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Separation and Characterization of Herpes Simplex Virus Type 1 Immediate-Early mRNA's

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Polyadenylated immediate-early transcripts of herpes simplex virus type 1, made in BHK cells infected and maintained in the presence of cycloheximide, have been separated on denaturing agarose gels containing methyl mercuric hydroxide. Three virus-specific mRNA bands of estimated sizes 4.7, 3.0, and 2.0 kilobases (kb) were detected, and these mRNA's were mapped on the virus genome and also used to direct protein synthesis in vitro. The 4.7- and 3.0-kb mRNA's hybridized predominantly to certain DNA fragments which are located in the short and long repetitive regions of the genome, respectively, whereas the 2.0-kb mRNA's mapped to three discrete regions of the virus DNA. In vitro translation of these separated mRNA size classes indicated that the 3.0-kb mRNA specified the synthesis of virus polypeptide V_{mw} 110, whereas the 2.0-kb mRNA's specified V_{mw} 68, 63, and 12. The synthesis of small amounts of V_{mw} 175 was specified by the 4.7-kb mRNA. In contrast with the mRNA's which specify these other immediate-early polypeptides, that specifying V_{mw} 12 is much larger than required for its coding sequences.

The locations on the genome of transcripts which accumulate in cells infected with herpes simplex virus type 1 (HSV-1) at various times postinfection, in the presence or absence of DNA or protein synthesis inhibitors, have been analyzed by hybridization to the fragments of HSV-1 DNA generated by digestion with a number of restriction endonucleases (3, 10). These, and previous studies (6, 22), have indicated that transcription of the HSV-1 genome is subject to temporal regulation.

Inhibition of virus protein synthesis with cycloheximide was found to result in accumulation of RNA sequences which hybridized to certain DNA fragments only (3, 10), and these restricted RNA sequences, transcribed by a preexisting α-amanitin-sensitive host RNA polymerase (4), have been defined as immediate-early (IE) RNA. IE RNAs accumulate also in cells infected at the nonpermissive temperature with certain HSV-1 temperature-sensitive (*ts*) mutants, and are the only virus transcripts detectable in *ts* K-infected cells (24). Release of the cycloheximide block (3), or shift-down of cells infected with *ts* K to the permissive temperature (24), was found to allow transcription from additional regions of HSV-1 DNA which map throughout the virus genome. These latter regions were also transcribed during the normal replicative cycle at early and late times postinfection (3), that is, before and after the onset of virus DNA replication.

The polypeptides specified by IE RNA, the IE polypeptides, form a subset of the virus proteins made during productive infection (8, 19). In our laboratory, the IE polypeptides identified have been designated by their apparent molecular weight and include V_{mw} 175, 136, 110, 87, 68, 63, and 12. The IE polypeptides include the α-polypeptides class as defined by Honess and Roizman (8). These authors have shown that functional α-polypeptides synthesis is required for the subsequent appearance of later classes, β and λ-polypeptides in their terminology, which are synthesized in the absence of virus DNA replication (8).

The observations detailed above suggest that IE RNA codes for one or more virus polypeptides which regulate a major event in virus transcription; that is, the transition from IE to early RNA synthesis. It is as yet unknown how IE polypeptides function in this regulation, but it is known that these polypeptides are modified and preferentially accumulated in the nucleus (17) and have DNA binding properties in vitro (R. T. Hay and J. Hay, manuscript submitted for publication).

We have previously mapped the locations on the HSV-1 genome of cytoplasmic and nuclear HSV-1 IE RNAs (3) and found that these transcripts hybridized predominantly to DNA fragments containing, or adjacent to, the inverted

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repeat regions of the virus genome. We report here the results of experiments designed to characterize the IE mRNA's. IE mRNA's have been resolved by electrophoresis through denaturing gels, and the separated size classes have both been mapped against HSV-1 DNA fragments and used to direct in vitro protein synthesis in a cell-free translation system.

MATERIALS AND METHODS

Cells and virus. BHK-21 (C13) cells were grown as monolayers in 80-oz. (ca 2.37-liter) roller bottles and infected with HSV-1 (Glasgow strain 17) at a multiplicity of infection of 50 PFU/cell as described previously (3).

Labeling RNA. Cells were pretreated with 200 μg of cycloheximide per ml for 1 h and either infected or mock infected (MI) with HSV-1. Cells were labeled with [^{32}P]orthophosphate from 0 to 6 h postinfection in the presence of cycloheximide as previously described (3).

Labeling DNA. HSV-1 DNA was ^{32}P-labeled in vitro by nick translation as previously described (3).

Cell fractionation and RNA extraction. Cytoplasmic RNA was prepared by lysis of cells with Nonidet P-40, removal of nuclei by low-speed centrifugation, and phenol–chloroform extraction of the cytoplasmic fraction as previously described (18).

Selection of polyadenylated RNA. Cytoplasmic RNA was recovered by low-speed centrifugation and dissolved in 2 ml of binding buffer (0.5 M KCl, 25 mM Tris-hydrochloride, pH 7.9). The RNA was applied to a column of oligo(dT)-cellulose (obtained from Collaborative Research Inc.) and washed with 3 volumes of binding buffer. Polyadenylated [poly(A)] RNA was eluted with distilled water, made 0.4 M with respect to sodium acetate, and precipitated with 3 volumes of ethanol by overnight storage at −20°C. RNA was recovered by low-speed centrifugation.

CH_3HgOH agarose gel electrophoresis. Preparative and analytical RNA electrophoresis was performed through 1.5% agarose gels containing 5 mM methyl mercuric hydroxide (CH_3HgOH) basically by the method of Bailey and Davidson (1). Pelleted poly(A) RNA was redissolved in 50 μl of E buffer (50 mM boric acid, 5 mM $Na_2B_4O_7 \cdot 10H_2O$, 10 mM Na_2SO_4, and 1 mM EDTA, pH 8.2), containing 25 mM CH_3HgOH, 10% glycerol, and sufficient bromophenol blue to act as a tracking dye. RNA was kept for 10 min at room temperature immediately preceding electrophoresis.

Preparative RNA electrophoresis (approximately 5 μg of RNA per gel) was performed on tube gels (12 by 0.6 cm) at 6 mA/gel for 5 h at room temperature. Gels were then soaked for 1 h in 10 mM β-mercaptoethanol–20 mM Tris-hydrochloride (pH 7.9) to remove CH_3HgOH bound to RNA. Gels were scanned with a Joyce-Loebel UV scanner to locate the endogenous, contaminating 28S and 18S rRNA's. Gels were cut into 2-mm slices with a Mickle gel slicer, and each slice was macerated in 1 ml of elution buffer (1 mM EDTA, 10 mM Tris, pH 7.9, containing 10 μg of yeast tRNA per ml), using a Dounce homogenizer. RNA was eluted by shaking at 4°C overnight. The agarose was pelleted by low-speed centrifugation, and the supernatant fractions were retained. A 10-μl sample of each fraction was counted in Aquasol to estimate the distribution of labeled RNA in the gel.

Analytical RNA electrophoresis was performed on slab gels (20 cm long). Electrophoresis was at 30 V overnight, and upon completion, gels were soaked in 0.5 M ammonium acetate containing 1 μg of ethidium bromide per ml for 1 h. RNA bands were visualized by UV fluorescence, and the gels were dried and subjected to autoradiography by using Kodirex film.

Hybridization of RNA to total HSV-1 DNA. Hybridization of [^{32}P]RNA to HSV-1 DNA bound to nitrocellulose filters was performed as described previously (9). Individual gel fractions were hybridized to one filter containing 2 μg of HSV-1 DNA and one filter containing 2 μg of bacteriophage λ DNA. Counts bound to λ DNA filters were subtracted from those bound to HSV-1 DNA filters to estimate HSV-1-specific RNA.

Quantitative estimates of the percentage of virus-specific labeled RNA in the total cytoplasmic poly(A) RNA and in each of the pooled fractions were made as above, except that filters contained 5 μg of DNA and reactions were allowed to proceed for 40 h. The efficiency of these reactions was estimated in a parallel reaction, using HSV-1 cRNA, made with *Escherichia coli* RNA polymerase as described previously (3). Under these conditions, it was found that approximately 45% of the HSV-1 cRNA input hybridized to the HSV-1 DNA filters.

Preselection of RNA against HSV-1 DNA fragments. Poly(A) RNA in 70% formamide–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was incubated for 20 h at 37°C, with 10-μg equivalents of the individual HSV-1 DNA fragments bound to nitrocellulose disks. Filters were extensively washed in 2× SSC, either with or without the addition of 25 μg of RNase A per ml. The filters were then incubated in iodoacetate solution (0.15 M sodium iodoacetate, 0.1 M sodium acetate in 2× SSC, pH 5.2) at 54°C for 40 min to destroy any residual RNase activity (23), and further washed in 2× SSC. Hybridized RNA was eluted from the filters by boiling in 0.1× SSC for 10 min in the presence of 10 μg of yeast tRNA carrier per ml.

Hybridization to blots. [^{32}P]RNA was hybridized to HSV-1 DNA fragments and immobilized to nitrocellulose membrane by the Southern blot technique (21), as described previously (3). Hybridization to fragments was detected by fluorography, using Kodak X-Omat H film and DuPont Cronex Lightning Plus intensifying screens. Films were allowed to expose for up to 3 weeks at −70°C.

In vitro translation. The pooled RNA fractions were made 0.5 M with respect to KCl and reselected on oligo(dT)-cellulose columns, as described above, to remove residual agarose. The RNA eluted from the columns was ethanol precipitated in the presence of 5 μg of *E. coli* rRNA carrier. RNA was recovered by low-speed centrifugation, dissolved in distilled water, and used to direct cell-free translation in the rabbit reticulocyte lysate system described previously (18).

Endogenous mRNA was destroyed by preincubation with micrococcal nuclease (16). The products of in vitro translation were separated by polyacrylamide gel electrophoresis (PAGE) as described by Marsden et al. (11), and were detected by autoradiography by using Kodirex film.

Preparation of in vivo-labeled IE polypeptides. Cells were infected with 50 PFU of HSV-1 per cell in medium containing 200 μg of cycloheximide per ml and incubated for 5 h in the presence of the drug. Cultures were then washed three times with prewarmed medium containing 5 μg of actinomycin D per ml and incubated for 1 h with phosphate-buffered saline containing 100 μCi of [^{35}S]methionine per ml. Cells were harvested and disrupted as described by Marsden et al. (11).

RESULTS

Analytical RNA electrophoresis. Cytoplasmic RNAs were isolated from infected (IE) and uninfected (MI) cells labeled for 6 h with [^{32}P]orthophosphate in the presence of cycloheximide, and the poly(A) RNAs were selected by chromatography on oligo(dT)-cellulose columns. This procedure did not result in the complete removal of ribosomal RNA, but the trace amounts of endogenous 28S and 18S rRNA's provided useful size markers on subsequent analyses.

IE and MI poly(A) RNA's were denatured with 25 mM CH_3HgOH and electrophoresed in parallel on an agarose slab gel containing 5 mM CH_3HgOH. After electrophoresis, the gel was stained with ethidium bromide, and the RNA was visualized by UV fluorescence (Fig. 1). In addition to the 28S and 18S rRNA bands seen in the MI RNA track, there were three virus-induced bands in the IE RNA track. These virus-induced bands were also detected by autoradiography of the gel (Fig. 2). By using the rRNA species as markers assumed to be 5.3 kilobases (kb) and 2.1 kb in length (12), the sizes of the virus-induced bands were estimated to be 4.7, 3.0, and 2.0 kb. Henceforth, these RNAs will be referred to by their size designation.

Preparative RNA electrophoresis. IE and MI poly(A) RNA preparations (approximately 5 μg each) were electrophoresed in parallel through agarose tube gels containing CH_3HgOH. After electrophoresis, the gels were soaked in 10 mM β-mercaptoethanol to remove CH_3HgOH (1) and UV scanned to detect the rRNA markers and the three virus-induced bands.

The gels were then cut into 2-mm slices, the slices were homogenized, and the RNA was eluted overnight in low-salt buffer. Most of the agarose was removed by low-speed centrifugation, and a sample of each supernatant was counted to determine the total distribution of labeled RNA in the gel. This is shown for IE RNA in Fig. 3.

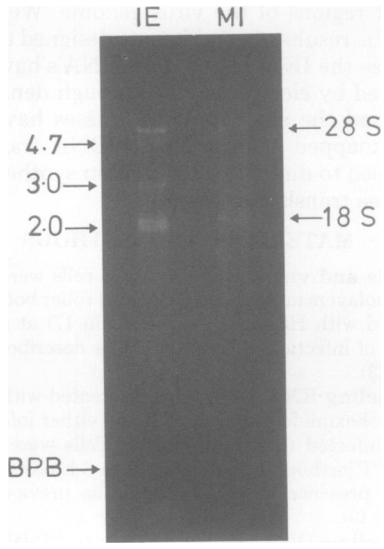

FIG. 1. *Poly(A) cytoplasmic RNAs isolated from HSV-1-infected (HSV) and MI BHK cells were separated by electrophoresis through CH_3HgOH agarose gels and detected by UV illumination after ethidium bromide staining. The positions of the endogenous 28S and 18S rRNA's, the 4.7-, 3.0-, and 2.0-kb virus-induced RNA species, and the bromophenol blue (BPB) dye front are indicated.*

Part of each eluted gel fraction was then hybridized to total filter-bound HSV-1 DNA to determine the distribution of virus-specific RNA (Fig. 3). The distribution of virus-specific RNA closely paralleled that of total RNA, and the peaks exactly coincided. This experiment did not allow accurate estimation of the percentage of total counts that were virus specific, since the eluate volumes varied somewhat and the hybridization reactions did not go to completion. More quantitative estimates were made by using, in parallel, the hybridization of HSV-1 cRNA (made with *E. coli* RNA polymerase) to measure the efficiency of the hybridization reactions; this showed that total labeled IE RNA was approximately 70% virus specific. These proportions for the virus-specific peak fractions were greater than 80%.

The portions of the IE RNA fractions remaining were pooled as indicated in Fig. 3 (fraction 1 contained the 4.7-kb species). The MI RNA fractions of equivalent size were likewise pooled, using the endogenous rRNA species to align the two gels. The pooled IE RNA fractions were divided; one half was hybridized to HSV-1 DNA fragments and the other was used to direct protein synthesis in vitro. The pooled MI RNA fractions were similarly translated in vitro.

Hybridization of gel fractions to HSV-1 DNA fragments. The pooled IE RNA fractions

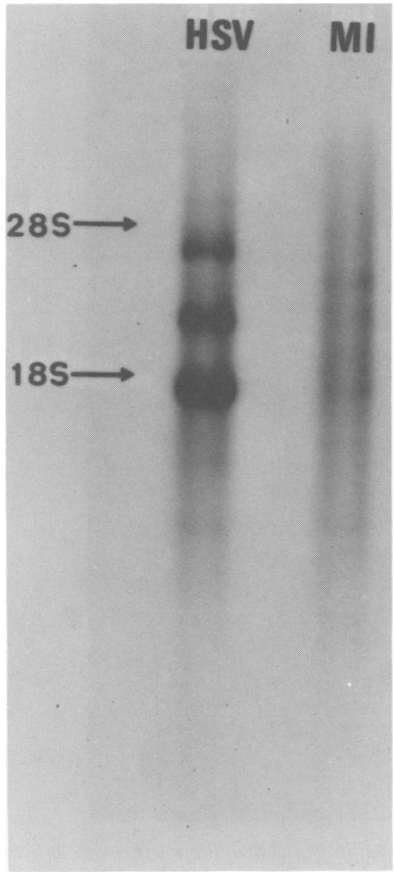

FIG. 2. *Autoradiograph of the gel shown in Fig. 1.*

were each hybridized to blot strips containing the DNA fragments generated by digestions with the *Bam*HI, *Hin*dIII, and *Hpa*I restriction endonucleases, and hybridization was visualized by fluorography. Interpretation of these RNA mapping data requires a knowledge of the HSV-1 genome structure.

As first described by Sheldrick and Berthelot (20), the genome has an unusual structure, consisting of two unique DNA regions (U_L and U_S), each flanked by inverted repeat regions (TR_L/IR_L, TR_S/IR_S) and joined at IR_L-IR_S. Four genome arrangements, resulting from the possible combinations of inversions of the two unique regions, are present in DNA preparations in approximately equal amounts (2, 7). A further consequence of this structure is that a number of fragments produced by digestion with a restriction endonuclease may contain sequences in common (25). Hence, hybridization of RNA to a fragment containing any part of the repeat regions does not necessarily imply that this RNA was transcribed from that particular fragment.

Physical maps for the DNA fragments generated by digestion with the enzymes used in these analyses have been obtained (25; A. J. Davison and N. M. Wilkie, personal communication). These maps are shown for one orientation of the unique regions (Fig. 4). Fluorographs of the pooled IE RNA fractions hybridized to the *Bam*HI, *Hin*dIII, and *Hpa*I digests of HSV-1 DNA are shown in Fig. 5, 6, and 7, respectively, and are compared to the total fragment patterns (revealed by hybridization of ^{32}P-labeled HSV-1 DNA to the blot strips) and the hybridization patterns of unresolved polyadenylated cytoplasmic IE RNAs.

It is apparent from hybridization to the *Bam*HI digest (Fig. 5) that good separation of RNA sequences has been obtained. Hence, with peak fraction 5 there was abundant hybridization to *Bam*HI-n, -x, and -z, as compared to little or no hybridization to these fragments with frac-

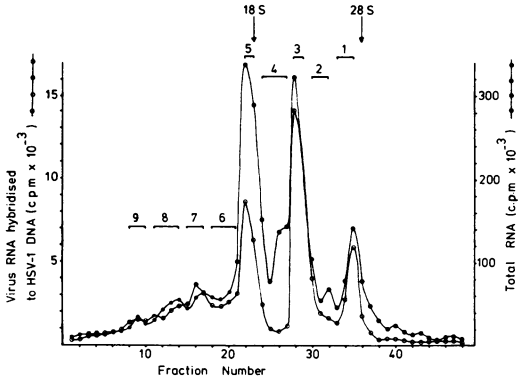

FIG. 3. *Distribution of ^{32}P-labeled total and virus-specific RNA after resolution of IE mRNA's on preparative gels. The positions of endogenous 28S and 18S rRNA's are indicated, as are the positions of the eluted RNA fractions (underlined numbers 1 through 9), which were pooled and used in subsequent analyses.*

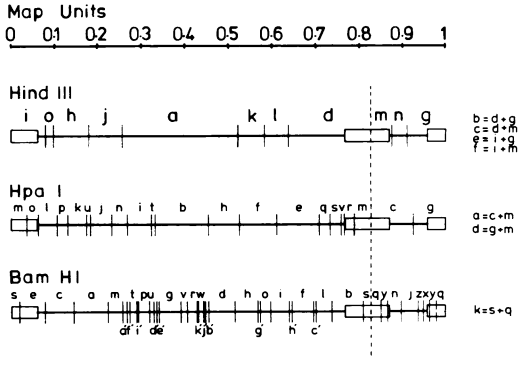

FIG. 4. *Physical maps for the fragments of HSV-1 DNA generated by restriction endonucleases HindIII, HpaI, and BamHI.*

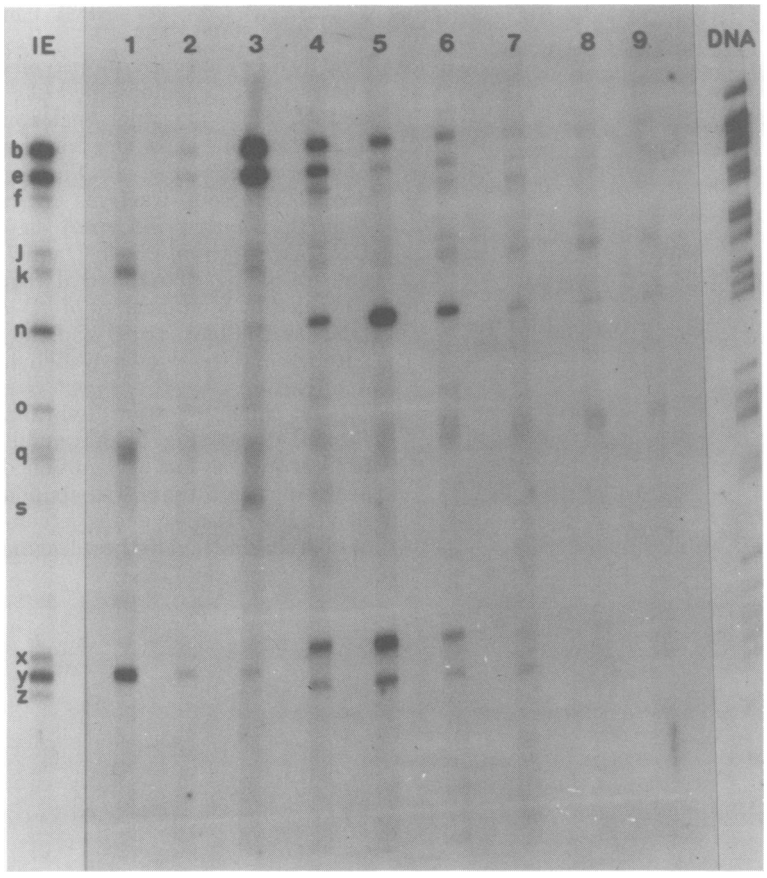

FIG. 5. *Fluorographs of the pooled IE RNA fractions indicated in Fig. 3 (fractions 1 through 9) hybridized to the BamHI fragments of HSV-1 DNA are compared to the hybridization patterns of unresolved cytoplasmic poly(A) IE RNAs (IE) and HSV-1 DNA labeled by nick translation (DNA). Fractions 1, 3, and 5 contained the 4.7-, the 3.0-, and the 2.0-kb RNA size classes, respectively.*

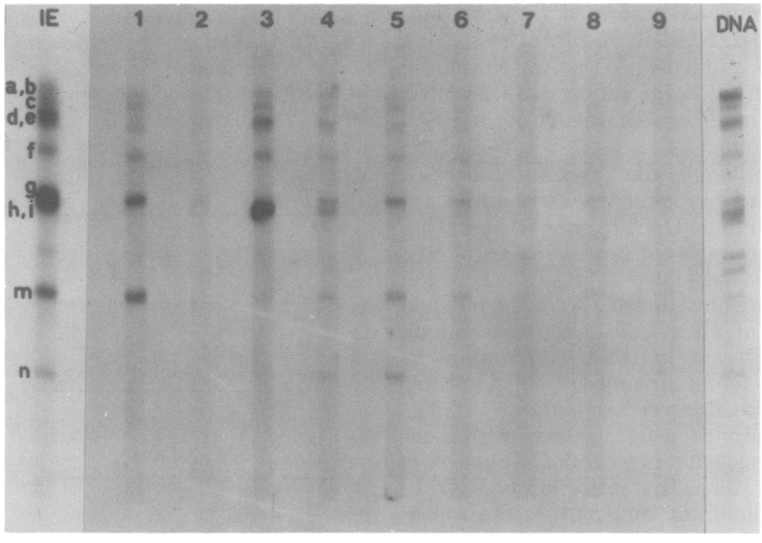

FIG. 6. *Fluorographs of the pooled IE RNA fractions (1 through 9) hybridized to the HindIII fragments of HSV-1 DNA are compared to the hybridization patterns of unresolved cytoplasmic poly(A) RNAs (IE) and HSV-1 DNA (DNA).*

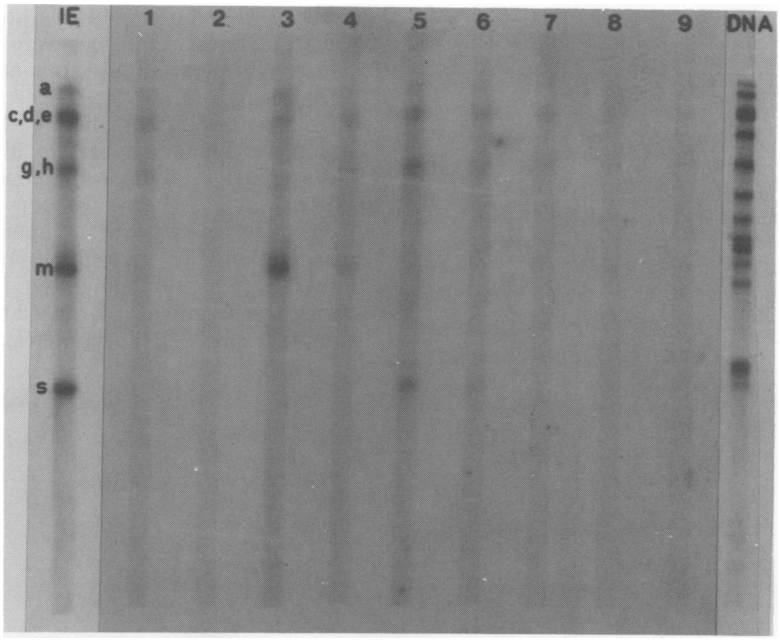

FIG. 7. *Fluorographs of the pooled IE RNA fractions (1 through 9) hybridized to the HpaI fragments of HSV-1 DNA are compared to the hybridization patterns of unresolved cytoplasmic poly(A) RNAs (IE) and HSV-1 DNA (DNA).*

tions 3 and 1. A small amount of material hybridizing to *Bam*HI-j, -k, and -q appears to be distributed throughout the gel, and this presumably reflects considerable size heterogeneity of RNA species containing these sequences. Little RNA complementary to *Bam*HI-o was detected after resolution of IE RNAs on these gels, and it is possible that these sequences were resolved outside the gel fractions analyzed.

The RNA mapping data for fractions 1, 3, and 5 (corresponding to the 4.7-, 3.0-, and 2.0-kb RNA species) have been summarized in Fig. 8. In making this summary, only abundant hybridization to fragments has been considered. This analysis may locate only the major sequences present in the separated RNAs. Hence, small noncontiguous or leader sequences may fail to be detected.

Fraction 1 hybridized predominantly to *Bam*HI-y and also to -k and -q. Fragments y and q are adjacent and map wholly within TR_S/IR_S (Fig. 4), whereas k is a fusion fragment of q and s. Because there was no hybridization to fragment s, the 4.7-kb RNA appears to hybridize exclusively to part of the TR_S/IR_S region.

Fraction 3 (the 3.0-kb RNA) hybridized predominantly to *Bam*HI-b and -e, and also to -s. *Bam*HI-s lies entirely within TR_L/IR_L, whereas fragments b and e contain the same sequences from TR_L/IR_L but differ in containing sequences from opposite ends of U_L. That hybridization was to the repetitive rather than the unique DNA sequences of *Bam*HI-b and -e may be deduced from the *Hpa*I digest (Fig. 7), where there was hybridization to *Hpa*I-m, but not to *Hpa*I-s or to any other fragment which does not contain sequences present in *Hpa*I-m. The 3.0-kb RNA, therefore, hybridized only to part of TR_L/IR_L.

Fraction 5 (the 2.0-kb RNA) hybridized predominantly to three distinct regions of the virus genome. There was hybridization to *Bam*HI-b, -n, -x, and -z (Fig. 5). Fragments x and z are adjacent and lie at the right end of U_S (in the orientation given in Fig. 4). Fragment z contains only U_S sequences, whereas x contains part of TR_S/IR_S in addition to U_S sequences. A priori, hybridization to *Bam*HI-n could, therefore, be due to sequences shared with x. However, since there was hybridization to *Hin*dIII-n (Fig. 6), which maps entirely within the left end of U_S, fraction 5 must contain sequences homologous to each end of U_S. Evidence that these transcripts are in part complementary to TR_S/IR_S is presented below.

The third region to which fraction 5 hybridized maps in *Bam*HI-b. The *Hpa*I digest (Fig. 7) indicates that this hybridization was to the U_L sequences of *Bam*HI-b, since there was hybridization to *Hpa*I-s, but not to *Hpa*I-m or -r.

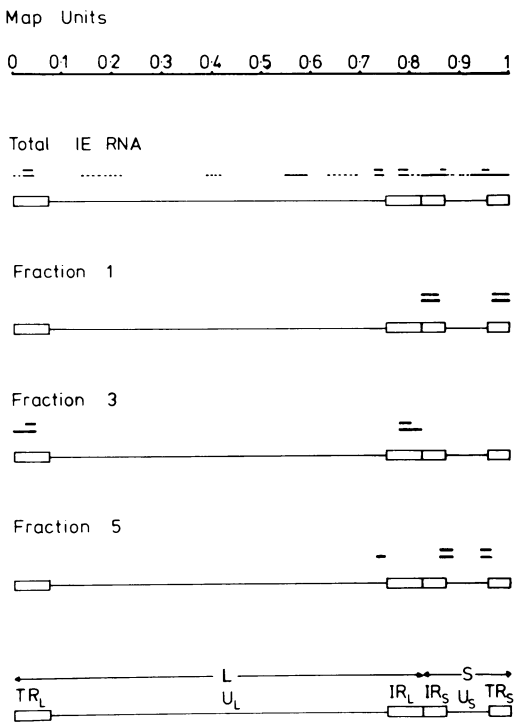

FIG. 8. *Summary of transcript mapping data. Map locations of the IE mRNA's contained in resolved fractions 1, 3, and 5 (the 4.7-, 3.0-, and 2.0-kb RNAs, respectively) are compared to those of unresolved cytoplasmic poly(A) IE mRNA's by the following criteria: (i) regions to which an abundance of RNA hybridized are indicated by a double continuous line; (ii) clearly detectable hybridization to a region is represented by a single continuous line; and (iii) low levels of hybridization are indicated by a discontinuous line.*

Selection of transcripts which overlap the junction between U_S and TR_S/IR_S. To provide evidence that the 2.0-kb transcripts which map at each end of U_S are also homologous in part to TR_S/IR_S, total cytoplasmic poly(A) IE RNAs were hybridized at low temperature to duplicate filters containing either *Bam*HI-n or -z. After hybridization, the filters were washed extensively. One of each duplicate was then treated with RNase under conditions in which RNA in an RNA/DNA hybrid is protected, but single-stranded RNA is susceptible to digestion. This treatment, therefore, eliminates tails of nonhomologous RNA sequences covalently linked to the hybridized RNA sequences. RNA sequences from the RNase-treated and untreated filters were hybridized to the total *Bam*HI DNA fragments (Fig. 9). RNA selected against *Bam*HI-n, both from treated and untreated filters, was homologous to *Bam*HI-n and -x. Nondigested filters selected, in addition, RNA sequences homologous to *Bam*HI-z. These sequences were susceptible to RNase treatment and are thus nonhomologous to fragment n. The RNase-resistant sequences which hybridized to fragment x, therefore, must be complementary to part of TR_S/IR_S, since these sequences alone are common to both -n and -x.

The RNase-resistant sequences selected against *Bam*HI-z hybridized only back to *Bam*HI-z (Fig. 9). Undigested RNA selected against this fragment hybridized to both -x and -n, in addition to -z. This implies that there is a

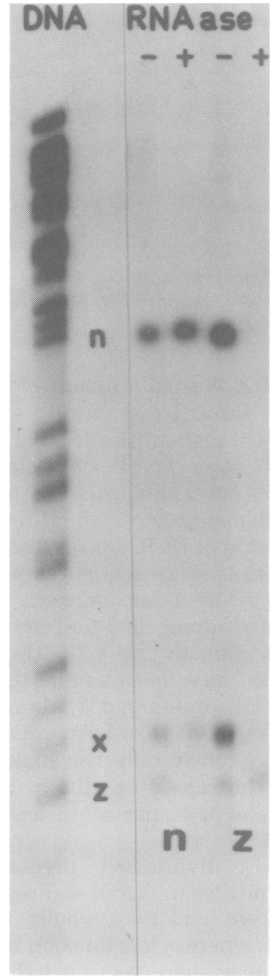

FIG. 9. *IE mRNA's selected by hybridization to filters containing HSV-1 DNA fragments BamHI-n or -z were either treated with RNase (+) or untreated (−), and the hybridized RNAs were eluted. Fluorographs of the eluted RNA samples hybridized to blot strips containing the BamHI DNA fragments (right) are compared to the total fragment pattern (left) revealed by hybridization of ^{32}P-labeled HSV-1 DNA to the fragments.*

transcript which spans the *Bam*HI cleavage site between -z and -x and which contains sequences present in TR_S/IR_S.

In vitro translation of pooled RNA fractions. The pooled IE and MI RNA fractions were used to direct protein synthesis in a rabbit reticulocyte cell-free translation system. For comparison, unfractionated IE and MI RNAs were translated in parallel. The products of in vitro translation were analyzed by PAGE, and the resultant autoradiographs are shown in Fig. 10 and 11. Also shown are IE and MI polypeptides labeled in vivo.

A number of virus-specific polypeptides were made when the cell-free translation system was programmed with total IE RNA, and attention is drawn to polypeptides V_{mw} 175, 136, 110, 87, 63, and 12 (Fig. 10). The synthesis, both in vivo and in vitro, of a virus-induced polypeptide of estimated molecular weight 38,000 was also noted (Fig. 10 and 11). This polypeptide has not been classified an IE polypeptide, since its synthesis is not invariably observed.

The fractionated IE RNA preparations, likewise, directed the synthesis of a number of virus polypeptides. Fraction 3 (the 3.0-kb mRNA band) specified primarily V_{mw} 110, although small amounts of V_{mw} 87 were also synthesized (Fig. 10). Synthesis of this latter polypeptide was also directed by fraction 4 RNA, which indicates that the major IE mRNA contained by fraction 3 specified V_{mw} 110.

Fraction 5 (the 2.0-kb mRNA's) specified the in vitro synthesis of V_{mw} 68, 63, and 12 (Fig. 10). The synthesis of small amounts of V_{mw} 63 and reduced amounts of V_{mw} 12 was also directed by fraction 4. The virus-induced polypeptide of estimated molecular weight 38,000 was primarily specified by fraction 7.

Few products of translation of fraction 1 mRNA (the 4.7-kb size class) were apparent (Fig. 10), and this presumably reflects the difficulty of isolating large mRNA's intact. A longer exposure of the gel shown in Fig. 10 (data not shown) indicated that small amounts of V_{mw} 175 were directed by fraction 1. Because synthesis of other high-molecular-weight polypeptides was also apparent at such an exposure, demonstration that the 4.7-kb virus mRNA specified V_{mw} 175 alone is equivocal.

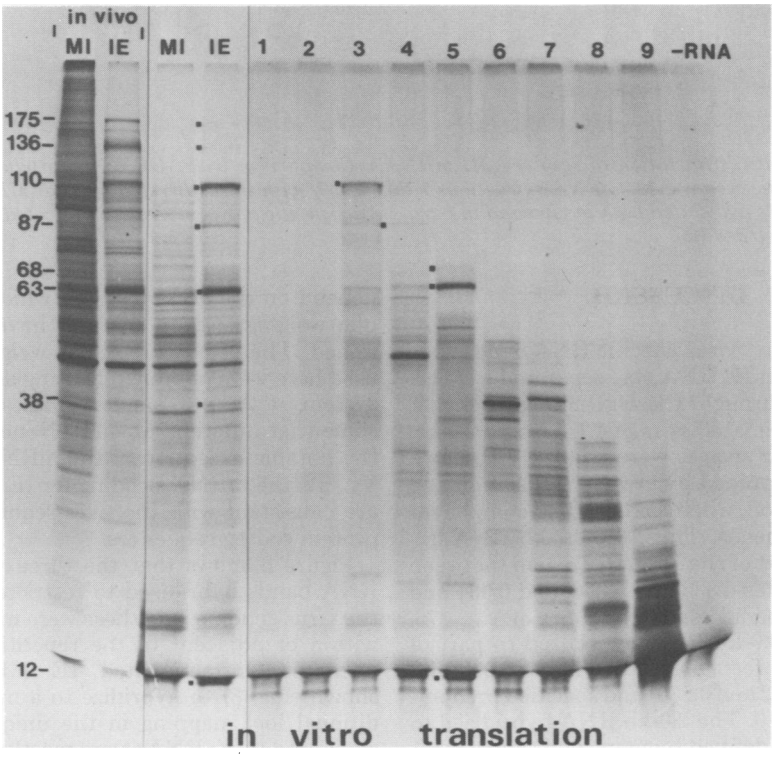

FIG. 10. *In vitro translation of resolved IE mRNA's. Comparison by PAGE of the products of in vitro translation of the resolved IE mRNA's (fractions 1 through 9) with those directed by total IE mRNA's and total MI mRNA's translated both in vivo and in vitro. Also shown are the products of translation of endogenous mRNA when the cell-free system was incubated in the absence of added RNA (−RNA). Virus-specific polypeptides are indicated to the left of the appropriate track* (■).

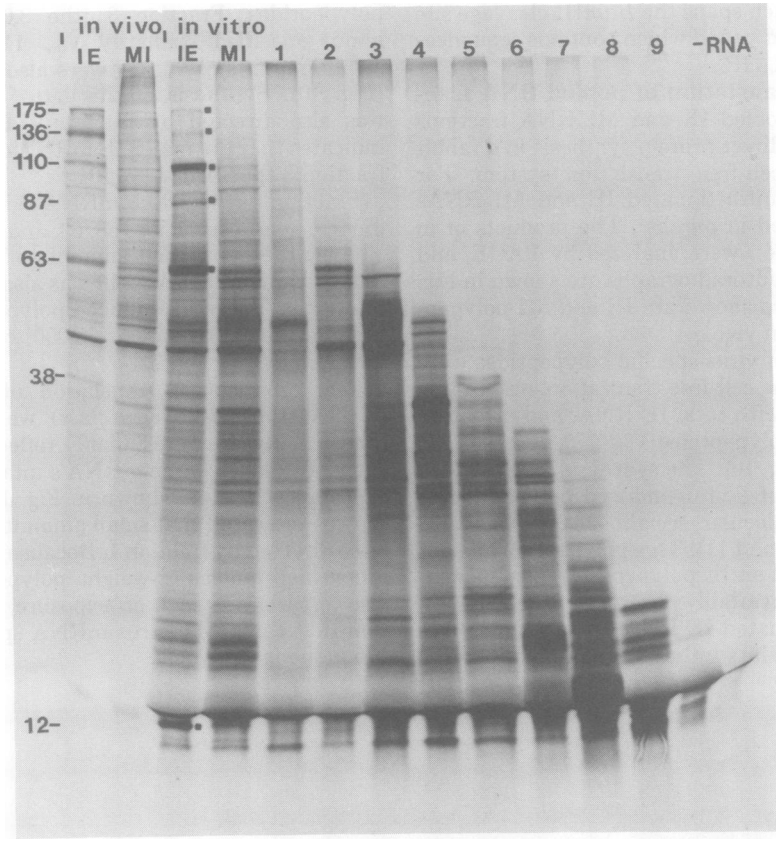

FIG. 11. *In vitro translation of resolved MI mRNA's. Comparison by PAGE of the products of in vitro translation of the resolved MI mRNA's (fractions 1 through 9) with those directed by total MI mRNA's and total IE mRNA's translated both in vivo and in vitro. Virus-specific polypeptides are indicated to the right of the appropriate track* (■).

DISCUSSION

Three distinct virus-specific RNA bands were observed when IE RNA was separated by electrophoresis through CH_3HgOH agarose gels. These had estimated sizes of 4.7, 3.0, and 2.0 kb. The two larger species were relatively homogeneous as determined by in vitro translation and genome location, whereas the 2.0-kb component was heterogeneous. Thus, the 4.7-kb RNA hybridized to part of the TR_S/IR_S region (between map coordinates 0.830 and 0.866, and 0.964 and 1.000) and specified small amounts of V_{mw} 175, whereas the 3.0-kb RNA hybridized to part of TR_L/IR_L (between map coordinates 0.000 and 0.038, and 0.792 and 0.830) and specified predominantly V_{mw} 110. The 2.0-kb RNA hybridized to three regions (defined by map coordinates 0.741 and 0.754, 0.865 and 0.898, and 0.938 and 0.964) and specified the synthesis of three polypeptides (V_{mw} 68, 63, and 12).

These virus RNA species were sized by using 28S and 18S rRNA's as markers, and estimates depend on the accuracy with which the molecular weights of these markers have been determined. The rRNA molecular weight estimates used here were obtained by using denaturing gel systems (12), but similar studies have given somewhat different sizes (1). Nonetheless, with the notable exception of the mRNA coding for V_{mw} 12, the mRNA's coding for IE polypeptides are consistent with the sizes required for their protein coding sequences.

Figure 8 shows that the three virus-specific RNA bands hybridized to restricted regions of the virus genome, and these were mostly located within or adjacent to the repetitive DNA sequences. Unfractionated IE mRNA's were shown (Fig. 8) to hybridize to a number of additional loci mapping in the unique DNA regions, and these RNAs were mostly of low abundance (Fig. 8). IE RNAs of low abundance were not considered when making the summaries of the DNA regions represented by the three virus mRNA size classes. Furthermore, certain transcripts, i.e., those hybridizing to *Bam*HI-j (Fig.

5), were heterodisperse in size and were not resolved by using this gel system. Therefore, these analyses allow characterization of the abundant IE mRNA's only.

HSV-1 IE polypeptides have previously been mapped by analyses of the virus genomes and the polypeptides expressed by intertypic recombinants between HSV types 1 and 2 (13, 19). These analyses depended upon small differences in mobility on PAGE between equivalent HSV types 1 and 2 polypeptides which allowed identification of the type-specific proteins expressed by recombinants. Strictly, this method maps the genome regions affecting polypeptide mobility rather than delineating the polypeptide coding regions. The technique used here directly maps the mRNA's coding for these polypeptides. The combination of both sets of data has allowed some of the IE polypeptides to be mapped on the HSV-1 genome more precisely.

Intertypic recombinant analyses have mapped V_{mw} 175, which is equivalent to ICP 4 (8), in TR_S/IR_S (13, 19) and V_{mw} 110, which is equivalent to ICP 0 (8), in TR_L/IR_L. Recombinants which are heterotypic for TR_L/IR_L express both the HSV type 1 V_{mw} 110 and its V_{mw} 118 type 2 equivalent (19). Because both TR_L and IR_L are expressed even in such recombinants which have a fixed, noninverting orientation of the L region (19), this indicates that V_{mw} 110-coding sequences lie entirely within repetitive DNA. This location is consistent with our data. Recombinants heterotypic for TR_S/IR_S made both the HSV-1 ICP 4 polypeptide and its HSV-2 equivalent (13). This suggests that these coding sequences map entirely within TR_S/IR_S. Our data are consistent with this location and indicate that the mRNA is homologous to only part of TR_S/IR_S (there was no hybridization of the 4.7-kb mRNA to *Bam*HI-n or -x).

Previous analyses of intertypic recombinants in our laboratory have mapped V_{mw} 63 between coordinates 0.562 and 0.790, and V_{mw} 68 and V_{mw} 12 in the S component (19). Correlation of these analyses with the data shown here gives a more precise map location for V_{mw} 63 (between map coordinates 0.741 and 0.754) and places V_{mw} 68 and 12, respectively, between coordinates 0.865 and 0.898, and 0.938 and 0.964. Similar analyses of intertypic recombinants reported by other workers (13) mapped ICP 27 (an IE polypeptide probably equivalent to V_{mw} 63) between map coordinates 0.790 and 0.830, which is part of TR_L/IR_L. The reason for the discrepancy between these data and our map location is unclear. However, our analyses were performed by labeling the IE proteins synthesized upon release of a cycloheximide block, whereas those of Morse et al. (13) were performed by pulse-labeling the polypeptides made at various times postinfection. Since ICP 27 was labeled when cells were pulsed from 9 to 10 h postinfection, a time at which IE polypeptide synthesis is negligible (8), it is unlikely that the protein mapped was equivalent to the IE polypeptide V_{mw} 63. It is also pertinent that recombinants heterotypic for TR_L/IR_L made only one type-specific ICP 27 (13).

The results presented here indicate that the two 2.0-kb transcripts which map in the S region overlap the junction between U_S and both TR_S and IR_S. The reasons for this conclusion are that only the 2.0-kb RNA (fraction 5) hybridized to fragments (*Bam*HI-n and -x) containing these junctions, and that IE transcripts homologous to *Bam*HI-n are also, in part, homologous to *Bam*HI-x. Data recently obtained in our laboratory (J. B. Clements and D. J. McGeoch, manuscript submitted for publication), by mapping cDNA made to the 3′ end of IE mRNA's against HSV-1 DNA fragments, have indicated that the 3′ ends of both these mRNA's lie in U_S. This implies that the 5′ ends of these mRNA's map within TR_S/IR_S, and it seems reasonable to presume that the two transcripts are transcribed from locationally distinct promoters of identical sequence. Two such mRNA's would contain identical 5′ ends, and distinct 3′ ends.

V_{mw} 12 is synthesized from a much larger mRNA than is required for its coding sequences. Such a situation has been described for the small T-antigen of simian virus 40 (14), which shares *N*-terminal peptides with the large T-antigen (15) and is terminated at a codon which appears to be excised from the large T-antigen mRNA (5). Polypeptides with identical *N*-terminal regions could arise with HSV-1 as a consequence of the genome structure. Hence, the mRNA's which code for V_{mw} 68 and V_{mw} 12 and which may map at either end of U_S could code for proteins with identical *N*-terminals (coded for by TR_S/IR_S sequences) but distinct C-terminals (coded for by U_S sequences). These two polypeptides chains would be terminated by nonidentical terminator codons (encoded by unique DNA sequences from opposite ends of U_S), and the maximum length encoded by the TR_S/IR_S DNA would be that required to specify V_{mw} 12.

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