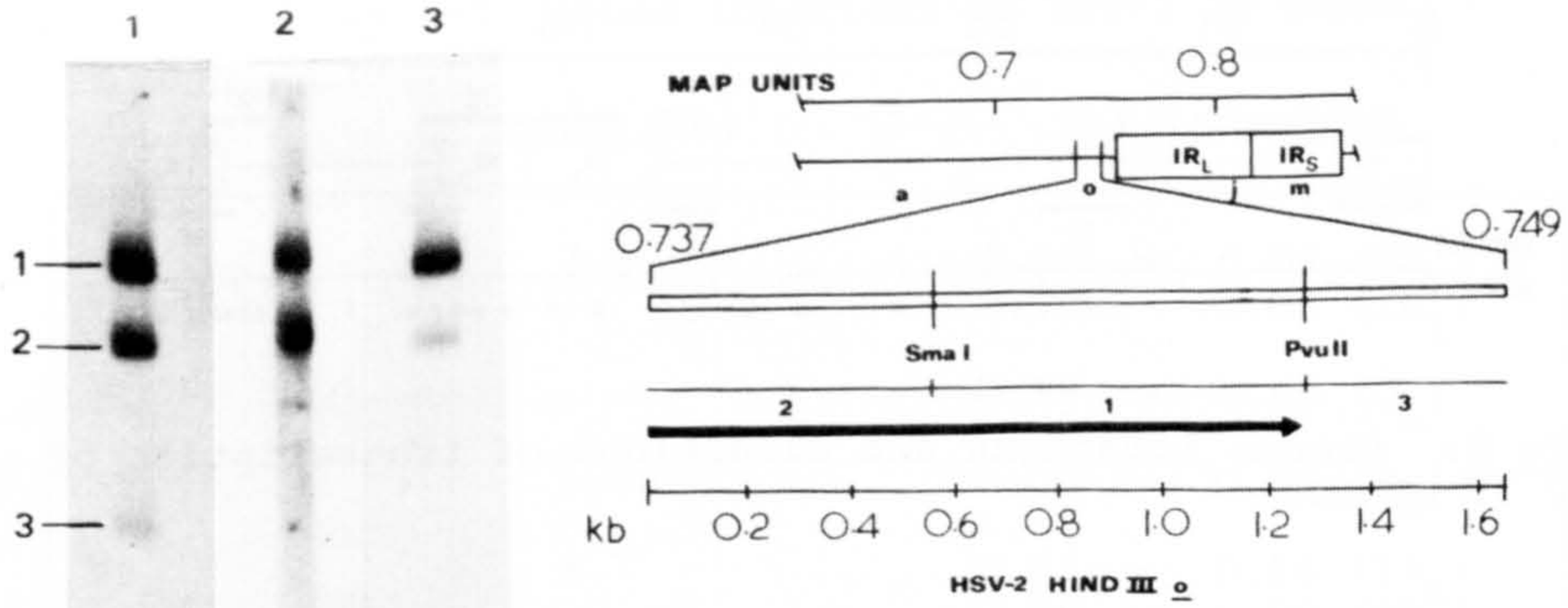


Figure 8: Fluorographs of IE cDNA hybridized to the Pvu II/Sma I fragments of the HSV-2 Hind III o DNA fragment, together with the physical map. 1. Nick-translated total HSV-2 DNA. 2. IE cytoplasmic RNA. 3. IE cDNA.

hybridization to fragment 1, with a small amount to fragment 2. This indicates that IE mRNA III is transcribed rightwards (Figs. 8 and 9).

IE mRNA IV IE cDNA hybridized to Bam HI u, but not to Bam HI g' or z (Fig. 7, track 6). This indicates that the direction of transcription of the 4.7 kb IE mRNA in IR_S is leftwards as shown in Fig. 9. Due to the structure of the HSV-2 genome, IE mRNA IV transcribed from the TR_S template is made from the complementary DNA strand, and therefore in a righwards direction (Fig. 9).

IE mRNA V IE cDNA hybridized to Bam HI e', but not to Bam HI z, (Fig. 7, track 6). Hence, IE mRNA V is transcribed rightwards from IR_S which serves as a template for IE mRNA IV (Fig. 9).

IE mRNA VI IE cDNA hybridized to Bam HI b', but not to Bam HI k' or a', and there was also abundant hybridization to Eco RI o, with little to Eco RI m (Fig. 7). The direction of transcription is therefore leftwards from TR_S to U_S from the strand complementary to that in TR_S which serves as a template for IE mRNA IV (Fig. 9).

3' termini of minor IE mRNAs Faint hybridization of IE cDNA was observed to Bam HI k, and also to Eco RI l (Figs. 2 and 7). As no in vivo labelled IE RNA was mapped to this region, this

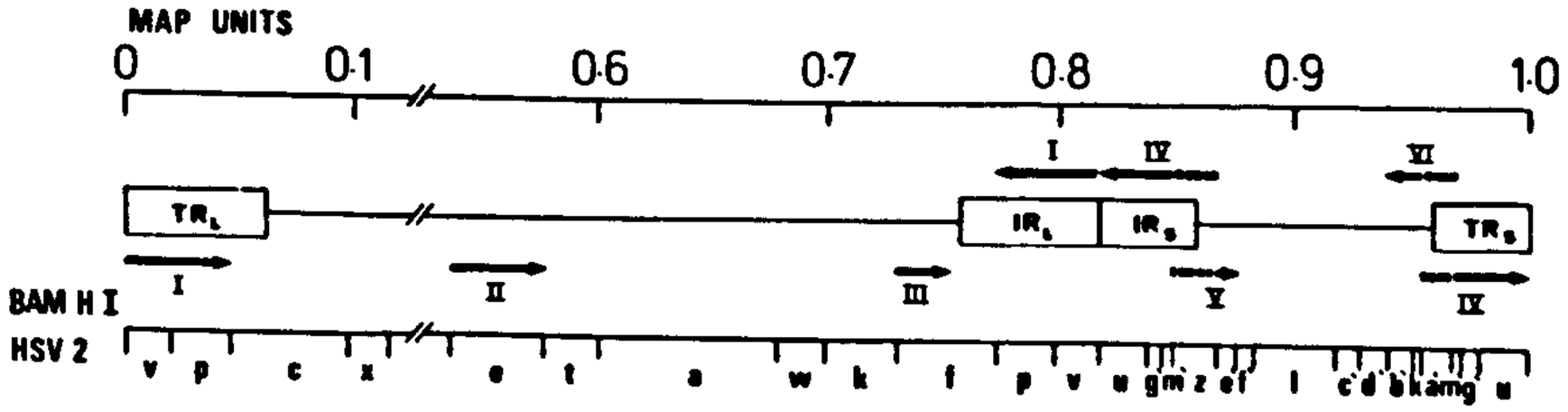


Figure 9: Genome locations and directions of transcription of HSV-2 IE mRNAs.

represents the 3' end of at least one previously undetected minor IE RNA.

As indicated earlier, the nuclear IE RNA hybridizing to Bam HI h i j was not detected in cytoplasmic IE RNAs (Fig. 1, track 7). However, cDNA prepared using a template of unlabelled cytoplasmic IE RNA did hybridize to Bam HI h i j and to Eco RI j, which overlaps with Bam HI h. This indicates that IE RNA from this region is present within the cytoplasm in low amounts, and also unambiguously locates it between 0.12 and 0.15 map units (Fig. 2).

DISCUSSION

The general pattern of HSV-2 transcription as determined by the Southern blot procedure appears directly analagous to that previously determined for HSV-1 using the same technique (33, 35, 37).

At early times, virus RNA hybridized to DNA fragments from all regions of the genome, as indicated by other studies (21, 22), and no major differences between the hybridization patterns of nuclear and cytoplasmic RNA were obtained. There were quantitative differences in the amount of early RNA that hybridized to the individual fragments, for example: the most abundant hybridization of early RNA was to Bam HI e and s t (0.54 to 0.60 map units), but little hybridization to the adjacent fragment Bam HI m, and also to Bam HI d was observed. This abundant hybridization to Bam HI e and s t is of interest, as this region is associated with morphological transformation of mouse cells and primary rat embryo cells cultured in vitro

TABLE 1. Sizes and genome locations of HSV-2 IE mRNAs.

IE mRNA SPECIES	SIZE (kb)	MAP LOCATIONS
I	3.4	0.00 - 0.04 (TR_L) 0.77 - 0.82 (IR_L)
II	-	0.54 - 0.60 (U_L)
III	1.75	0.73 - 0.74 (U_L)
IV	4.7	0.82 - 0.86 (IR_S) 0.96 - 1.00 (TR_S)
V	1.75	0.85 - 0.88 (IR_S/U_S)
VI	1.75	0.94 - 0.97 (TR_S/U_S)

(40; I. Cameron and J.C.M. Macnab, personal communication). We have determined the orientation of an IE mRNA (IE mRNA II) which maps in this region (Fig. 9).

At late times, virus transcripts also hybridized to DNA fragments located throughout the genome consistent with HSV-1 and HSV-2 liquid hybridization experiments (15, 31, 34) and with HSV-1 blot hybridization experiments (33). Unlike the early situation, obvious differences in the hybridization patterns of late nuclear and cytoplasmic RNAs were observed. There was little late cytoplasmic RNA which hybridized to both sets of repetitions as compared with late nuclear RNA. This effect could be due either to sequestration of transcripts complementary to these repetitive sequences within the nucleus, or to their specific degradation in the cytoplsm. This has also been observed for HSV-1 (33, 34).

The early and late patterns are distinguished by quantitative differences in the relative abundance of RNA sequences hybridizing to the individual DNA fragments.

In contrast with the early and late RNAs, HSV-2 IE RNA hybridized to only certain DNA fragments, located in both repetitive regions, and in both unique regions. No major differences in the hybridization patterns of IE nuclear and cytoplasmic RNAs were observed, and this is consistent with the

observations reported for HSV-1 (33, 35). Similar restricted immediate early transcription has been observed for pseudorabies virus, a related herpesvirus (38).

The IE RNA results are not consistent with liquid hybridization data previously reported for HSV-2, which indicated that 45% of the genome was represented in total IE RNA (15). Liquid hybridization data described for HSV-1 indicated that 50% (32) to 30% (34) of the genome was represented in IE nuclear RNA, whereas only 13% was represented in the cytoplasm (34). Our current knowledge of the genome map locations, size and polypeptides specified by both HSV-1 (13, 33, 36) and HSV-2 IE mRNAs (Fig. 3 and 9), together with the molecular weights of those IE polypeptides shown to be unrelated due to breakdown (D. MacDonald and H.S. Marsden, personal communication), indicates that at least 20% of the HSV genome must be represented in the cytoplasm at the immediate early stage.

No additional IE nuclear sequences were detected with a DNAase treated RNA sample which was not fractionated on a Cs_2SO_4 gradient, or with cytoplasmic RNA labelled in vitro. These controls therefore demonstrate that the restricted hybridization patterns of nuclear and cytoplasmic IE RNA are not due to artefacts introduced by the methods of RNA isolation or purification.

HSV-2 IE mRNAs separated on denaturing gels showed three bands of similar, but not identical, size to those found with HSV-1 (36). The HSV-2 IE mRNAs mapped to genome regions equivalent to those of the respective HSV-1 IE mRNAs of a similar size.

The difference in size of the 3.4 kb HSV-2 IE mRNA I and its 3.0 kb HSV-1 equivalent may be related to the differences in molecular weight of the two equivalent polypeptides shown to map to this location (13). These are 118 x 10^3 for HSV-2 and 110 x 10^3 for HSV-1 (the polypeptides translated in vitro, and therefore unmodified, have molecular weights of 116 x 10^3 and 109 x 10^3 respectively (20)). The 3.4 kb HSV-2 IE mRNA I is sufficient to encode a polypeptide of 116 x 10^3 while the 3.0 kb HSV-1 mRNA is sufficient to encode a polypeptide of 110 x 10^3. The 4.7 kb IE mRNAs of HSV-2 and HSV-1 are both capable of encoding polypeptides of at least 182 x 10^3.

The orientations of the HSV-2 IE mRNAs are identical to those of the equivalent HSV-1 mRNAs (17). In addition, using a cloned DNA fragment, it was possible to orientate HSV-2 IE mRNA III, which was not orientated for HSV-1 (17). IE mRNA III is transcribed from the opposite DNA strand to that of IE mRNA I in IR_L, therefore this mRNA would appear to have a separate promoter. Hence it is unlikely that all the major HSV-2 IE mRNAs are transcribed using a single promoter located in TR_S/IR_S as suggested for HSV-1 (17).

In conclusion, the HSV-2 transcriptional programme closely parallels that of HSV-1. This is particularly evident in the immediate early phase, where individual mRNAs have been orientated on the genome. Late in infection there is sequestration of specific RNA sequences within the nucleus. Control of transcription appears to be exerted by an off/on mechanism, as in the switch from the immediate early to the early phase, and by abundance controls, as evidenced by differences in the relative abundance of RNAs which hybridize to various parts of the genome, both within, and between, the three replication phases studied. The data indicates that in HSV-2 there must be at least two distinct virus promoters which are recognised by an unmodified host cell RNA polymerase and we suggest that the same is likely to be true for HSV-1.

ACKNOWLEDGEMENTS

We wish to thank Professor J.H. Subak-Sharpe for his advice and interest in this work. We also wish to thank Dr N.M. Wilkie and Mr A.J. Davison for providing physical maps prior to publication, Dr C.M. Preston for assistance with *in vitro* translation, and Dr F.J. Rixon for providing HSV-1 RNA. A.J.E. was in receipt of a Medical Research Council grant for training in research techniques.

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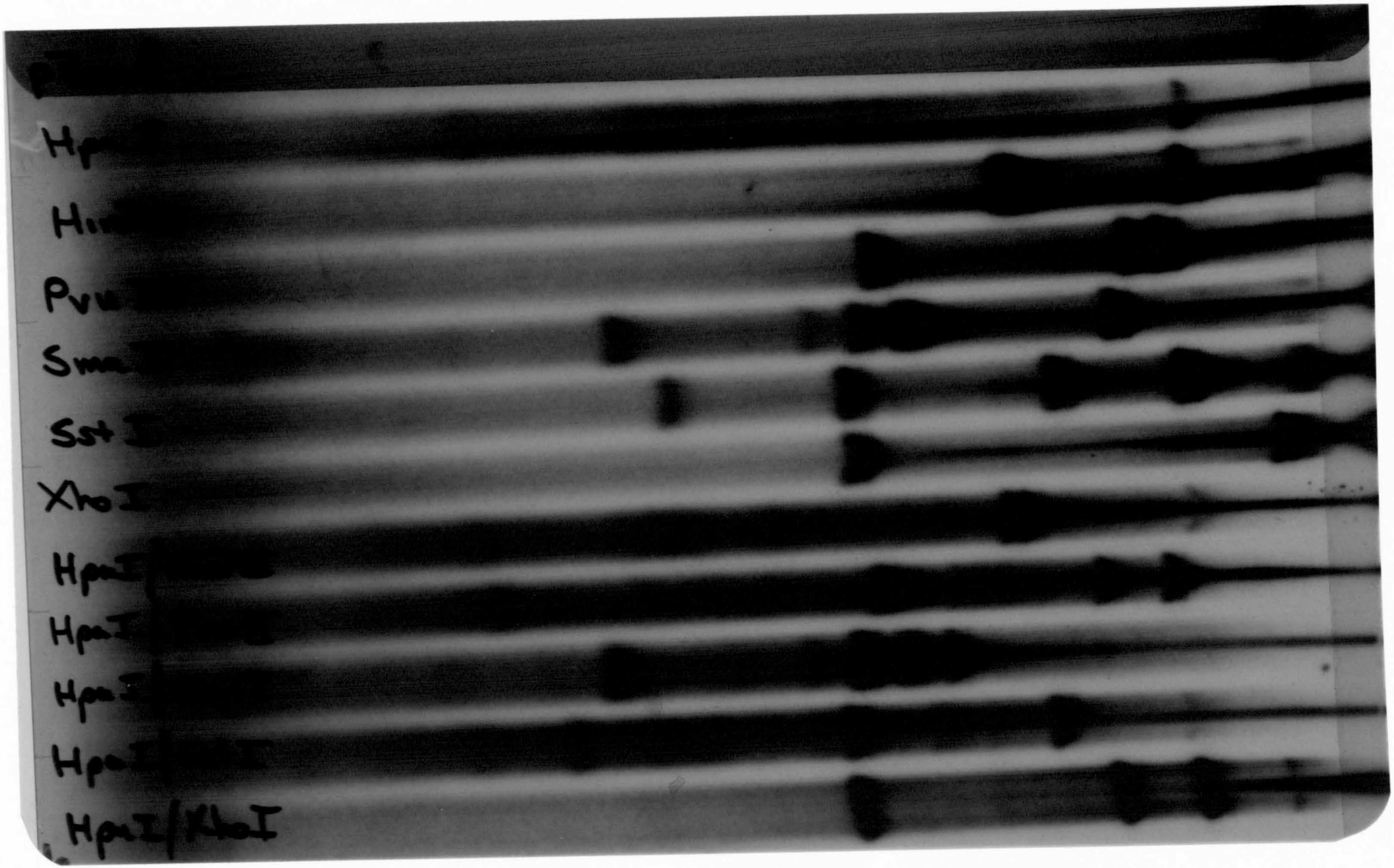
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