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Detailed Characterization of the mRNA Mapping in the *Hin*dIII Fragment K Region of the Herpes Simplex Virus Type 1 Genome

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We have isolated as recombinant DNA clones, in the plasmid pBR322, regions of the herpesvirus type 1 genome spanning the region between 0.53 and 0.6 on the prototypical arrangement. This 11,000-base-pair region corresponds to 10% of the large unique region and encodes five major and several minor mRNA species abundant at different times after infection, which range in length from 7 to 1 kilobase. In this report, we have used RNA transfer blots and S1 nuclease digestion of hybrids between viral DNA and polyribosomal RNA to precisely localize (±0.1 kilobase) these mRNA's. Comparison of neutral and alkaline gels of S1 nuclease-digested hybrids indicates no internal introns in the coding sequences of these mRNA's, although noncontiguous leader sequences near (ca. 0.1 kilobase) the 5′ ends of any or all mRNA's could not be excluded. The 5′ ends of several late mRNA's that are encoded opposite DNA strands map very close to one another, and the 3′ ends of a major late and a major early mRNA, which are partially colinear, terminate in the same region. In vitro translation of the viral mRNA's isolated by hybridization with DNA bound to cellulose and fractionation of mRNA species on denaturing agarose gels allowed us to assign specific polypeptide products to each of the mRNA's characterized. Among other results, it was demonstrated unequivocally that two major late mRNA's, which partially overlap, encode the same polypeptide.

The genome of herpes simplex virus type 1 (HSV-1) is large and has a rather complex arrangement, which has been reviewed by Roizman (33). One arrangement has been generally accepted as prototypical (P), and this, too, is described in this review (33). Transcription of the genome of this virus is regulated throughout the infectious cycle. Before de novo protein synthesis, a very restricted class of viral mRNA species and polypeptides is synthesized from the α or immediate-early genes of the virus (3, 10, 11, 17-22, 31, 32, 46). These gene products are presumably regulatory in function, and several appear to be synthesized throughout infection (3, 32). At least one seems to be required continuously for normal later viral gene expression (32).

After the synthesis and function of this first group of gene products, but before viral DNA replication, a relatively large number of true early or β mRNA species and their corresponding polypeptides are expressed (16-19, 21, 36, 37, 39, 40, 44, 45; Wagner et al. *in* Y. Becker, ed., *Herpesvirus DNA: Recent Studies on the Internal Organization and Replication of the Viral Genome,* in press); a number of these are either directly or indirectly involved in initiating and maintaining HSV-1 DNA replication.

After viral DNA replication, another large group of viral genes is expressed. These are the late or γ genes (4, 5, 16, 18, 19, 21, 36; Wagner et al., in press). A large number of these code for virion structural proteins. These late viral genes can be roughly divided into two groups. The first includes those genes which are marginally expressed in the absence of viral DNA replication, and the second group includes those that are only detectable after viral DNA replication (16, 21, 36, 39, 40, 44, 45; Wagner et al., in press).

To reduce the gross phenomenology of HSV-1 mRNA expression to manageable proportions, we concentrated our studies on certain regions of the viral genome that display features of general interest. One such region is that roughly encompassed by *Hin*dIII fragment K, a piece of DNA 9.8 kilobases (kb) in length mapping from 0.527 to 0.592 from the left end of the (P) arrangement of the HSV-1 genome (4, 17; Wagner

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et al., in press). We previously reported the results of experiments which indicate that a 5.2-kb mRNA homologous to this region is marginally expressed as mRNA in the α or immediate-early phase of replication (3, 17). We also reported (4, 16, 17) that this mRNA is a major early mRNA and encodes a 140,000-dalton (d) polypeptide. After viral DNA replication, further abundant mRNA species, 7, 3.8, and approximately 2 kb in size, encoding at least two other polypeptides (122,000 and 54,000 d), are seen along with the continual synthesis of the 5.2-kb mRNA. Further, preliminary mapping data indicated that the 7- and 5.2-kb and a small mRNA species must overlap.

In the present paper, we report the results of experiments with recombinant DNA technology, S1 nuclease analysis, and in vitro translation of highly resolved mRNA species. We have precisely mapped (±0.1 kb) five major mRNA species and have assigned specific polypeptides to each. Further, two other mRNA species, whose 3′ ends map to the left of 0.6 on the (P) arrangement of the HSV-1 genome, but whose 5′ ends extend to the right of this coordinate, have been partially characterized. Our data indicate that the details of gene expression in this region are of a sufficient complexity to warrant even more detailed studies in the future.

MATERIALS AND METHODS

Cells and virus. Monolayer cultures of HeLa cells were grown at 37°C in Eagle minimal essential medium containing 10% calf serum and no antibiotics. Plaque-purified virus of the KOS strain of HSV-1 was used for all infections.

Enzymes. All restriction endonucleases were obtained from New England Biolabs, and digestion was carried out in buffers recommended by that supplier. Each microgram of DNA was digested with 0.5 U of the appropriate enzyme for 3 h at 37°C. Phage T4 ligase was obtained from the Bethesda Research Laboratories. The ligation reaction was essentially as recommended by the supplier. A solution of 10 μg of restricted HSV-1 and 1 μg of pBR322 DNA in 25 μl of solution was incubated with each unit of ligase in 66 mM Tris (pH 7.6)–6.6 mM $MgCl_2$–10 mM dithiothreitol–0.4 mM ATP for 6.5 h at 14°C. Then the solution was diluted to 250 μl, and incubation was continued for a further 20 h. Reverse transcriptase from avian myeloblastosis virus was supplied by Joseph Beard. The incubation was carried out as described previously (3–5, 16, 17) with a 1,000-fold excess of random oligodeoxyribonucleotides primer (42) or a 100-fold excess of oligodeoxythymidylic acid [oligo(dT_{12-18})] (Collaborative Research, Inc.). Incubation mixtures were 50 μl and contained 50 mM Tris (pH 8.1); 50 mM KCl; 8 mM $MgCl_2$; 50 μCi of [^{32}P]TTP (350 Ci/mM; New England Nuclear Corp.); 0.2 mM each of dATP, dCTP, and dGTP; 4 mM dithiothreitol, 10 μg of actinomycin per ml; 0.1 to 1 μg of denatured DNA or RNA template primer; and 2 U of reverse transcriptase. Incubation was for 60 min at 37°C. For random primed reactions, an additional unit of enzyme and 0.1 μmol each of dGTP, dCTP, and dATP were added after 1 h, and incubation was carried out for a further hour at 37°C. The S1 nuclease was obtained from Boehringer-Mannheim as a lyophilized powder. Best results were obtained with lot no. 1409409, although usable results occurred with several earlier lots. Samples of 5×10^5 U, as supplied, were dissolved in 0.5 ml of S1 buffer (0.25 M NaCl, 0.03 M NaOAc [pH 4.6], 0.001 M $ZnCl_2$) containing 50% glycerol. For each digestion, 1×10^4 U was used, and samples containing 15 μg of polyadenylic acid [poly(A)] RNA, 10 μg of carrier tRNA, and 3 μg of [^{32}P]DNA (specific activity, 50,000 to 60,000 Cerenkov cpm/μg) were digested in S1 buffer containing 5% glycerol for 30 min at 45°C.

Isolation, labeling and size fractionation of polyribosomal RNA. Monolayer cultures of HeLa cells (2×10^7 cells/flask) were infected for 30 min at a multiplicity of 10 PFU of virus per cell in phosphate-buffered saline containing 0.1% glucose and 1.0% fetal calf serum. For ^{32}P-labeled RNA, the cells were overlaid with 200 μCi of $^{32}P_i$ per ml (2.5 to 3.0 mCi per culture; New England Nuclear Corp.) in Eagle minimal essential medium containing a one-tenth normal concentration of phosphate and 5% dialyzed calf serum. Time of infection was measured after the 30-min absorption period. Viral RNA synthesized in the absence of HSV-1 DNA synthesis (early RNA) was prepared from cells pretreated for 1 h and incubated for 6 h postinfection with 1.5×10^{-4} M adenosine arabinoside and 3.7×10^{-6} M pentostatin, as described previously (16). The drugs were a gift of C. Shipman of the University of Michigan.

Polyribosomes were isolated from the cytoplasm of HSV-1-infected cells by the magnesium precipitation method of Palmiter (29). Polyribosome-associated RNA was purified by phenol-chloroform extraction after proteinase K digestion of the polyribosomes. Poly(A)-containing mRNA [poly(A) RNA] was isolated from total rRNA by the use of (oligo(dT)-cellulose. Details of this procedure have been presented elsewhere (3–5, 16, 17).

RNA was size fractionated by electrophoresis on 1.2% agarose gels containing 10 mM methylmercury hydroxide (6), as has been previously described (3–5, 16, 17). Electrophoresis was in 1-cm tracks on vertical slabs (12 by 15 by 0.15 cm) at 25 mA/gel for 4 to 5 h. After electrophoresis, the gels were soaked in 50 mM β-mercaptoethanol for 20 min before further work-up. RNA was eluted from appropriate gel slices by dissolving the agarose in 2 to 3 ml of phenol at 60°C and then extracting twice with equal volumes of 0.1 M NaCl, 0.05 M Tris (pH 7.4), and 0.001 M EDTA. The RNA in the aqueous phase was then ethanol precipitated and repurified by oligo(dT) chromatography. Overall yields of RNA were in the range of 50%.

Recombinant DNA and isolation of restriction fragments. All HSV-1 restriction fragments are referred to by their accepted letter designations where appropriate (i.e., *Hin*dIII fragment K [0.527–0.592]) and their coordinates in map units on the (P) arrangement of the HSV-1 genome. All restriction fragments used are listed in Table 1. All recombinant DNA experiments were done in accordance with current NIH guidelines.

TABLE 1. *Size and map location of restriction fragments in the region 0.527–0.602 of HSV-1*

Map location	Fragment name	Size[a] (base pairs)	Source
0.527–0.592[b]	H·K[c]	9,800	1[d]
0.527–0.537	H–X·K–A'[e]	1,500	2[f]
0.527–0.543	H–S·K–M	2,430	3[g]
0.527–0.570	H–B·K–H	6.500	3
0.537–0.566	X·T	4,400	2
0.543–0.578	S·L	5,200	3
0.566–0.582	X·Y	2,300	2
0.570–0.576	B·H'	840	2
0.576–0.592	H–B·K–O	2,430	2
0.578–0.588	S·C'	1,550	2
0.588–0.592	H–S·K–P	620	3
0.592–0.602	H–B·L–O	1,480	4[h]

[a] Size of restriction fragments based on comigration with *Eco*RI and *Hin*dIII digest of phase λ (C1857S7) DNA (43).

[b] Map location on the (P) arrangement of HLV-1 was based on setting the *Hin*dIII site at the left at 0.527 and a total size of the KOS strain of HSV-1 of 150,000 base pairs (37). Each 1,000 base pairs is then 0.00667 map units.

[c] Fragment names are based on the following references and sources: *Hin*dIII (33, 37); *Bam*HI (46); *Sal*I (24); *Xho*I (R. H. Watson and N. Wilkie, personal communication).

[d] (1): cloned in pBR322 from an *Hin*dIII digest of the KOS strain of HSV-1.

[e] Names of fragments derived from double digests are as described (33, 37).

[f] (2): derived from fractionation of an *Hin*dIII and appropriate restriction enzyme digest of *Hin*dIII fragment K cloned in pBR322.

[g] (3): subcloned in PBR322 from a fragment derived from the *Hin*dIII fragment K clone.

[h] (4): subcloned in pBR322 from a fragment derived from *Hin*dIII fragment L (0.592–0.651; 8,850 base pairs) cloned in pBR322 from an *Hin*dIII digest of HSV-1 (KOS) DNA.

*Hin*dIII fragments K (0.527–0.592) and L (0.592–0.651) were cloned in pBR322 in our laboratory. The procedure is essentially that of Bedbrook et al. (7); pBR322 was restricted with *Hin*dIII, mixed with *Hin*dIII-restricted HSV-1 DNA, and ligated. About 10 μl of ligated mixture containing ~0.2 μg of DNA was transfected into 5×10^8 cells of *Escherichia coli* strain X-1776 using the $CaCl_2$ method (13). Transfected cells were spread on detergent-free nitrocellulose filters (Millipore Corp., Bedford, Mass.) and grown on 1.5% agar plates containing X-1776 broth (X-1776 broth is [per liter]: 25 g of tryptone, 7.5 g of yeast extract, 20 mM $MgSO_4$, 50 mM Tris [pH 7.5], 100 mg of diaminopimelic acid, and 5 g of thymidine) with 40 μg of ampicillin per ml. After overnight growth, colonies were replica plated onto fresh filters, and these were subjected to colony hybridization (15). We carried out colony hybridization using 2×10^6 to 3×10^6 Cerenkov cpm (34) of ^{32}P-labeled complementary DNA (cDNA) made to isolated *Hin*dIII fragments K (0.527–0.592) and L (0.592-0.651). Hybridization conditions are described below. Positive colonies were located by autoradiography, respread, and replated for low-density colony hybridization. After the second round of colony hybridization, individual colonies were picked and streaked on 1.5% agarose containing X-1776 broth with ampicillin, and individual colonies were picked for growth in 50-ml liquid cultures. The plasmid was isolated from these cultures by the method of Clewell and Helinski (12); about 5 μg of plasmid was isolated. This was characterized by restriction endonuclease digestion and electrophoresis against appropriate size standards. When intactness of the cloned HSV-1 DNA fragment was confirmed, the plasmid was used to transfect *E. coli* LE392 (a gift of B. Moss). The colony of transfected bacteria then was treated as above to confirm the presence of the proper fragment; 100- to 200-ml cultures were grown in Luria broth, and the plasmid was isolated by the procedure of Tanaka and Weisblum (41) for chloramphenicol amplification. Strain LE392 grown in 40 μg of ampicillin yielded between 0.5 and 1 μg of plasmid DNA per ml of culture. Each preparation was rechecked by restriction digestion before further use. A *Bam*HI restriction fragment (*Bam*HI fragment H [0.516–0.570]) was isolated by cloning a *Bam*HI digest of HSV-1 in an identical manner.

Individual restriction fragments of the DNA clones were prepared by restriction enzyme digestion followed by fractionation by electrophoresis in 40 mM Tris (pH 7.8)–5 mM sodium acetate–2 mM EDTA on horizontal 0.7% agarose slabs (20 by 20 by 0.7 cm) at 30 mA per gel, as described previously (3–5, 16, 17). Agarose bands containing DNA of interest were dissolved in phenol at 65°C, and agarose was removed by repeated extraction of the phenol with a buffer containing 0.1 M NaCl and 2 mM EDTA (pH 7.4). Recovery of DNA was between 50 to 60% of theoretical yield.

Subclones of the original cloned DNA fragments were made by isolating the specific DNA restriction fragment (0.5 to 2 μg) and ligating a 10-fold excess of this with appropriately digested pBR322 followed by transfection directly into LE392 and colony screening as described.

Isolation of restriction-fragment-specific mRNA. Restriction-fragment-specific mRNA was isolated from poly(A) polyribosomal RNA by preparative hybridization to the appropriate DNA covalently coupled to cellulose. Details of coupling of DNA to cellulose and preparative hybridization were as described previously (3–5, 16, 17). Hybridization was in 80% recrystallized formamide containing 0.4 M Na^+, 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0), and 0.005 M EDTA at 58°C. Rinsing of cellulose and elution of hybridized RNA were as described previously (5). In the experiments described here, samples of 50 to 60 μg of ^{32}P-labeled late poly(A) polyribosomal mRNA were hybridized with 100-μg equivalents of a given restriction fragment for 4 h (a microgram equivalent is that amount of a specific fragment obtainable from 1 μg of HSV-1 DNA).

Synthesis of cDNA. The cDNA to particular RNA or DNA sequences was synthesized as described in previous reports (3–5, 16, 17). Total cDNA was synthesized by using random oligodeoxyribonucleotides

prepared from calf thymus DNA as a primer for reverse transcriptase (42). The 3′ cDNA was made by use of oligo(dT)$_{12-18}$ as a primer. Partial alkaline digestion of the RNA and reselection on oligo(dT)-cellulose columns were as described previously (4, 17).

Preparation and hybridization of DNA (Southern) blots. Southern blots of DNA were prepared as described previously (3–5, 16, 17) according to the basic method of Southern (35). Hybridization of RNA to Southern blots was carried out in 65% formamide–0.4 M Na^+–0.1 M HEPES (pH 8.0)–5 mM EDTA (hybridization buffer) at 45°C for 24 to 48 h. Hybridization of cDNA to blots was carried out at 37°C for 24 to 48 h in hybridization buffer containing Denhardt solution (0.02% Ficoll, 0.02% bovine serum albumin, and 0.02% polyvinylpyrrolidone [14]). All blots were rinsed twice for 1 h in hybridization buffer under hybridization conditions at 37°C and subjected to autoradiography with Kodak X-Omat R film.

Preparation and hybridization of RNA transfer blots. After fractionation of 8 to 15 μg of RNA per track of polyribosomal poly(A) RNA by electrophoresis in methylmercury-containing agarose gels, RNA was blotted onto diazotized paper by the method of Alwine et al. (2). The cDNA was hybridized to such RNA blots exactly as described for hybridization to Southern blots.

S1 nuclease analysis of HSV-1 RNA. S1 nuclease analysis of RNA was carried out essentially as described by Berk and Sharp (8, 9). Cultures (100 ml) of *E. coli* LE392 containing the appropriate restriction fragment of HSV-1 DNA in pBR322 were grown in low-phosphate morpholinepropanesulfonic acid medium (28, 38) containing 2 to 3 μCi of $^{32}P_i$. The DNA had a specific activity of 50,000 to 70,000 Cerenkov cpm/μg. The DNA was linearized by digestion with *Hin*dIII and *Eco*RI, and 3- to 4-μg samples were added to 20-μg infected-cell polyribosomal poly(A) RNA and precipitated with ethanol. The mixed pellets were dissolved in 100 μl of hybridization buffer containing 80% recrystallized formamide, denatured by incubation for 10 min at 78°C, and immediately transferred to a 58°C water bath for 4 h of hybridization. The hybridization was terminated by addition of 0.9 ml of ice-cold buffer (pH 4.6) containing 250 mM NaCl, 30 mM sodium acetate, 1 mM $ZnCl_2$, and 5% glycerol. A 10-μl sample (~10^4 U) of S1 nuclease was added, and digestion was carried out for 30 min at 45°C. At this time, 20 μg of tRNA was added as carrier to each sample, and the material was ethanol precipitated.

Samples were fractionated by electrophoresis on horizontal 0.9 to 1.2% neutral agarose gels (20 by 20 by 0.5 cm) and run in a buffer (pH 8.3) containing 50 mM Tris, 40 mM sodium acetate, and 2 mM disodium EDTA, or on the same size alkaline gels in 30 mM NaOH–2 mM disodium EDTA. The latter buffer was found to completely degrade ^{32}P-labeled RNA run as a test. Electrophoresis for 16 h at 40 mA was carried out with restriction endonuclease-digested ^{32}P-labeled cloned DNA in parallel tracks as size markers. The gels were then neutralized if needed and dried in vacuo, and bands were visualized by autoradiography on Kodak X-omat R film.

In addition to specific bands, which were reproducibly seen, there was a variable amount of background radioactivity migrating heterogeneously throughout the gels. This background was specific for added infected-cell RNA since control experiments using as much as 100 μg of carrier HeLa cell RNA showed no residual radioactivity after mock hybridization and S1 digestion. Shorter digestion times with the enzyme increased this background, but longer digestion times resulted in reduction of specific bands, especially larger ones. The background was usually reduced in alkaline gels and increased when hybridization was with DNA, which could be expected to be nicked due to prolonged storage or manipulation. These observations led us to conclude that a major portion of the background was due to some nicking of DNA-RNA duplexes by the S1 nuclease. The digestion times chosen were the best compromise between loss of bands and too much background, and the bands discussed in the text were reproducibly seen. In some cases, however, photography of smaller bands clearly visible in the autoradiographs was complicated by this background.

In vitro translation. Translation of viral mRNA was carried out in vitro using a micrococcal nuclease-treated rabbit reticulocyte system (New England Nuclear Corp.) with [^{32}S]methionine (675 Ci/mM) as the radioactive amino acid. Details of the procedure and fractionation of polypeptides in sodium dodecyl sulfate-acrylamide gels by the method of Laemmli (23) have been described in several previous papers (3, 4, 16). Gels were dried with vacuum, and radioactive bands were localized with autoradiography using Kodak NS-2T film.

RESULTS

Restriction endonuclease map of the region 0.527–0.602. Restriction endonuclease sites on *Hin*dIII fragment K (0.527–0.592) and the left end of *Hin*dIII fragment L (0.592–0.651) were determined by appropriate restriction endonuclease digestion of the cloned material in pBR322 and comigration with digested phage λ DNA. The location of *Bam*HI, *Xho*I, and *Sal*I sites are shown in Fig. 1, and the specific size of restriction fragments in kilobase pairs is shown in Table 1. Table 1 also indicates those fragments which were subcloned in pBR322 for further studies. As described in the legend to Fig. 1, the coordinates for each restriction site were based on setting the left-hand *Hin*dIII site of fragment K at 0.527. The location of restriction sites and the sizes of specific fragments are fully consistent with data from this and other laboratories (6, 11, 24, 25, 27, 33, 37, 46).

Localization of specific mRNA species within the region 0.527–0.602. We used RNA transfer blots of either 15 μg of early polyribosomal poly(A) mRNA or 8 μg of such RNA isolated from normal cells at 6 h postinfection (late) to grossly localize the viral mRNA species encoded by this region. The RNA blots were hybridized to 2 × 10^6 to 3 × 10^6 Cerenkov cpm of cDNA made to the appropriate restriction

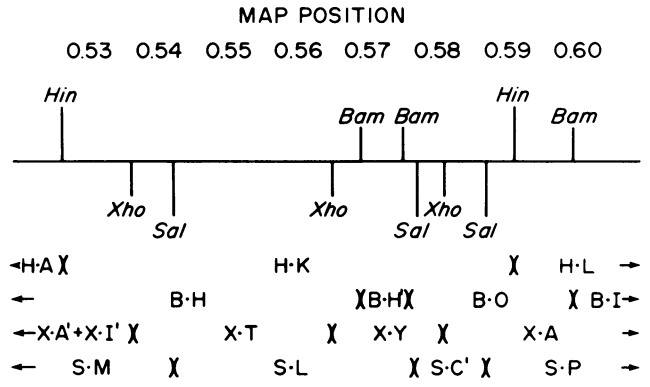

FIG. 1. *Restriction endonuclease cleavage sites in the region 0.53–0.6 on the (P) arrangement of HSV-1 (KOS). HindIII fragments K (0.527–0.592) and L (0.592–0.651) were cloned in pBR322, and these cloned DNA fragments were digested with HindIII (Hin), BamHI (Bam), SalI (Sal), and XhoI (Xho) restriction endonucleases in various combinations to determine the positions of the sites shown. Fragments were sized by electrophoresis on 0.7 and 1.2% neutral agarose gels versus standard bands derived by either HindIII or EcoRI digestion of phage λ (CI857S7) DNA (see the text). The left-hand HindIII site was set at 0.527, and all other sites were based on the size of the restriction fragments generated (1,000 base pairs = 0.00667 map units). Names of restriction fragments are based on the following references: HindIII (33, 37), BamHI and SalI (24, 46), XhoI (R. Watson and N. Wilkie, personal communication; the site between their fragments A' and I' is missing in our KOS strain of HSV-1).*

fragment (see above), and the results of a number of experiments are shown in Fig. 2 and summarized in Table 2. All RNA sizes were based on the location of 28S (5.2 kb; 26) and 18S (2.0 kb; 47) run in parallel gels.

Early mRNA homologous to *Hin*dIII fragment K (0.527–0.592) showed a major band at 5.2 kb and smaller amounts of mRNA 3.8 and 1.5 kb in size. Early mRNA homologous to the region 0.592–0.602 (the 1.485-kb fragment *Hin*dIII-*Bam*HI·L-O) migrated at 5.2 and 1.5 kb, with very small amounts of mRNA migrating at 1.9 and 1.0 kb also visible. After DNA replication (late), the complexity of the mRNA homologous to these regions increased markedly; mRNA species 7.0 and 1.9 kb were seen as major species in the region 0.527–0.592 (*Hin*dIII fragment K), and the 3.8-kb species became a major one. The late 7.0-kb mRNA was also seen to be a major species hybridizing to the region 0.592–0.602, and the 1.9- and 1.0-kb bands in this region became readily detectable.

We used RNA transfer blots of late polyribosomal poly(A) mRNA to further localize the mRNA species seen. The 3.8-kb species was seen to map between 0.527 and 0.566, whereas the 7- and 1.9-kb species in *Hin*dIII fragment K (0.527–0.592) map to the right of 0.543 since they were not seen hybridizing to cDNA probe made to the region 0.527–0.543, but did hybridize to probe made to the region 0.537–0.566. The 1.9-kb mRNA maps to the left of 0.566 since it was not seen when RNA blots were hybridized to cDNA made to regions to the right of 0.566. The 7-kb mRNA maps through the region from 0.566 to 0.602 since it was seen using all cDNA probes to individual regions within these coordinates. It will be shown below that the 1.9-kb mRNA mapping to the right of 0.592 is a second species of this size unrelated to the 1.9-kb species seen in the region 0.537–0.566.

The 5.2-kb early mRNA species maps to the right of 0.566 since it was not readily detectable in RNA transfer blots hybridizing to cDNA probe made to the region 0.537–0.566. This RNA species maps between 0.566 and 0.602 since it was seen when cDNA probes made to any region between these coordinates are used. The 1.5-kb early mRNA species maps between 0.588 and 0.602 since it was only seen with RNA transfer blots hybridized to cDNA probes made to regions between these coordinates. Finally, the 1.0-kb minor mRNA species, as well as the second 1.9-kb mRNA species, map to the right of 0.592 since they were only seen in RNA transfer blots hybridized to regions to the right of 0.592.

Localization of the 3' ends of the mRNA species mapping between 0.527 and 0.602. We isolated specific HSV-1 mRNA species mapping within the region of interest by hybridization of late poly(A) mRNA to the appropriate restriction fragment bound to cellulose (see above). Specific bands were fractionated by electrophoresis on methylmercury-containing agarose gels and localized by determining the radioactivity in 2-mm slices of the gel track. Typical

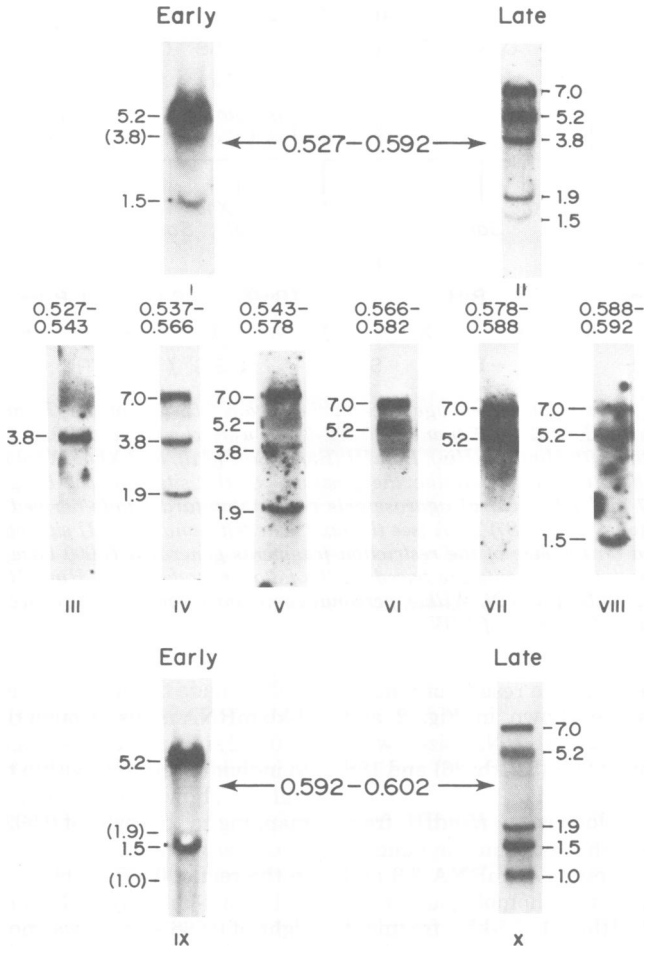

FIG. 2. *RNA transfer (Northern) blots of early and late HSV-1 polyribosomal poly(A) mRNA homologous to the region 0.527–0.602. Samples (15 or 8 μg) of early or late poly(A) mRNA, respectively, were fractionated on methylmercury hydroxide agarose gels by electrophoresis and transferred onto diazotized paper (see the text). Blots were hybridized to 3 × 10⁶ to 5 × 10⁶ Cerenkov cpm of [³²P]cDNA made to: HindIII fragment K (0.527–0.592, tracks i and ii); HindIII-SalI fragment K-M (0.527–0.543, track iii); XhoI fragment T (0.537–0.566, track iv); SalI fragment L (0.543–0.578, track v); XhoI fragment Y (0.566–0.582, track vi); SalI fragment C' (0.578–0.588, track vii); HindIII-SalI fragment K-P (0.588–0.592, track viii), HindIII-BamHI fragment H-L (0.592–0.602, track ix and x). Sizes of these fragments are seen in Table 1, and their location is shown in Fig. 1. Sizes of mRNA were based on the migration of 28S and 18S rRNA markers run in parallel tracks (5.2 and 2.0 kb; 26, 47).*

size fractionation patterns of HSV-1 mRNA isolated with *Hin*dIII fragment K (0.527–0.592), *Sal*I fragment L (0.543–0.578), and *Hin*dIII-*Bam*HI fragment L-O (0.592–0.602) are shown in Fig. 3A–C. The RNA from appropriate gel slices was typically partially degraded with alkali and then eluted as described. The 3′ end of such RNA was reselected on oligo(dT)-cellulose and used for synthesis of 3′ cDNA via use of an oligo(dT)-primed reaction with reverse transcriptase (see above). The 3′ cDNA was hybridized to Southern blots of either (i) *Hin*dIII, *Eco*Ri, and *Sal*I digests of *Hin*dIII fragment K (0.527–0.592) or (ii) an *Hin*dIII and *Bam*HI digest of *Hin*dIII fragment L (0.592–0.651) cloned in pBR322. Typical hybridization patterns, along with guide strips of the digested restriction fragments, are shown in Fig. 4.

The 3′ cDNA made to 7- and 5.2-kb mRNA isolated with *Hin*dIII fragment K (0.527–0.592) bound to cellulose (Fig. 3A) hybridized to the fragment *Hin*dIII-*Bam*HI fragment L-O (0.592–0.602; Fig. 4A). There is no evidence of this 3′ cDNA hybridizing to the right of 0.602; there-

TABLE 2. *Size of mRNA species detected by Northern blot analysis with [^{32}P]cDNA made to different regions between 0.527 and 0.602 on the HSV-1 genome*

Location of cDNA probe[a]	Size of RNA seen[b]
0.527–0.592	7 5.2[c] 3.8[d] 1.9 1.5[e]
0.527–0.543	3.8
0.537–0.566	7 3.8 1.9
0.543–0.578	7 5.2 3.8 1.9
0.566–0.582	7 5.2
0.578–0.588	7 5.2
0.588–0.592	7 5.2 1.5
0.592–0.602	7 5.2[c] 1.9[f] 1.5[e] 1.0[f]

[a] See Fig. 1 for location of restriction sites and Table 1 for the size of the fragments.

[b] Size of RNA based on comigration with 28S (5.2 kb; 26) and 18S (2.0 kb; 47) HeLa cell rRNA (see the text). These RNA sizes include poly(A) tails 200 and 300 bases in length (36).

[c] Major early species (see the text).

[d] Minor early but major late species.

[e] Minor species early and late.

[f] Detectable early species which are more abundant late.

fore, we conclude that the 3′ ends of these two mRNA species map wholly in this region. Since these mRNA species extended well to the left of 0.592 (Fig. 2 and Table 2), these results confirm our earlier report that these mRNA species are transcribed from left to right on the (P) arrangement of the HSV-1 genome (4, 17). In contrast to these results, the 3′ cDNA made to the 3.8-kb mRNA maps solely in *Hin*dIII-*Sal*I fragment K-M (0.527–0.543; Fig. 4B). Since this mRNA extended to the right of 0.543 (Fig. 2 and Table 2), we conclude that this mRNA is transcribed from right to left (i.e., from the opposite strand from the 7- and 5.2-kb species) on the (P) arrangement of the HSV-1 genome.

The 3′ cDNA to the 1.9 kb isolated with either *Hin*dIII fragment K (0.527–0592; Fig. 4C) or *Sal*I fragment L (0.543–0.578; not shown) bound to cellulose (Fig. 3A and B) hybridized to the region 0.543–0.578. The 3′ end data do not rigorously establish a direction of transcription for this mRNA species. However, S1 mapping data, as well as in vitro translation studies (below), suggest that this mRNA is colinear with the 5′ end of the 7-kb mRNA and is transcribed in the same direction (see below).

The 3′ cDNA made to the 1.5-kb mRNA isolated with *Hin*dIII fragment K (0.527–0.592; Fig. 4D) or *Hin*dIII-*Bam*HI fragment L-O (0.592–0.602; Fig. 4E) bound to cellulose (Fig. 3A and C) hybridized to the region 0.592–0.602. In the latter experiment, the cDNA was made to undegraded RNA to make larger 3′ cDNA (see below), and this cDNA also hybridized very faintly to the region 0.588–0.592 (Fig. 4E). This and the fact that the 1.5-kb mRNA extended to the left of 0.592, but not beyond 0.588 (Fig. 2 and Table 2), indicates that this 1.5-kb early mRNA is transcribed from left to right.

The 1.9-kb mRNA isolatable with *Hin*dIII-*Bam*HI fragment L-O (0.592–0.602; Fig. 3C) was isolated intact to make 3′ cDNA long enough to extend beyond the most proximal region near this mRNA's 3′ end. Such 3′ cDNA hybridized strongly to the region 0.592–0.602 and faintly to DNA to the right of 0.602 (Fig. 4E). These data allow us to tentatively conclude that this mRNA is transcribed from right to left with its 5′ end to the right of 0.602. The in vitro translation experiments described below further demonstrate that this mRNA is not related to the 1.9-kb species mapping between 0.537 and 0.566. The 3′ cDNA made to the 1.0-kb mRNA also isolated with *Hin*dIII-*Bam*HI fragment L-O (0.592–0.602) bound to cellulose hybridized mainly to this region, with a small amount of radioactivity seen hybridizing to the fragment encompassing the region 0.602–0.618. From this, we conclude that this minor mRNA species also is transcribed from right to left and is at least partially colinear with the 1.9-kb species in this region. The small amounts of this mRNA obtainable have precluded further characterization.

Lack of internal introns in the coding sequences for the major HSV-1 mRNA's homologous to the region 0.527–0.602. The availability of un-nicked, pure HSV-1 DNA restriction fragments via recombinant DNA allowed us to carry out a detailed S1 nuclease analysis of the major HSV-1 mRNA species grossly mapped with the data summarized in Fig. 2 and Table 2. We first compared neutral and alkaline S1 analysis of *Hin*dIII fragment K (0.527–0.592) DNA protected by early and late polyribosomal poly(A) mRNA. Samples of RNA were hybridized to ^{32}P-labeled DNA, digested with S1 nuclease, and subjected to neutral and alkaline agarose gel electrophoresis (see above).

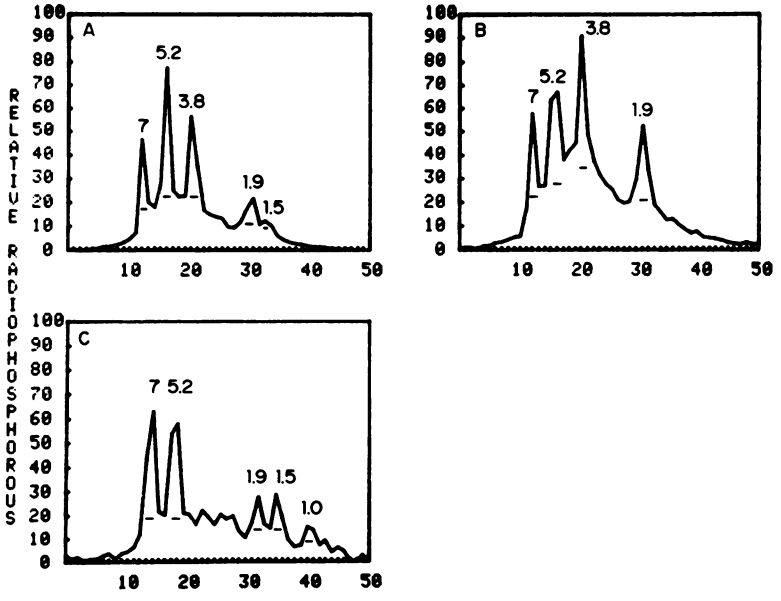

FIG. 3. *Isolation of specific HSV-1 mRNA species homologous to the region 0.527–0.602. Late ^{32}P-labeled polyribosomal poly(A) mRNA was hybridized with (A) HindIII fragment K (0.527–0.592) bound to cellulose; (B) SalI fragment L (0.543–0.578) bound to cellulose; and (C) HindIII-BamHI fragment L-O (0.592–0.602) bound to cellulose. The hybridized mRNA was fractionated by electrophoresis on agarose gels containing methylmercury hydroxide, and tracks were cut into 2-mm slices. Radioactivity was determined by determining Cerenkov radiation in a Beckman LS-230 scintillation counter. Sizes of the mRNA species were confirmed from the migration of 28S and 18S rRNA (5.2 and 2.0 kb; 26, 47) in a parallel gel. The sizes of individual peaks in kilobases are indicated above them.*

As seen in Fig. 5, we observed the presence of readily detectable, sharply migrating bands, as well as the diffusely migrating background material discussed above.

Early mRNA in both neutral and alkaline gels protected a 4-kb portion of *Hin*dIII fragment K (0.527–0.592) DNA, as well as a lesser amount of a 3.6-kb portion (Fig. 5A). The sizes of these bands and their relative amounts suggested that the 4-kb major band corresponded to the portion of the 5.2-kb major early mRNA mapping in this region, and the 3.6-kb band was due to the 3.8-kb mRNA seen in less abundance early. There was no detectable change in the migration rate of these two bands between neutral and alkaline gels, and we concluded that, within the confines of this region (0.527–0.592), no introns are present in either of these coding regions for HSV-1 mRNA unless they are very near (ca. 100 bases) one end of the coding sequence.

Late mRNA (Fig. 5B) protected the same 4- and 3.6-kb bands, although the latter was seen in greater abundance, as expected from our conclusion that it was due to the 3.8-kb mRNA, which is an abundant late species. In addition to these bands, a band migrating with a size corresponding to 5.9 to 6 kb in the neutral and alkaline gels and one migrating with a size of 1.7 kb were also seen. These bands were assigned to the portion of this region protected by the 7- and 1.9-kb mRNA's, respectively. As was the case with mRNA early, no detectable change in the migration of these bands was seen between the neutral and alkaline gels, again demonstrating the lack of detectable internal introns in the coding sequences for these mRNA's.

To exclude the possibility that one of the larger coding sequences could contain an intron that would yield protected regions of DNA comigrating with smaller bands seen in the S1 gels, we carried out a two-dimensional gel analysis of later mRNA hybridized to *Hin*dIII fragment K (0.527–0.592) and digested with S1 nuclease. The digested material was run in one direction at neutral pH and then turned 90° and run for an equal amount of time under alkaline conditions. It is seen (Fig. 5C) that the four readily detectable bands fall on a strict diagonal and that none of the bands in the neutral gels gives rise to detectable smaller bands under alkaline conditions. These results again confirm that any interruptions in the DNA coding sequences for these RNA species in the region 0.527–0.592 must be very close to the ends of the RNA chains.

The 3′ ends of the 7-, 5.2-, and minor 1.5-kb

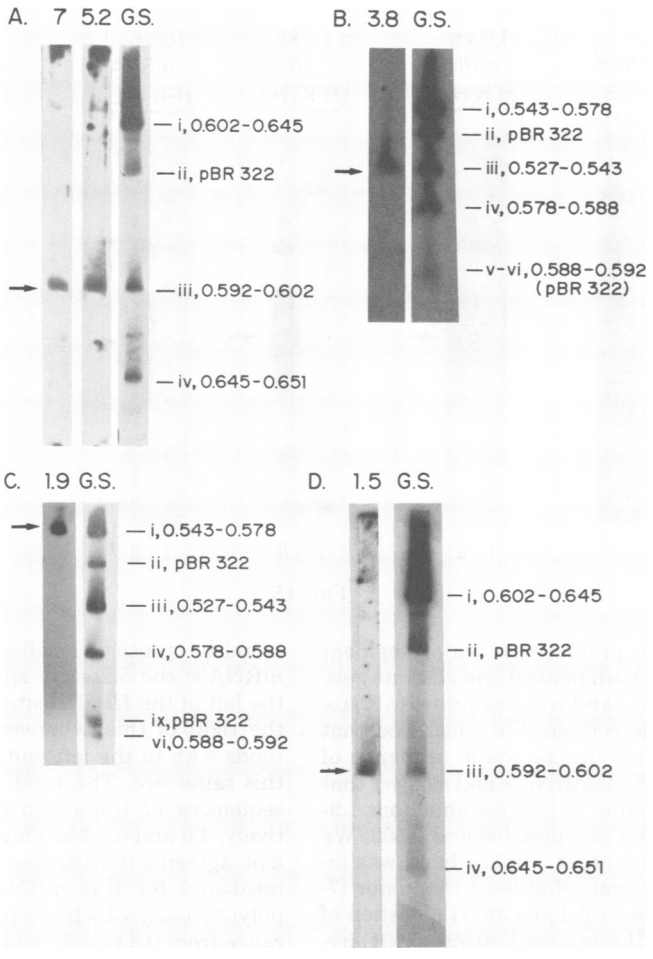

FIG. 4. *Hybridization of 3' cDNA made to specific HSV-1 mRNA mapping between 0.527 and 0.602 to Southern blots of restriction fragments from that region. (A) 3' cDNA (75,000 Cerenkov ^{32}P cpm) to 7- and 5.2-kb mRNA was hybridized to Southern blots of an HindIII-BamHI double digest of HindIII fragment L (0.592–0.651) cloned in pBR322. A parallel blot was hybridized to 200,000 ^{32}P cpm of total cDNA made to the total cloned DNA (G.S., guide strip). Bands are from the top: (i) 0.602–0.645, BamHI·I; (ii) pBR322; (iii) 0.592–0.602, HindIII-BamHI·L-O; (iv) 0.645–0.652, HindIII-BamHI·L-H'. (B) 3' cDNA (50,000 ^{32}P cpm) to 3.8-kb mRNA was hybridized to a Southern blot of an HindIII-SalI double digest of HindIII fragment K (0.527–0.592) cloned in pBR322. A parallel blot was hybridized to 100,000 ^{32}P cpm of total cDNA made to the total cloned DNA (G.S.). Bands are from the top: (i) 0.543–0.578, SalI·L; (ii) pBR322; (iii) 0.527–0.543, HindIII-SalI·K-M; (iv) 0.578-0.588, SalI·C'; (v and vi) 0.588–0.592, HindIII-SalI·K-P and pBR322. (C) 3' cDNA to the 1.9-kb mRNA isolated with HindIII fragment K (0.527–0.592) bound to cellulose (75,000 ^{32}P cpm) was hybridized to a Southern blot of an HindIII-SalI double digest of HindIII fragment K cloned in pBR322. The guide strip is as in (B). (D) 3' cDNA (50,000 cpm) made to 1.5-kb mRNA isolated with HindIII fragment K (0.527–0.592) bound to cellulose was hybridized to a Southern blot of an HindIII-BamHI double digest of HindIII fragment L cloned in pBR322. The guide strip is as in (A). (E) 3' cDNA made to intact 1.9- (120,000 cpm of cDNA), 1.5- (100,000 cpm of cDNA), and 1.0-kb (50,000 cpm of cDNA) mRNA isolated with HindIII-BamHI fragment L-O (0.592–0.602) bound to cellulose were hybridized to Southern blots of HindIII-BamHI digests of HindIII fragment L in pBR322 (tracks marked I) or HindIII-SalI digests of HindIII fragment K in pBR322 (tracks marked II). Guide strips are as in (A) and (B). The bands marked (P) in the guide strip for HindIII fragment L (0.592–0.651) are due to partial digestion products.*

mRNA's mapping in *Hin*dIII fragment K (0.527–0.592) have been shown to map between 0.592 and 0.602. We did two experiments to determine how far the major mRNA species extended and whether there were any detectable interruptions in the 3' coding sequences for them. As seen in Fig. 6A, total late poly(A) polyribosomal RNA, when hybridized to *Hin*dIII-*Bam*HI fragment

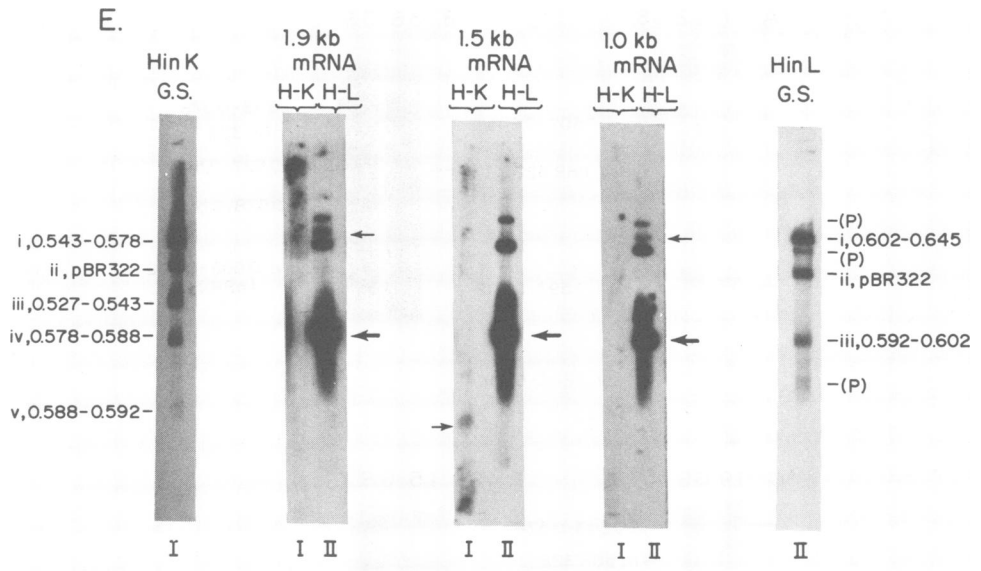

FIG. 4E.

L-O (0.592–0.602), protected a major segment 0.9 kb in length in both neutral and alkaline gels. In addition, a faint band corresponding to a size 0.6 kb was also detectable. We concluded that the major band was due to the 3′ sequence of both the 5.2- and 7-kb mRNA's and suggest that the 0.6-kb band is due to the less abundant 1.9- and 1.0-kb mRNA's mapping beyond 0.602. We confirmed that the larger (0.9-kb) band was indeed due to the 3′ ends of at least the major (7- and 5.2-kb) mRNA's by doing an S1 digestion of ^{32}P-labeled *Hin*dIII fragment L (0.592–0.651) hybridized to 1 μg of *Hin*dIII fragment K (0.527–0.592) specific RNA prepared by hybridization to DNA cellulose. The only band detectable in such an experiment was one 0.89-0.9 kb in size (Fig. 6B), a result in excellent agreement with the 0.9-kb value determined in the experiment of Fig. 6A. The fact that there was no change in migration of this band between neutral and alkaline gels (neutral gel not shown) demonstrates that no introns are present in the 3′ end sequences of either of these major mRNA species.

We cannot conclude that the minor 1.5-kb mRNA species mapping between 0.588–0.602 has its 3′ end at the same site on the basis of these data since a minor band could readily be obscured by the rather high background. However, the length of the 5′ end of this mRNA species (see below) suggests that it too extends 0.9 kb to the right of 0.592.

High-resolution mapping of the major HSV-1 mRNA species homologous to the region 0.527–0.592 by S1 nuclease analysis. The data described in the previous section allow a fairly precise localization of two major mRNA's: the 5.2 early species extends 4 kb to the left of the *Hin*dIII site at 0.592 and 0.9 kb to the right of this, whereas the 7-kb species extends 6 kb to the left and 0.9 kb to the right of this same site. The total lengths of the coding sequences of these two mRNA's are, respectively, 4.9 and 6.9 kb. These values are in excellent agreement with the sizes of 5.2 and 7 kb measured for this mRNA species with their poly(A) tails, which we previously reported to range from 0.2 to 0.3 kb in length (36).

To confirm these values and to more precisely localize the 3.8- and 1.9-kb mRNA species mapping entirely to the left of 0.592, we analyzed S1 nuclease digestion of hybrids formed with late polyribosomal poly(A) mRNA and selected subclones of DNA in the region 0.527–0.592. We used alkaline gel values for the sizes of the bands seen, and data for such gels are shown in Fig. 7 and are summarized in Table 3.

Where DNA from the region 0.527–0.543 (*Hin*dIII-*Sal*I fragment K-M) was used for hybridization, a single band (2.4 kb) was protected. This size is the full length of the fragment in question, indicating that the 3′ end of the 3.8-kb mRNA previously shown to be located between 0.527 and 0.566 (Fig. 2, Table 2) extends at least to 0.527, if not to the left of this *Hin*dIII site. When DNA from the region 0.527–0.570 (*Hin*dIII-*Bam*HI fragment K-H) was used for hybridization, a band 3.6 kb in size was seen. The same size band was seen when *Bam*HI fragment H (0.516–0.570) was used (data not shown); we have therefore concluded that the 3′

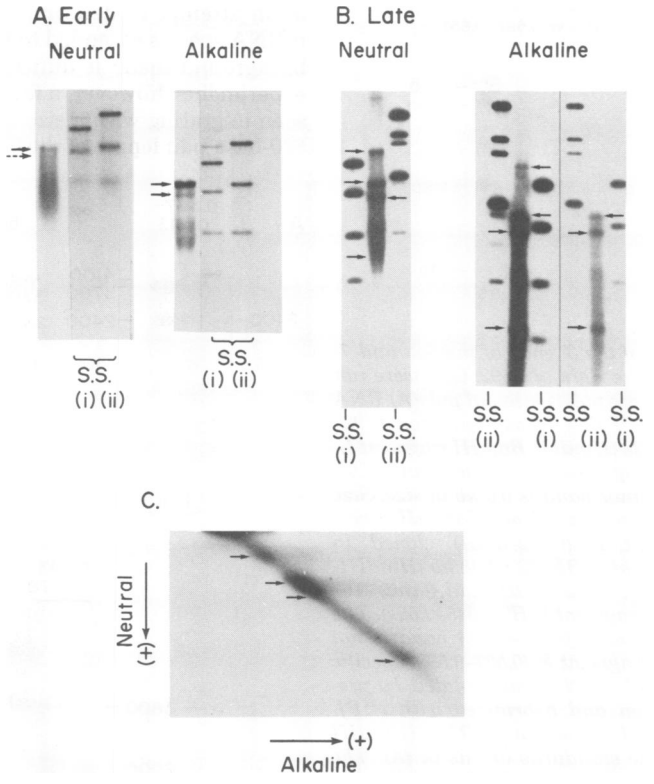

FIG. 5. *S1 nuclease analysis of the major mRNA species homologous to DNA in the region 0.527–0.592. (A) Early polyribosomal poly(A) RNA was hybridized with ^{32}P-labeled HindIII fragment K (0.527–0.592) DNA in pBR322, digested with S1 nuclease, and subjected to neutral or alkaline gel electrophoresis in a 0.9% agarose gel (see the text). The arrows indicate a major and a minor band migrating at 4 and 3.6 kb, respectively. Sizes were determined from the migration of size standards (S.S). obtained by digesting the cloned DNA with EcoRI, HindIII, and SalI enzymes (i) and EcoRI, HindIII, and BamHI enzymes (ii). Bands in track (i) are: 5.2 kb (SalI fragment L, 0.543–0.578); 3.7 kb (pBR322); 2.4 kb (HindIII-SalI fragment K-M, 0.527–0.543); 1.55 kb (SalI fragment C', 0.578–0.588). The faint band larger than the 5.2-kb band is a partial digestion fragment. Bands in track (ii) are: 6.5 kb (HindIII-BamHI fragment K-H, 0.527–0.570); 3.98 kb (pBR322); 2.4 kb (HindIII-BamHI fragment K-O, 0.576–0.592). Other smaller bands are not readily visible in this exposure. (B) A similar experiment was carried out with late polyribosomal-associated poly(A) RNA. Arrows indicate bands migrating with sizes of 6, 4, 3.6, and 1.7 kb. Two exposures of the alkaline gel are shown to better emphasize specific bands. The size markers are as described in (A); the track marked (i) is an HindIII, EcoRI, SalI digest of HindIII fragment K in pBR322; and the track marked (ii) is an HindIII, EcoRI, BamHI digest of this clone. In the latter case, there are a number of partial digestion bands migrating more slowly than the pBR322 band. (C) An S1 digest of later poly(A) mRNA hybridized to HindIII fragment K (0.527–0.592) was first subjected to agarose gel electrophoresis at neutral pH; then the gel was turned 90° and subjected to electrophoresis at alkaline pH. The arrows indicate the four major bands corresponding to those in the gel of (B). The material at the top of the gel is undigested material.*

end of the 3.8-kb mRNA does not extend appreciably to the left of 0.527.

In addition to the band migrating in the size of 3.6 kb, we found bands corresponding to DNA sizes of 2.6, 1.7, and 0.9 kb when the DNA from the region 0.527–0.570 was used for hybridization. The 2.6-kb fragment is expected as the 5′ end of the 7-kb mRNA, and the 0.9-kb fragment corresponds to the 5′ end of the 5.2-kb early mRNA. The 1.7-kb DNA fragment is due to the 1.9-kb mRNA mapped in the region 0.537–0.566 (Fig. 2, Table 2). The lack of a restriction site between 0.537 and 0.566 for the enzymes presently available to us has precluded a more precise localization of this mRNA at this time. But the fact that its 3′ end mapped to the left of 0.578 (Fig. 4) and that it encoded the same polypeptide as the 7-kb mRNA (see below) indicate that it is colinear with the 5′ end of this latter mRNA.

When DNA from the region 0.543–0.578 (*Sal*I fragment L) was used to hybridize to late poly-

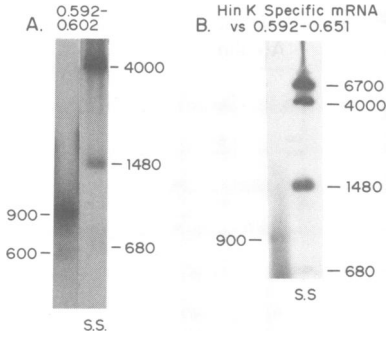

FIG. 6. *Extension of the 3' ends of the 5.2- and 7-kb mRNA species to the right of 0.592. Gels were run at alkaline pH. (A) Late polyribosomal poly(A) RNA was hybridized with [^{32}P]DNA from the region 0.592–0.602 cloned in pBR322 (HindIII-BamHI fragment L-O). The gel was 0.9% agarose. The major band is 0.9 kb in size, and the minor band is 0.6 kb in size. Size standards (S.S.) are from an HindIII-BamH digest of HindIII fragment L (0.592–0.651) cloned in pBR322. Bands are 4 kb (pBR322); 1.48 kb (HindIII-BamHI fragment L-O, 0.592–0.602); and (faint) 0.68 kb (HindIII-BamHI fragment L-H', 0.645–0.651). The 6.7-kb BamHI fragment I (0.602–0.645) band is not shown. (B) HindIII fragment K (0.527–0.592) specific poly(A) polyribosomal mRNA was isolated by preparative hybridization and hybridized with [^{32}P]-DNA from 0.592–0.651 cloned in pBR322 (HindIII fragment L). The size standards are as in (A). The pBR322 band at 530 bases is not visible in either.*

ribosomal poly(A) RNA, S1 digestion yielded bands migrating with sizes corresponding to 3.8, 2.0, 1.7, and 1.2 kb. The 1.2-kb DNA fragment corresponds to the extension of the coding sequence of the 5' end of the 3.8-kb mRNA to the right of the *Sal*I site at 0.543. The 3.8-kb DNA fragment is due to the coding sequence for the 5' end of the 7-kb mRNA extending leftwards of the *Sal*I site at 0.578; similarly, the 2.0-kb fragment is from the 5' end of the 5.2-kb mRNA. The 1.7-kb band is from the 1.9-kb mRNA mapping wholly in the region 0.543–0.578. The two fainter bands migrating between 1,700 and 1,200 bases were not reproducibly seen in our experiments and, therefore, are not discussed further.

The 1.5-kb minor mRNA species has not been identified with any readily detectable DNA fragment protected when *Hin*dIII fragment K (0.527–0.592) was hybridized with late poly(A) mRNA and digested with S1 nuclease (Fig. 5). This was due to the rather large amount of heterogeneously migrating background radioactivity and to the low level of radioactivity to be expected in such a DNA fragment, since the 5' end of the mRNA should not extend more than 0.6 kb to the left of 0.592. We used the *Hin*dIII-*Sal*I fragment K-P (0.588-0.592) to carry out hybridizations, followed by S1 nuclease analysis, in an attempt to determine how far this minor mRNA species extended to the left of 0.592. The background made it difficult to interpret such experiments; however, a fairly evident band was seen migrating with a size corresponding to the 620-base-pair length of the fragment, as expected

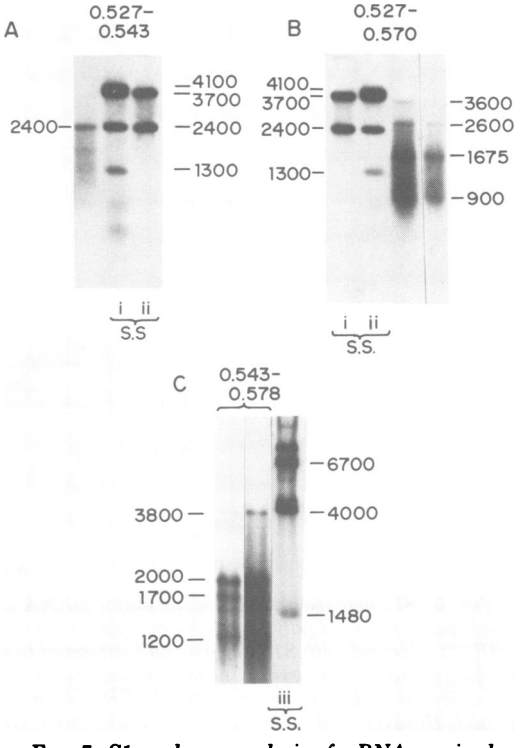

FIG. 7. *S1 nuclease analysis of mRNA species homologous to various regions within the region 0.527–0.592. All gels shown were run at alkaline pH. (A) Late polyribosomal poly(A) mRNA was hybridized with ^{32}P-labeled DNA from the region 0.527–0.543 (HindIII-SalI fragment K-M) cloned in pBR322 and digested with S1 nuclease. The major band is 2.4 kb, as judged by comigration with size standards (S.S.). Track (i) is an HindIII, BamHI, SalI digest of BamHI fragment H (0.516–0.570). Bands are: 4.1 kb, BamHI-SalI fragment H-P (0.543–0.570); 3.7 kb, pBR322; 2.4 kb, HindIII-SalI fragment K-M (0.527–0.543); 1.3 kb, BamHI-HindIII fragment H-K (0.516–0.527). Track (ii) is an HindIII, SalI digest of HindIII-SalI fragment K-P (0.527–0.543). Bands are: 3.7 kb, pBR322; 2.4 kb, HindIII-SalI fragment K-P (0.527–0.543). (B) Hybridization of late mRNA was with HindIII-BamHI fragment K-H (0.527–0.570). The size standards are as in (A). (C) Hybridization of late mRNA was with SalI fragment L (0.543–0.578). Two exposures are shown to emphasize particular bands. The size standard (track iii) is an HindIII, BamHI digest of HindIII fragment L (0.592–0.651) cloned in pBR322. Indicated bands are 6.7 kb, BamHI fragment I (0.602–0.645); 3.98 kb, pBR322; and 1.5 kb, HindIII-BamHI fragment L-O (0.592–0.602).*

TABLE 3. *Size of DNA fragments protected by major HSV-1 mRNA species mapping between 0.527–0.6*

Map position of ^{32}P-labeled DNA[a]	Size of DNA fragments protected (kb)[b]	Putative mRNA species responsible (kb)[c]
0.527–0.592	6.0	7
	4.0[d]	5.2
	3.6[e]	3.8
	1.7	1.9
0.527–0.543	2.4	3.8
	3.6	3.8
0.527–0.570	2.6	7.2
	1.7	1.9
	0.90	5.2
0.543–0.578	3.8	7
	2.0	5
	1.7	1.9
	1.2	3.8
0.588–0.592	0.62	7
		5.2
	0.35[f]	1.5
0.592–0.602	0.9	7
		5.2
		1.5
	0.6[f]	1.9
		1.0

[a] See Fig. 1 and Table 1.

[b] All sizes were based on migration of restriction endonuclease-digested ^{32}P-labeled HSV-1 DNA fragments in alkaline gels (see the text). The sizes of the markers are shown in Table 1 and indicated in Fig. 1. All values were the average of at least two determinations and are ±50 bases of the individually determined ones.

[c] Based on the Northern blot data of Fig. 2 and Table 2.

[d] Present as a major band when early RNA was used in hybridization.

[e] Present as a minor band when early RNA was used in hybridization.

[f] Faint band.

from the presence of the 7- and 5.2-kb mRNA. Another faint band was regularly detected migrating, corresponding to a size of 350 bases. This band cannot readily be seen in photographs of the X-ray film and, therefore, the data are not shown. We tentatively conclude that this minor band corresponds to the 5′ end of the 1.5-kb mRNA species.

Polypeptide products encoded by HSV-1 mRNA species homologous to the region 0.527–0.602. In our preliminary studies, we used mRNA homologous to *Hin*dIII fragment K (0.527–0.592) isolated by DNA-cellulose hybridization. We than translated mRNA, size fractionated on a sucrose velocity gradient to determine what size polypeptides were encoded by the different-sized mRNA (4). We concluded that a 54,000-d polypeptide was encoded by both the 7-kb mRNA and an mRNA species smaller than 2 kb, presumbaly the 1.9-kb species, whereas the 5.2-kb mRNA encoded a 140,000-d polypeptide, and the 3.8-kb mRNA yielded both a 122,000-d and an 85,000- to 86,000-d polypeptide upon translation. The precise localization of specific mRNA species to different regions between 0.527 and 0.602, as well as the high resolution attainable by denaturing gel electrophoresis of preparatively hybridized mRNA populations, was used in the present paper to confirm and expand our original observations.

We used *Sal*I fragment L (0.543–0.578) bound to cellulose to purify the 7-, 5.2-, 3.8-, and 1.9-kb mRNA species homologous to this region. Typical size fractionations of such RNA by methylmercury-containing agarose gel electrophoresis are shown in Fig. 3B. The mRNA from slices was eluted and reselected on oligo(dT)-cellulose. Samples containing about 0.1 μg of each were then translated in vitro. The translation products of the size-fractionated mRNA species are shown in Fig. 8A. It is completely clear that the 7- and 1.9-kb mRNA encode the same size (54,000-d) polypeptide. As was found previously (4), the 5.2-kb mRNA encodes at 140,000-d polypeptide, whereas translation of the 3.8-kb mRNA yields a major polypeptide band migrating at 122,000 d and another prominent band migrating at 85,000 d.

To fully confirm that translation of the 3.8-kb mRNA indeed yields both 122,000-d and 85,000-d polypeptides, we isolated this mRNA by hybridizing total late poly(A) mRNA to *hin*dIII-*Sal*I fragment K-M (0.527–0.543) bound to cellulose. Analytical gels of such mRNA showed only the 3.8-kb species (data not shown), as expected from the RNA transfer blot experiments shown in Fig. 2. In vitro translation of this mRNA (Fig. 8B) in the rabbit reticulocyte system again showed a major product migrating at 122,000 d and significant material migrating at 86,000 d. We cannot, at this time, determine whether this consistent finding of at least two polypeptides efficiently translatable from this mRNA is due to an artifact of the in vitro translation system. Similar results were seen when the translation products were only heated to 85°C, instead of 100°C, in the presence of sodium dodecyl sulfate before electrophoresis; therefore, we do not feel that this result is due to artifactual degradation of the in vitro translation product. It is clear, however, that our RNA transfer blot and S1 mapping data do not indicate the presence of two mRNA's in this region unless they are identical in size and the relationship between their coding sequences is unusual.

We purified and fractionated mRNA homol-

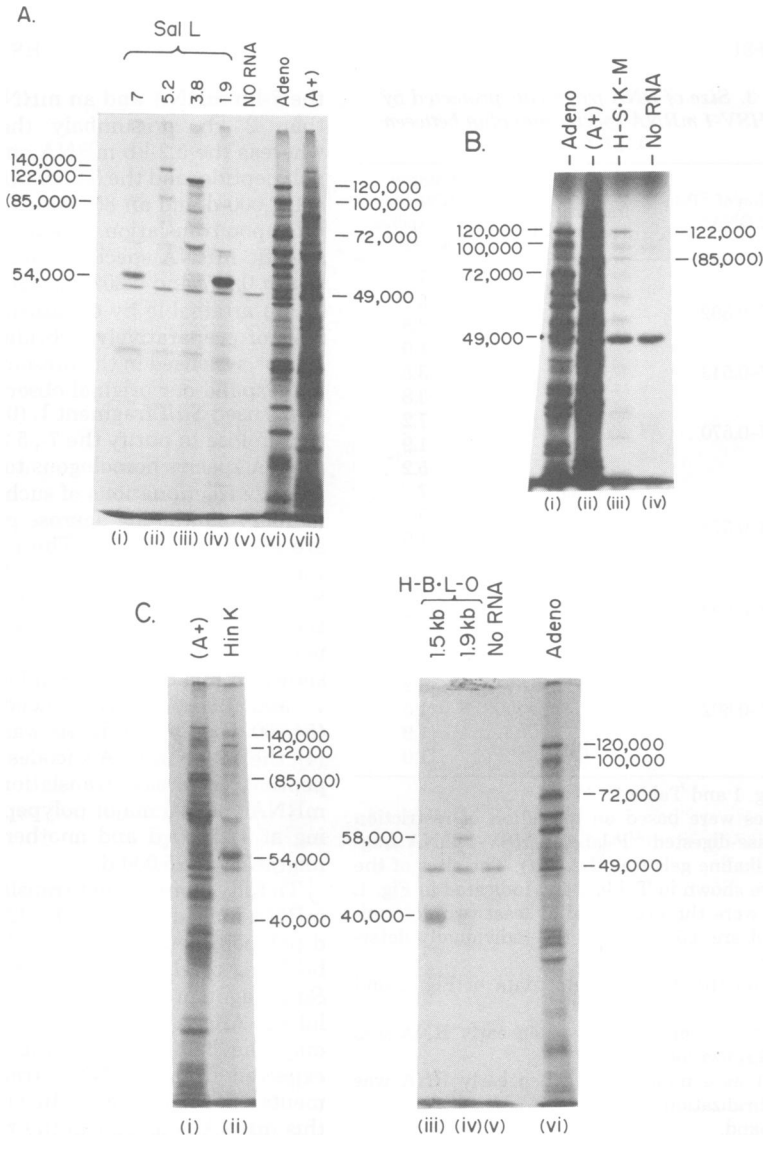

FIG. 8. *In vitro translation of specific mRNA's homologous to different regions in the area 0.527–0.602. Specific mRNA samples (0.05-0.1 μg) were isolated after denaturing agarose gel electrophoresis by phenol extraction and reselection on oligo(dT)-cellulose. This mRNA was translated with [^{35}S]methionine in a commercial reticulocyte lysate system, and translation products were fractionated by electrophoresis on 9% acrylamide gels (see the text). Molecular weights of polypeptides indicated in the figure were based on the migration of adenovirus-specific bands as described previously (3, 4). (A) Translation of SalI fragment L (0.543–0.578) specific mRNA. Tracks (i) through (iv) are the products of the 7-, 5.2-, 3.8-, and 1.9-kb mRNA, respectively; track (v) is the no RNA control; track (vi) is the translation of an adenovirus standard mRNA included in the kit and used as a primary size standard (3, 4, 16); finally, track (vii) contains translation products of 0.5 μg of total late poly(A) mRNA from HSV-1-infected cells. (B) Translation of HindIII-SalI fragment K-M (0.527–0.543) specific mRNA. Track (i) is identical to track (vi) in (A); track (ii) is the HSV poly(A) control, identical to track (vii) in (A); track (iii) contains the translation products of 0.05 μg of the specific mRNA isolated by preparative hybridization and reselection on oligo(dT)-cellulose; track (iv) is the no RNA control. (C) Translation of HindIII-BamHI fragment L-O (0.592–0.602) specific mRNA. Track (i) contains the products of translation of 0.5 μg of total late HSV-1 poly(A$^+$) mRNA; track (ii) is the translation products of 0.15 μg of HindIII fragment K (0.527–0.592) specific mRNA; track (iii) is the translation products of 0.05 μg of 1.5-kb mRNA; track (iv) contains the translation products of 0.05 μg of 1.9-kb mRNA specific for this region; track (v) is no RNA control; and track (vi) contains the translation products of adenovirus mRNA. The sharp doublet migrating at about 66,000 d in tracks (ii) and (iv) is seen in many, but not all, mRNA preparations and may be an artifact of our work-up procedures. The band at 49,000 d is endogenous to the system.*

ogous to the region 0.592–0.602 by hybridization with *Hin*dIII-*Bam*HI fragment L-O bound to cellulose. A typical size fractionation of such mRNA is shown in Fig. 3C. Translation of 0.05 μg each of the 1.9- and 1.5-kb mRNA species is shown in Fig. 8C. The 1.9-kb species encoded a polypeptide migrating, corresponding to a size of 58,000 d, clearly different from the size of the polypeptide product of in vitro translation of the 1.9-kb mRNA mapping to the left of 0.566 (Fig. 8A). Translation of the 1.5-kb mRNA results in the synthesis of polypeptide products which migrate somewhat heterogeneously, corresponding to an average size of 40,000 d. Material of this size can also be seen in the translation products of the total *Hin*dIII fragment K (0.527–0.592) specific mRNA shown in Fig. 8C and has been observed in the translation of unfractionated region specific mRNA isolated with early poly(A) mRNA (4). Translation of the 7- and 5.2-kb mRNA isolated from the gel gave the same translation products as seen for these mRNA's in Fig. 8A; Therefore, the data are not shown.

DISCUSSION

The techniques described in this report have been used in concert to map rather precisely a number of relatively abundant HSV-1 mRNA species in the region 0.527–0.602. Our use of RNA transfer (Northern) blot hybridization to grossly localize the mRNA species before S1 nuclease analysis precludes possible confusion caused by the rather complex arrangements of the mRNA's, as well as the sometimes high background obtained with the S1 gels (see above). We have summarized the data obtained for the position, direction of transcription, and (where known) size of polypeptides encoded by the mRNA's described in this paper (Fig. 9). The precise localization of the 1.9- and 1.5-kb mRNA's must be regarded as most probable because of the lack of a convenient restriction site cutting the coding sequence of the former, and because the small amounts of the latter have made it difficult to obtain good S1 mapping data. The indication that the 3′ coding sequences of the 1.9- and 1.0-kb mRNA's mapping around the *Bam*HI site at 0.602 actually extend 0.58 kb to the left of this site must also be taken as tentative at this time.

The dense packaging of the coding sequences for the mRNA species described in this paper and the fact that mRNA's functional at different stages of infection overlie each other indicate that this region will be of continuing interest in developing a complete molecular description of HSV-1 mRNA biogenesis. Higher-resolution maps of these mRNA's could be obtained by successive refinements of the methods described; however, the present data are of sufficient resolution to indicate areas of interest for the study of nuclear precursors of these mRNA's and to begin sequence analysis. Such studies will lead to highest resolution as direct corollary of the data obtained.

Agreement between the size of the DNA sequences protected by the various mRNA species and the sizes of those mRNA's as determined by RNA transfer blot hybridization is excellent (cf. Tables 2 and 3). Based on the various S1 analyses and the location of specific restriction sites

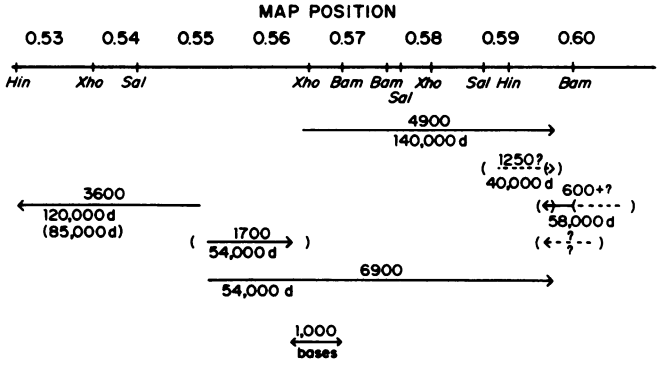

FIG. 9. *Location of mRNA's mapping between 0.527 and 0.602 on the (P) arrangement of the HSV-1 genome. The mRNA's are shown with their coding sequence lengths in bases on top, and the sizes of the polypeptide(s) that they encode are shown below. The arrows indicate the direction of transcription. The location of four mRNA's was inferred by information additional to the S1 data described in this paper. These are the 1.9-kb (1,700 bases) mRNA mapping to the left of 0.566, the exact 3′ end of the 1.5-kb (1,250 bases) mRNA mapping around 0.59, and the 5′ ends of the 1.9- and 1-kb mRNA's mapping around 0.6. The positional uncertainties are discussed in the text and are indicated with parentheses. Restriction sites are as described in the legend to Fig. 1.*

(Table 1), the size of the coding sequence for the 7-kb mRNA is 6.8 to 6.9 kb; that of the 5.2-kb mRNA is 4.9 to 5 kb; that of the 3.8-kb mRNA is 3.5 to 3.6 kb; that of the 1.9-kb mRNA is 1.7 kb; and that of the 1.5-kb mRNA is 1.25 kb. Since the size of the poly(A) tails of HSV-1 mRNA late after infection averages 0.2 to 0.3 kb (36), these values suggest upper limits for the sizes of any noncontiguous "leader" sequences to these mRNAs as being 0.1 kb or less. Such sequences, if they exist, might not protect their coding sequences under our S1 conditions if their base composition was low enough to allow limited S1 digestion.

There are no readily detectable introns in the interior of the coding sequences for the major mRNA species characterized in this report. Other work in progress in this laboratory suggests that this is the case for a number of major mRNA's mapping in the long unique region. Although many early adenovirus mRNA species contain internal introns in their coding sequences (8), some late ones do not, aside from the noncontiguous tripartite leader sequence (30). This, and the fact that at least one functional adenovirus gene (IX) contains no introns at all (1), indicates that a large amount of splicing need not be obligate in the maturation of viral mRNA. There is no doubt, however, that some HSV-1 mRNA species do contain splices as genes for two immediate-early mRNA's, whose 5′ ends map in the short repeat region, contain a 150-base intron 260 bases in from their 5′ ends (46a). This suggests that splicing may be involved in the biogenesis of this mRNA before viral DNA replication, at least.

Although the exact biological function of the genes encoding the viral mRNA's described in this report is largely unknown, the size of the polypeptide products from in vitro translation studies correlates well with the size of viral polypeptides found using intertypic recombinants in vivo (25, 27). The ability to isolate the mRNA's encoding these polypeptides should allow appropriate in vitro studies of their function as these become indicated through in vivo studies.

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