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Herpes Simplex Virus Type 1 *Hin*dIII Fragment L Encodes Spliced and Complementary mRNA Species

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We have used DNA bound to cellulose to isolate and translate in vitro herpes simplex virus type 1 (HSV-1) mRNA's encoded by *Hin*dIII fragment L (mapping between 0.592 and 0.647), an 8,450-base-pair (8.45-kb) portion of the long unique region of the viral genome. Readily detectable, late mRNA's 2.7 and 1.9 kb in size encoding 69,000- and 58,000-dalton polypeptides, respectively, were isolated. A very minor late mRNA family composed of two colinear forms, one 2.6 kb and one 2.8 kb, was isolated and found to encode only an 85,000-dalton polypeptide. A major early mRNA, 1.8 kb in size encoding a 64,000-dalton polypeptide, was also isolated. High-resolution mapping of these mRNA's by using S1 nuclease and exonuclease VII digestion of hybrids between them and 5′ and 3′ end-labeled DNA fragments from the region indicated that the major early mRNA contained no detectable splices, and about half of its 3′ end was complementary to the 3′ region of the very minor 2.6- to 2.8-kb mRNA's encoded on the opposite strand. These mRNA's also contained no detectable splices. The major late 2.7-kb mRNA was found to be a family made up of members with no detectable splices and members with variable-length (100 to 300 bases) segments spliced out very near (ca. 50 to 100 bases) the 5′ end.

Like all herpesviruses, herpes simplex virus type 1 (HSV-1) is characterized by an unusual arrangement of its genome. The viral DNA has a molecular weight of 95×10^6 to 100×10^6 daltons (d; reviewed in reference 44), which corresponds to a length of 150,000 base pairs (150 kb; 50). The linear HSV-1 genome is segmented into a long unique region (U_L, ca. 105 kb, 70%) and a short unique region (U_S, ca. 15 kb), each bounded by different-length, inversely reiterated sequences (R_L, 9 kb; R_S, 6 kb). Such a structure results in four equimolar populations of the viral DNA differing in the relative orientations of the long and short segments (25, 47, 56, 64, 65). In view of this fact, one arrangement has, by convention, been chosen as the prototypical (P) configuration.

There are three readily recognizable stages of RNA expression during HSV-1 replication, as there are for many other herpesviruses. Each stage is characterized by an increasing complexity of viral mRNA expressed (reviewed in references 48 and in E. Wagner, K. Anderson, R. Costa, G. Deni, B. Gaybid, L. Holland, J. Stringer, and L. Tribble, *in* Y. Becker, ed., *Herpesvirus DNA: Recent Studies on the Internal Organization and Replication of the Viral Genome*, in press). In the first stage, α or immediate-early mRNA species are seen. These can be expressed abundantly without de novo protein synthesis, i.e., in an unmodified host cell nucleus (31, 43). Abundant members of this class of mRNA are quite limited, map in regions of the HSV-1 genome at or near the R_L and R_S regions, and encode only a limited number of polypeptides in vivo and in vitro (3, 14, 15, 27-29, 33, 42, 61, 62). Two α mRNA's encoding different polypeptides are chimeric in that their 5′ ends map in the S_R region, but their 3′ ends map in different ends of the U_S region (5, 14, 61). The coding sequence for their identical 5′ ends contains the to date best-characterized HSV-1 intron, which is about 150 bases in length and occurs approximately 260 bases from the 5′ ends of the mRNA's (62).

After expression of one or several HSV-1 α proteins, a more complex population of viral mRNA becomes abundant before viral DNA replication (31, 38, 51, 52, 57, 59). mRNA's which are expressed after the α mRNA's, but before viral DNA replication, comprise the β or early class. These β viral mRNA's map throughout the HSV-1 genome in noncontiguous regions, but only a limited number of readily resolvable species are found (27, 30, 50). To date, three β mRNA's have been rigorously mapped. These

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are the 1.5-kb thymidine kinase *tk* mRNA at about 0.3 in the U_L region (20, 35, 41); a 5.2-kb mRNA encoding a 140,000-d polypeptide mapping between 0.55 and 0.6 in the U_L region (4, 5); and as described here, a 1.8-kb mRNA mapping at about 0.615 in the U_L region and encoding a 64,000-d polypeptide. None of these mRNA's appears to be spliced, although a very short splice (<50 bases) extremely near the end of this last mRNA cannot be rigorously excluded at this time.

Many β mRNA's are involved with priming the cell for viral DNA replication. We, as well as other workers, have shown that, without viral DNA replication, the β viral mRNA and protein population appears to persist (26, 40, 51, 52, 57, 59, 60). In the rigorous absence of viral DNA replication, the specific viral mRNA species and the polypeptides they encode in vitro are virtually indistinguishable from those seen in the infected cell cytoplasm before the onset of viral DNA replication (26).

Concomitant with viral DNA replication is the appearance of late HSV-1 mRNA (57). Two subclasses of late mRNA can be readily distinguished. These are a group which is detectable in the absence of viral DNA replication, the "leaky late" or $\beta\gamma$ mRNA's (19, 27) and a group which cannot be seen at all in the cytoplasm in the absence of viral DNA replication, the "true late" or γ mRNA's (26, 30). Both groups of mRNA species encode a large number of polypeptides (26), many of which presumably are structural proteins of the virion. We have precisely mapped a number of $\beta\gamma$ and γ mRNA's. These include a very abundant 6-kb $\beta\gamma$ mRNA mapping between 0.225 and 0.265 in the U_L region (18). This mRNA encodes a 155,000-d polypeptide which appears to be the major capsid polypeptide of the virus. Other $\beta\gamma$ and γ mRNA's have been mapped in *Hin*dIII fragment K (mapping between 0.527 and 0.592) (4–6); and as described in this paper, in *Hin*dIII fragment L (mapping between 0.592 and 0.647). These latter mRNA's share properties of many of the mRNA's already described; however, they display some novel features not previously seen in HSV-1 mRNA mapping in the U_L region. For example, we have found a family of mRNA's of which some members appear to have spliced 5′ ends. We also have found that this U_L region contains the coding sequence of an abundant β mRNA which is partially complementary to the coding sequence of a less abundant family of γ mRNA's transcribed from the opposite DNA strand. Whether the unusual features of HSV-1 mRNA's encoded in this region reflect some unusual biological functions of the polypeptides they encode is unknown.

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 enzymes except reverse transcriptase (a gift of J. Beard) were obtained from Bethesda Research Laboratories; digestion was carried out in buffers recommended by that supplier. For restriction digests, each microgram of DNA was digested with 0.5 U of the appropriate enzyme for 3 h at 37°C. Phage T4 ligase was used essentially as recommended by the supplier and as described previously (4, 18). Phage T4 polynucleotide kinase was used for 5′ phosphate exchange as described by Maxam and Gilbert (34).

Isolation, labeling, and size fractionation of polyribosomal RNA. Monolayer cultures of HeLa cells (2×10^7 cells per 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 overlayered 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 1/10 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 (26). 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 (39, 49). Polyribosome-associated RNA was purified by phenol-chloroform extraction after proteinase K digestion of the polyribosomes. Polyadenylic acid-containing mRNA [poly(A) RNA] was isolated from total ribosomal RNA by the use of oligodeoxythymidylic acid-cellulose (Collaborative Research, Inc.). Details of this procedure have been presented elsewhere (3–6, 18, 26, 27).

RNA was size fractionated by electrophoresis on 1.2% agarose gels containing 10 mM methylmercury hydroxide (8) as previously described (6).

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 L for the fragment mapping between 0.592 and 0.647), and their coordinates are given parenthetically in map units on the P arrangement of the HSV-1 genome. All recombinant DNA experiments were covered under Section III-O of the January 1980 National Institutes of Health guidelines. *Hin*dIII fragment L (0.592–0.647) and *Bam*HI fragment I (0.602–0.643) were cloned in pBR322 in our laboratory by using *Escherichia coli* LE392. The procedure has been described previously (4, 18) and is essentially that of Bedbrook et al. (10).

Transfected cells (17) were replica plated onto filters, and these were subjected to colony hybridization by using 2×10^6 to 3×10^6 Cerenkov cpm (45) of [^{32}P]cDNA made to isolated *Hin*dIII fragment L

(0.592–0.647) and *Bam*HI fragment I (0.602–0.643). Hybridization conditions are described below. Plasmid from positive colonies was isolated by the method of Clewell and Helinski (16); 100- to 200-ml cultures were grown in Luria broth, and the plasmid was isolated by the procedure of Tanaka and Weisblum (53) for chloroamphenicol amplification. The LE392 grown in 40 μg of ampicillin per ml yields between 0.5 and 1 μg of plasmid DNA per ml of culture. Each preparation was rechecked by restriction digestion before further use.

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 previously (4, 18).

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 are as described previously (3–6, 18, 26, 27). Hybridization was in 80% recrystallized formamide containing 0.4 M Na^+, 0.1 M *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 (6).

Synthesis of cDNA. The cDNA to particular DNA sequences was synthesized as described in previous reports (3–5, 18, 26, 27). Total cDNA was synthesized by using random oligodeoxyribonucleotides prepared from calf thymus DNA as a primer for reverse transcriptase (54).

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 in 65% formamide, 0.4 M Na^+, 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 8.0), 5 mM EDTA, and Denhardt solution (21) at 45°C for 48 h as described previously (4, 18).

Nuclease mapping of HSV-1 RNA. S1 nuclease and exonuclease VII analysis of RNA was carried out essentially as described by Berk and Sharp (11–13). Appropriate HSV-1 DNA clones (5 μg) were restricted at the desired site with the appropriate enzyme. The DNA then was either 5′ or 3′ labeled with ^{32}P as described below. Samples containing 2 μg of DNA and 10 μg of infected cell polyribosomal poly(A) RNA were precipitated with ethanol. The mixed pellets were dissolved in 50 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 the addition of 0.9 ml of ice-cold pH 4.6 buffer containing 250 mM NaCl, 30 mM sodium acetate, 1 mM $ZnCl_2$, and 5% glycerol. S1 nuclease was added, and digestion was carried out for 40 min at 37°C. The material was then ethanol precipitated. When exonuclease VII digests were to be done, a sample containing 0.7 μg of DNA and 4.5 μg of RNA was hybridized separately in 15 μl of hybridization buffer at 58°C for 4 h.

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

Specific end labeling of DNA. Clones of the DNA of interest were 5′ end labeled by digesting the clone with the proper enzyme and then using the 5′ phosphate exchange reaction described by Maxam and Gilbert (34) with 100 μCi of [α-^{32}P]ATP (>2,000 Ci/mmol; Amersham Corp.). The DNA was between 2,000 and 4,000 cpm (Cerenkov units) per μg. We used reverse transcriptase to 3′ end label. Cloned DNA was digested with an appropriate enzyme which leaves a protruding 5′ end. The ends were denatured by incubation at 65°C for 10 min, and then the DNA was incubated with 1 U of reverse transcriptase and 50 μCi of α-^{32}P-labeled deoxynucleoside triphosphate chosen from the known next position to the 3′ end of the restriction site. Incubation buffer did not contain the other deoxynucleoside triphosphates; incubation was for 30 min at 37°C. The specific activity of the DNA was comparable to that of the 5′-labeled material.

In vitro translation. Translation of viral mRNA was carried out in vitro by using a micrococcal nuclease-treated rabbit reticulocyte system (New England Nuclear Corp.) with [^{35}S]methionine (675 Ci/mmol) as the radioactive amino acid. Details of the procedure and fractionation of polypeptides in sodium dodecyl sulfate-acrylamide gels by the method of Laemmli (32) have been described in several previous papers (3–5, 18, 26). Gels were dried with vacuum, and radioactive bands were localized by autoradiography with Kodak NS-2T film.

RESULTS

General characteristics of the mRNA species encoded by *Hin*dIII fragment L (0.592–0.647). Restriction endonuclease sites for *Bam*HI, *Sal*I, *Hpa*I, and *Eco*RI in the region 0.592–0.647 are shown in Fig. 1. We have previously shown that several mRNA's mainly mapping to the left of 0.592 have their 3′ ends in *Hin*dIII-*Bam*HI fragment L-O (0.592–0.602) (4); therefore, we used cDNA made to *Bam*HI fragment I (0.602–0.643) DNA to hybridize to an RNA transfer (Northern) blot of late-infected cell polyribosomal poly(A) mRNA's to see what specific mRNA's mapped to the right of these. These data are shown in Fig. 2A (track 1) where major bands of 2.7 and 1.8 to 1.9 kb are seen, as well as some minor ones.

There are actually three readily detectable and several very-low-abundance mRNA species responsible for these bands. Multiple mRNA species were graphically demonstrated by the

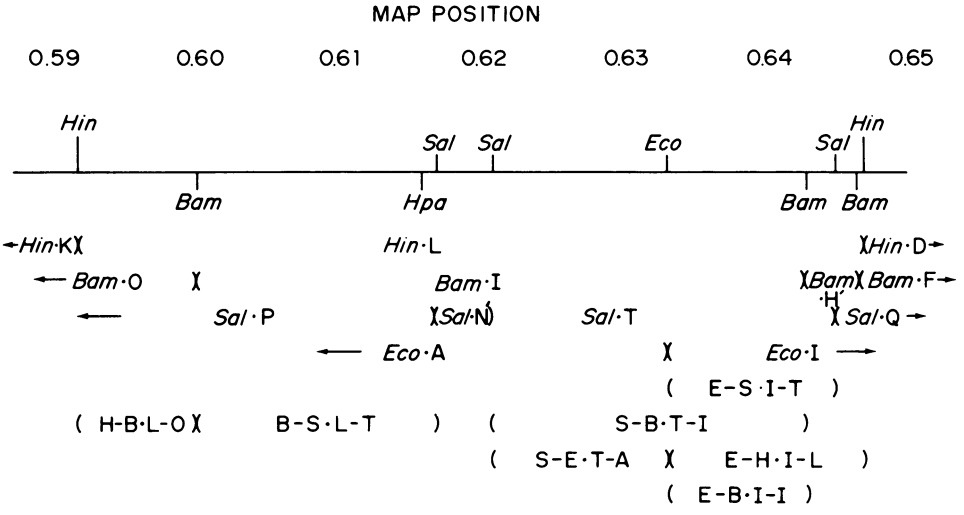
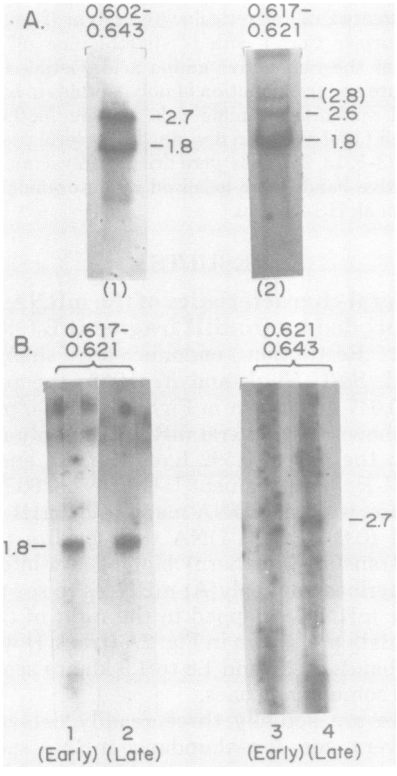


FIG. 1. *Restriction endonuclease cleavage sites and locations of HSV-1 DNA fragments cloned in pBR322 in the region 0.59 through 0.65 on the P arrangement of HSV-1 (KOS). HindIII fragment L (0.592–0.647) and BamHI fragment I (0.602–0.643) were cloned in pBR322, and these cloned DNA fragments were digested with HindIII (Hin), BamHI (Bam), SalI (Sal), EcoRI (Eco), or HpaI (Hpa) restriction endonucleases in various combinations to determine the position of the sites shown. Fragments were sized by electrophoresis on 0.7 and 1.2% neutral agarose gels versus standard size DNA fragments derived by HindIII and EcoRI digestion of phage λ (cI857S7) DNA (55). The left-hand HindIII site was set at 0.592. All other sites were based on restriction fragment sizes (1,000 base pairs = 0.00667 map units). Names of fragments are as described (4, 18).*



analysis of the translation products of *Bam*HI fragment I (0.602–0.643)-specific mRNA (Fig. 3A, track i). When 0.2 μg of total late region-specific mRNA selected by hybridization to *Bam*HI fragment I (0.602–0.643) DNA bound to cellulose was translated in vitro, major polypeptides of 69,000 and 64,000 d were seen as well as

FIG. 2. *RNA transfer (Northern) blots of mRNA encoded by the region 0.602 through 0.645. (A) Samples of 8 μg of late poly(A) mRNA were fractionated on agarose gels containing methylmercury hydroxide and blotted onto diazotized paper (see text). Blots were hybridized to 5 × 10^6 Cerenkov cpm of [^{32}P]-cDNA made to (track 1) BamHI fragment I (0.602–0.643) or (track 2) SalI fragment N′ (0.617–0.621). The location of the restriction fragments are shown in Fig. 1. Sizes of mRNA were based on the migration of 28S and 18S rRNA run in parallel tracks (5.2 kb and 2.0 kb; 36, 63). (B) Demonstration of a major early mRNA mapping in the region 0.617 through 0.621. Early poly(A) mRNA (15 μg) was obtained from adenosine arabinoside-treated cells (see text) and fractionated as described for panel (A) above. Details of hydridization are also as described above. Tracks 1 and 2 show early and late mRNA hybridized to [^{32}P] cDNA made to SalI fragment N′ (0.617–0.621) and tracks 3 and 4 show early and late mRNA hybridized to [^{32}P] cDNA made to SalI-BamHI fragment T-I (0.621–0.643).*

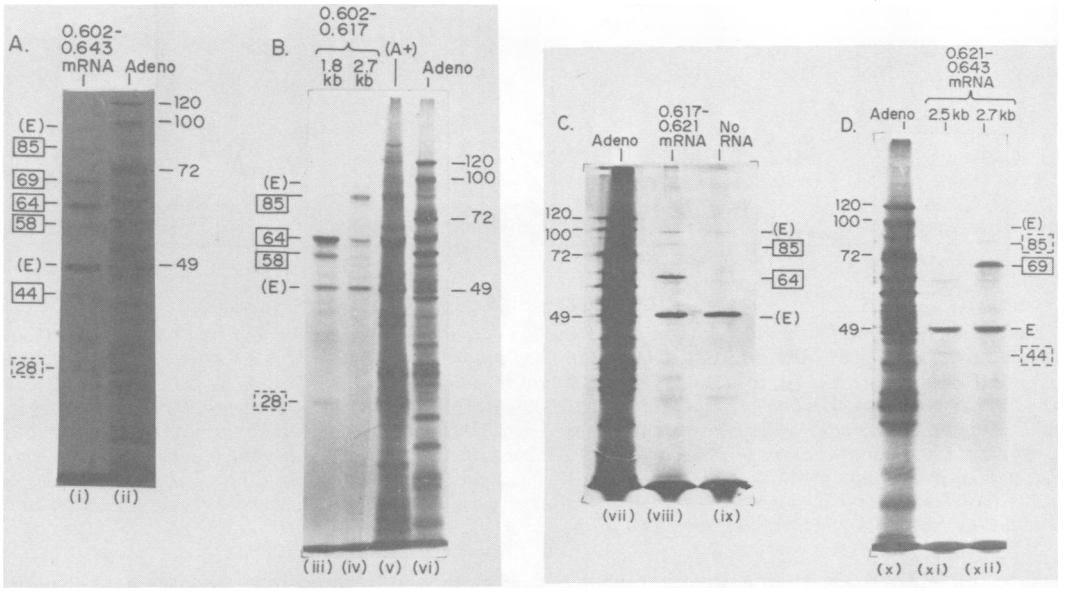


FIG. 3. *In vitro translation of mRNA encoded in the region 0.602 through 0.643. Specific mRNA samples (0.03 to 0.05 μg) in the region were isolated by DNA cellulose hybridization. These were either reselected on oligodeoxythymidylic acid-cellulose and translated in a reticulocyte lysate system directly or size fractionated by denaturing agarose gel electrophoresis (see Fig. 4) and eluted by phenol extraction followed by reselection on oligodeoxythymidylic acid-cellulose (see text). Translation was with [^{35}S]methionine, and the translation products were fractionated by electrophoresis on 9% acrylamide gels. Molecular masses (in thousands of daltons) of polypeptides indicated in the panels were determined from the migration of adenovirus-specific bands as described previously (3–5, 18, 26). (A) Translation of total BamHI fragment I (0.602–0.643)-specific mRNA (track i). The bands marked (E) of approximately 100,000 and 49,000 d are endogenous translation products of the system as evidenced by translation of a non-RNA control (see panel C, track ix). Polypeptides migrating with rates corresponding to molecular masses of 85,000, 69,000, 64,000, 58,000, 44,000, and 28,000 d were reproducibly seen. Track (ii) contains the translation products of the adenovirus control mRNA. (B) Translation of BamHI-SalI fragment I-P (0.602–0.617)-specific mRNA. Track (iii) contains the translation products of the 1.8-kb mRNA fractionated in Fig. 3A and bands of 64,000 and 58,000 d are seen. Track (iv) contains the translation products of the 2.7-kb mRNA of Fig. 3A, and the 85,000-d polypeptide is seen as a major product. Track (v) contains the products of translation of a 0.1-μg sample of total late infected cell poly(A) mRNA; and track (vi) is as track (ii). (C) Translation of SalI fragment N′ (0.617–0.621)-specific mRNA (track viii). Polypeptides of 85,000 and 64,000 d are the only bands seen beyond endogenous products seen also in the non-RNA control, track (ix). Track (vii) is as track (ii) of panel (A). (D) Translation of SalI-BamHI fragment T-I (0.621–0.643)-specific mRNA. Track (x) is as tracks (ii), (vi), and (vii). Track (xi) contains the products of translation of the 2.5-kb shoulder of Fig. 3B. Faint bands of 85,000 and 44,000 d can be seen with difficulty. Track (xii) contains the products of translation of the major 2.7-kb mRNA band of Fig. 3B. A major polypeptide migrating at a rate corresponding to a size of 69,000 d is seen in addition to a faint band at 85,000 d.*

lesser amounts polypeptides of 85,000, 58,000, 44,000, and 28,000 d.

The gross localization of mRNA's encoding these polypeptides was accomplished by use of subclones of *Bam*HI fragment I (0.602–0.643) DNA and *Hin*dIII fragment L (0.592–0.647) DNA. We have previously shown (4) that a 1.9-kb βγ mRNA of moderate abundance which encodes the 58,000-d polypeptide has its 3′ end to the left of 0.602 and its 5′ end extending into *Bam*HI-*Sal*I fragment I-P (0.602–0.617). When this latter fragment bound to cellulose was used for isolation of specific mRNA's followed by their fractionation on a denaturing agarose gel, the pattern shown in Fig. 4A was obtained. A minor mRNA species approximately 2.7 kb in size and a major band of mRNA migrating with a rate corresponding to a size of approximately 1.8 kb were readily resolvable. These bands were eluted and translated. As shown in Fig. 3B (track iii), the 1.8-kb mRNA encodes the 64,000- and 58,000- polypeptides and a very small amount of the 28,000-d polypeptide. The mRNA for this latter polypeptide has not been identified; however, since it is known that the 58,000-d polypeptide is encoded by the 1.9-kb mRNA with its 3′

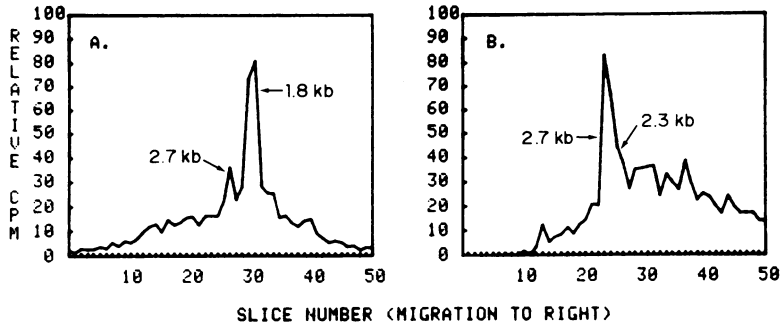


FIG. 4. *Isolation and size fractionation of specific HSV-1 mRNA species homologous to the regions 0.602 through 0.617 and 0.621 through 0.643. Late ^{32}P-labeled polyribosomal poly(A) mRNA was hybridized with (A) BamHI-SalI fragment I-P (0.602–0.617) DNA bound to cellulose or (B) SalI-BamHI fragment T-I (0.621–0.643) DNA 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 measuring Cerenkov radiation in a Beckman LS-230 scintillation counter. Sizes of the mRNA species were determined from the migration of 28S and 18S rRNA (5.2 and 2.0 kb; 36, 63) in a parallel gel. The sizes of individual bands (in kb) eluted for in vitro translation are indicated above them. Details of elution and translation of mRNA fractions are described in the text.*

end to the left of 0.602, then a second mRNA of about the same size (1.8 kb) encodes the 64,000-d polypeptide. S1 data (below) indicate that this 1.8-kb mRNA is more abundant than the 1.9-kb mRNA. The very minor 2.7-kb mRNA encodes the 85,000-d polypeptide (Fig. 3B, track iv).

The 1.8-kb mRNA and the very minor 2.7-kb mRNA extend into *SalI* fragment N′ (0.617–0.621) since mRNA specific to this fragment encodes both the 85,000- and the 64,000-d polypeptide when translated in vitro (Fig. 3C, track viii). Further, RNA transfer (Northern) blots of late polyribosomal poly(A) mRNA hybridized with cDNA made to *SalI* fragment N′ (0.617–0.621) DNA show a major 1.8-kb mRNA band; interestingly, use of this method can resolve the very minor 2.7-kb band seen in Fig. 4A into two bands, one about 2.6 kb and one (fainter) about 2.8 kb (Fig. 2A, track 2).

The 1.8-kb mRNA spanning the *SalI* site at 0.617 does not extend beyond 0.621 since no readily resolvable band of this size is seen in RNA transfer (Northern) blots hybridized with cDNA made to *SalI-BamHI* fragment T-I (0.621–0.643) DNA (Fig. 2B, tracks 3 and 4). In support of this, we found that total mRNA encoded by *SalI-BamHI* fragment T-I (0.621–0.643) DNA cannot be translated to give a 64,000-d polypeptide band (data not shown). Finally, this 1.8-kb mRNA is seen to be a β (early) mRNA since *SalI* fragment N′ (0.617–0.621) cDNA lights a 1.8-kb band when hybridized to RNA transfer blots of early polyribosomal poly(A) mRNA isolated from cells treated with ara-A and pentostatin (Fig. 2B, track 1).

When HSV-1 mRNA homologous to *SalI-BamHI* fragment T-I (0.621–0.643) DNA is fractionated on a denaturing agarose gel, a major band of 2.7 kb is seen along with a shoulder about 2.3 kb in size and small amounts of poorly resolved material ranging in size between 2.3 to <1 kb (Fig. 4B). The 2.7-kb mRNA encodes the 69,000-d polypeptide as shown by its in vitro translation products in Fig. 3D (track xii). A small amount of the 85,000-d polypeptide is also translated with this size material. This indicates that the very minor 2.7-kb mRNA found mapping to the left of 0.617 into *SalI* fragment N′ (0.617–0.621) extends beyond 0.621. This is confirmed by the S1 mapping data described below.

The abundant 2.7-kb mRNA appears to be a γ mRNA since it cannot be seen at all when *SalI-BamHI* fragment T-I (0.621–0.643) cDNA is hybridized to RNA transfer (Northern) blots of early polyribosomal poly(A) mRNA (Fig. 2B, track 3). The 2.3-kb shoulder to the 2.7-kb mRNA seen in Fig. 4B was translated also, and only small amounts of the 85,000-d and the 44,000-d polypeptides were seen (Fig. 3D, track xi). This suggests that *SalI-BamHI* fragment T-I (0.621–0.643) also encodes the mRNA for this latter polypeptide.

The experiments discussed above then allowed us to identify at least four mRNA's encoding discrete polypeptides in the region 0.602–0.643. The properties of these mRNA's are summarized in Table 1. To simplify the discussion of our nuclease mapping data of the next section, we have arbitrarily numbered these mRNAs as follows: the $\beta\gamma$ mRNA encoding the 58,000-d polypeptide with its 3′ end to the left of 0.602 is species 1; the β mRNA encoding the 64,000-d polypeptide is species 2; the very minor 2.7-kb doublet γ mRNA encoding the 85,000-d polypep-

TABLE 1. *General properties of isolable HSV-1 mRNA species mapping between 0.602 and 0.647*

mRNA		Major restriction fragment(s) encoding it	Time of appearance and abundance[a]	Size of polypeptide encoded
Species[b]	Size[c]			
1	1.9	*Bam*HI-*Sal*I fragment I-P (0.602–0.617), *Hin*dIII-*Bam*I fragment L-O (0.592–0.602)	$\beta\gamma$[d]	58,000 d[e]
2	1.8	*Bam*HI-*Sal*I fragment I-P (0.602–0.617), *Sal*I fragment N′ (0.617–0.621)	β	64,000 d[f]
3	2.6, 2.8	*Bam*HI-*Sal*I fragment I-P (0.602–0.617), *Sal*I fragment N′ (0.617–0.621), *Sal*I-*Bam*HI fragment T-I (0.621–0.643)	$\gamma^{(-)}$	85,000 d[g]
4	2.7	*Sal*I-*Bam*HI fragment T-I (0.621–0.643)	γ	69,000 d[h]

[a] β (Fig. 2B, tracks 1 and 2) indicates a major species seen in the presence of adenosine arabinoside (a viral DNA synthesis inhibitor); $\beta\gamma$ is a minor species seen in the absence of viral DNA synthesis and is significantly more abundant after this; γ (Fig. 2B, tracks 3 and 4) is a major species seen only after viral DNA synthesis; γ (−) (Fig. 2A, track 2; Fig. 4A) is a minor species seen after viral DNA synthesis.

[b] The species have been arbitrarily numbered as described in the text.

[c] Size is based on comigration with 28S and 18S HeLa cell rRNA (5.2 and 2.0 kb; 36, 63) and includes the lengths of the poly(A) tail which averages ca. 200 bases (49).

[d] Not as abundant as mRNA species 2 (4).

[e] Figure 3B, track iii (6).

[f] Figure 3B, track iii; Fig. 3C, track viii.

[g] Figure 3B, track iv; Fig. 3C, track viii.

[h] Figure 3D, track xii.

tide is species 3; and the major γ mRNA encoding the 69,000-d polypeptide is species 4.

Detailed nuclease mapping of mRNA's encoded by the region 0.602 through 0.647. Our approach toward the precise mapping of the mRNA species of interest was straightforward. The data discussed in the previous section allowed the rough localization of the mRNA species spanning one or more of the restriction sites shown in Fig. 1. We then followed procedures adapted from those described by Berk and Sharp (11–13). Late polyribosomal poly(A) mRNA was hybridized with 3′ or 5′ end-labeled DNA. The position of the end label was at a restriction endonuclease site known to cut the DNA in a region encoding a specific mRNA. After hybridization, the material was digested with either S1 nuclease or exonuclease VII, and the protected DNA was size fractionated by gel electrophoresis. The direction of transcription of the mRNA could be deduced from whether a specific 3′ or 5′ end-labeled DNA fragment hybridized, and the sizes of the protected end-labeled DNA fragments provided a very precise measure of the extent of the length of coding sequences both to the left and to the right of the restriction site.

Our data are summarized in Fig. 5. Some actual data are shown in Fig. 6, 7, and 8, and all data are fully summarized in Table 2. Results for each region of the DNA encoding a different mRNA species were as follows.

mRNA species 1. $\beta\gamma$ mRNA species 1 protected 510 bases of DNA from S1 digestion when hybridized with *Hin*dIII-*Bam*HI fragment L-O (0.592–0.602) DNA 3′ labeled at the *Bam*HI site (0.602; Fig. 6, track ii). Thus, the 3′ end of this mRNA extends 510 bases to the left of 0.602. When late polyribosomal poly(A) mRNA was hybridized to *Bam*HI-*Sal*I fragment I-P (0.602–0.617) DNA 5′ labeled at the *Bam*HI site (0.602) and digested with S1 nuclease, a DNA-RNA hybrid band 1,125 pairs in length was seen on neutral gels (Fig. 7A, track i). Alkaline gel electrophoresis yielded a major band migrating with a length corresponding to 1,075 bases (Fig. 7B, track iii). This slight difference in length may indicate a very short noncontiguous region at the 5′ end of this mRNA (ca. 50 bases) since exonuclease VII digestion yielded a band 1,175 bases in length (Fig. 7B, track v). Such differences were reproducible but are at the limits of resolution of the present methods, and the presence or absence of such a short intron in the coding sequences can only be confirmed by nucleotide sequence analysis.

There are some very faint minor bands also seen in the neutral and alkaline S1 gel profiles of this 5′-labeled DNA hybridized with late polyribosomal poly(A) mRNA (Fig. 7A, track i; Fig. 7B, track iii). We suggest that these are caused by the presence of other very-low-abundance mRNA's not subject to characterization by our present methods.

mRNA species 2. Total late polyribosomal poly(A) mRNA protected a 1,000-base length of DNA when hybridized to *Bam*HI-*Sal*I fragment I-P (0.602–0.617) DNA 5′ labeled at the *Sal*I site (0.617) and digested with either S1 nuclease or exonuclease VII (data not shown). This indicated that mRNA species 2 has its 5′ end 1,000 bases to the left of 0.617. The identity of lengths of DNA protected from both S1 and exonuclease VII indicates that there are no detectable introns in the 5′ end of this mRNA.

The 3′ end of mRNA species 2 protects a 440-base length of *Sal*I fragment N′ (0.617–0.621). This was inferred from the fact that, when polyribosomal poly(A) mRNA was hybridized to the 600-base-pair *Sal*I fragment N′ (0.617–0.621)

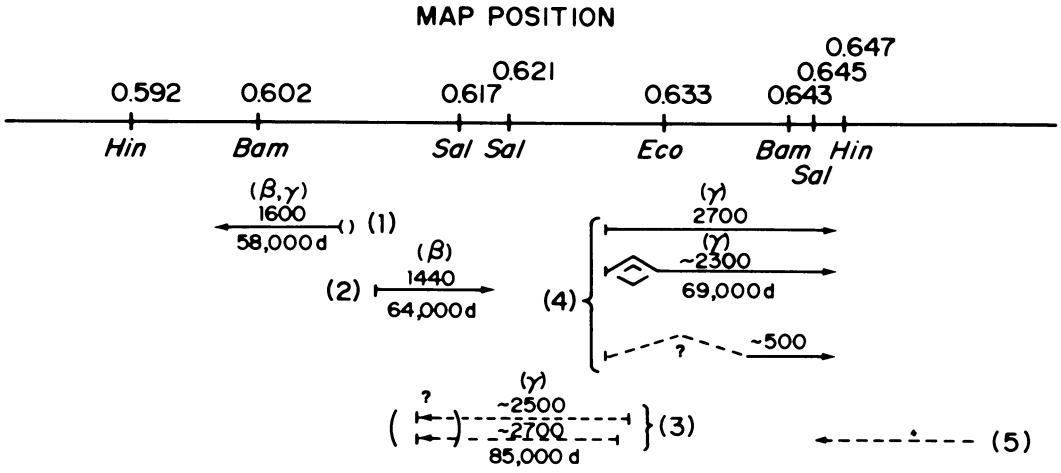


FIG. 5. *Schematic localization of HSV-1 mRNA species mapping between 0.602 and 0.645 on the (P) arrangement of the HSV-1 genome. This figure is based on data summarized in Table 2 and shown in Fig. 6, 7, and 8. The arrows indicate direction of transcription. The total length of the mRNA's and their time of appearance are shown above them and the size of the polypeptide encoded are shown below. The numbers at the 5′ ends of the RNAs refer to Tables 1 and 2.*

DNA 3′ end labeled at both *SalI* sites, both a faint band of 600 bases and a major band of 440 bases were seen after S1 nuclease digestion and alkaline gel fractionation (Fig. 6, track vi). We concluded that the more abundant 440-base band was protected by mRNA species no. 2 by virtue of its relative abundance.

mRNA species 3. The RNA transfer blot data (Fig. 2A, track 2) suggests that low-abundance γ mRNA species 3 is actually a doublet or a family of related forms. We found that the 5′ end of this mRNA family mapped to the right of 0.621 by hybridizing late polyribosomal poly(A) mRNA with *SalI-EcoRI* fragment T-A (0.621–0.633) DNA 5′ end labeled at the *SalI* site (0.621) and digesting with S1 nuclease or exonuclease VII. Alkaline gel fractionation of S1 digests of such hybrids revealed a faint band 1,400 bases in length; an even fainter band migrating at 1,600 bases could be seen with difficulty (data not shown). The existence of two species of mRNA differing in length by about 200 bases was also suggested by alkaline gel fractionation of exonuclease VII digests of the hybrids (data not shown). Here again a minor band of 1,400 bases and a fainter band of 1,600 bases could be seen. We conclude, then, that mRNA family 3 is probably made up of two colinear species differing in the position of their 5′ ends. Whether one or both encode the 85,000-d polypeptide is unclear at this time.

We found that the coding sequence for mRNA family 3 extends through the 600-base *SalI* fragment N′ (0.617–0.621) since S1 digest of hybrids between late polyribosomal poly(A) mRNA and this fragment labeled at both 3′ ends yield a faint fragment 600 bases in length (Fig. 6, track vi), and the same experiment using DNA labeled at both 5′ ends gives only one faint band 600 bases in length (data not shown). The 440-base *SalI* fragment N′ (0.617–0.621) protected by mRNA species 2 would not be detectable since a 5′ end labeled at 0.617 is not on its DNA coding strand.

The preparative hybridization and in vitro translation data of Fig. 3B (track iv) and Fig. 4A (summarized in Table 1), and the fact that the 5′ end of mRNA family 3 is to the right of 0.621, indicated that its 3′ end extends to the left of 0.617. We did experiments hybridizing late polyribosomal poly(A) mRNA to *BamHI-SalI* fragment I-P (0.602–0.617) DNA 3′ labeled at the *SalI* site (0.617). S1 nuclease digestion yielded a faint diffuse band migrating between 400 and 500 bases (data not shown), but no sharp bands. We do not know whether this failure to resolve sharp bands corresponding to the 3′ coding sequence for mRNA family 3 is caused by some length heterogeneity or by the fact that the 3′ end is complementary to the much more abundant mRNA species 2 encoded on the opposite DNA strand. The degree of complementary overlap between mRNA species 2 and family 3 is the most yet seen between two HSV-1 mRNA species.

mRNA species 4. The preparative hybridization data of Fig. 4B and the RNA transfer (Northern) blot data of Fig. 2B (tracks 3 and 4) indicate that the abundant 2.7-kb γ mRNA is the only major mRNA encoded in *SalI-BamHI*

TABLE 2. *Size of DNA fragments protected by HSV-1 mRNA species mapping between 0.592 and 0.647*

Map position of DNA[a]	Type of label[b]	pH of separation[c]	Nuclease treatment[d]	Size of DNA fragment protected[e] (base pairs)	mRNA species responsible[f]	Data shown[g]
0.592–0.602	3′ at 0.602	A	S1	510	1	Fig. 6, ii
0.602–0.617	5′ at 0.602	N	S1	1,125	1	Fig. 7A, i
		A	S1	1,075	1	Fig. 7B, iii
		A	X	1,175	1	Fig. 7B, v
	5′ at 0.617	N	S1	1,000	2	NS
		A	S1	1,000	2	NS
		A	X	1,000	2	NS
	3′ at 0.617	A	S1	(400 to 500)[h]	3	NS
0.617–0.621	5′ at 0.617 and 0.621	A	S1	600[i]	3	NS
	3′ at 0.617 and 0.621	A	S1	600[i]	3	Fig. 6, vi
				440	2	Fig. 6, vi
0.621–0.633	5′ at 0.621	A	S1	1,400[i] (1,600)[j]	3	NS
		A	X	1,400[i] (1,600)[j]	3	NS
	5′ at 0.633	A	S1	600 250 175, etc.	4	Fig. 8
		A	X	600	4	NS
0.621–0.643	5′ at 0.643	N	S1	2,100 1,500 to 1,600	4	Fig. 7D, ix
		A	S1	2,100 1,400 to 1,500	4	Fig. 7C, vi
		A	X	2,100	4	Fig. 7C, vii
0.633–0.645	5′ at 0.645	A	S1	1,750 500[i]	4	NS
0.633–0.647	5′ at 0.647	A	S1	None found		NS
0.645–0.647	3′ at 0.645	A	S1	340	4	Fig. 6, iii
	3′ at 0.647		S1	200	5	Fig. 6, iv

[a] See Fig. 1.
[b] The 5′ end labeling was carried out with phage T4 kinase; 3′ end labeling was done with avian myoblastosis virus reverse transcriptase (see text).
[c] A, Alkaline (denaturing) gel; N, neutral (nondenaturing) gel.
[d] S1, S1 nuclease; X, exonuclease VII.
[e] See Fig. 5.
[f] See text, Table 1, and Fig. 5.
[g] The lower case roman numerals indicate track number; NS, not shown.
[h] Diffuse, faint band.
[i] Faint band.
[j] Very faint band.

fragment T-I (0.621–0.643). When late polyribosomal poly(A) mRNA was hybridized with DNA from this region that was 5′ labeled at the *Bam*HI site (0.643), and the hybrids were then digested with exonuclease VII, alkaline gel electrophoresis yielded a major band 2,100 bases in length (Fig. 7D, track vii). This suggests that mRNA species 4 had its 5′ end about 600 bases to the left of the *Eco*RI site at 0.633. Exonuclease VII digests of hybrids between late polyribosomal poly(A) mRNA and *Sal*I-*Eco*RI fragment T-A (0.621–0.633) DNA 5′ end labeled at the *Eco*RI site (0.633) confirmed this by yielding a band 600 bases in length (data not shown).

The S1 nuclease analysis of hybrids between late polyribosomal poly(A) mRNA and DNA 5′ labeled at 0.643 yielded a considerably more complex pattern. Neutral gel analysis of S1 nu-

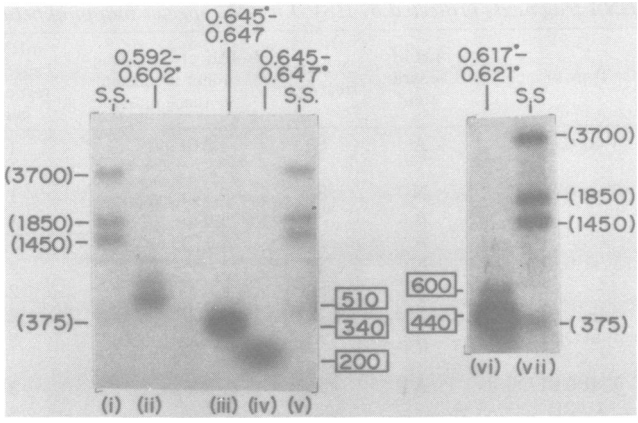


FIG. 6. *S1 nuclease mapping of the 3' ends of mRNA species encoded in the region 0.602 through 0.647. All gels shown were run at alkaline pH. DNA clones labeled at the 3' ends indicated by the dot (•) were hybridized with late poly(A) mRNA and digested with S1 nuclease, and the protected DNA fragments were fractionated by electrophoresis. Tracks (i), (v), and (vii) are size standards obtained from a 5' ^{32}P-labeled EcoRI-BamHI-SalI digest of SalI-BamHI fragment T-I (0.621–0.643) DNA cloned in pBR322. This digest yields fragments 3,700 and 375 bases in length from the pBR322; and bands 1,850 nucleotides (SalI-EcoRI fragment T-A; 0.621–0.633) and 1,450 nucleotides (EcoRI-BamHI fragment I-I; 0.633–0.643) from the HSV-1 clone (Fig. 1). Track (ii) is the protected DNA after hybridization of RNA with HindIII-BamHI fragment L-O (0.592–0.602) DNA 3' labeled at the BamHI site (0.602). The band is 510 bases in length. Track (iii) is protected DNA after hybridization of RNA with SalI-HindIII fragment Q-L (0.645–0.647) DNA 3' labeled at the SalI site (0.645). The band is 340 bases long. Track (iv) is protected DNA after hybridization of RNA with the same fragment 3' labeled at the HindIII site (0.647). The band is 200 bases long. Track (vi) is protected DNA after hybridization of RNA with SalI fragment N' (0.617–0.621) DNA 3' labeled at both SalI sites. The bands are 600 (faint) and 440 bases long.*

clease-protected DNA yielded a major band 2,100 bases in length and a significant amount of material migrating at about 1,600 to 1,500 bases and smaller (Fig. 7D, track ix). Alkaline gel analysis yielded major bands 2,100 bases and between 1,400 and 1,500 bases in length (Fig. 7C, track vi). When correlated with the exonuclease data described above, this indicates that mRNA species 4 is a mixture of at least two forms. Both begin about 600 bases to the left of the *Eco*RI site at 0.633, but half of the mRNA has a segment about 400 bases long spliced out, whereas the other half has only a very short or no region at all spliced out. The mRNA species 4, then, is a family of related forms.

These observations were confirmed and expanded by using alkaline gel analysis of S1 nuclease-digested hybrids between late polyribosomal poly(A) mRNA and *Sal*I-*Eco*RI fragment T-A (0.621–0.633) DNA 5' labeled at the *Eco*RI site (0.633). Alkaline agarose gels of S1 nuclease and exonuclease VII digests demonstrated a major band 600 bases in size (data not shown). This shows again that the largest member of mRNA family 4 has no splice or, at most, an extremely small one. In the S1 digests, a diffusely migrating band smaller than 300 bases could be seen on the original X-ray film. We used denaturing Maxam-Gilbert (34) 12% acrylamide sequencing gels to greatly improve the resolution of the S1 nuclease-resistant DNA fragments smaller than 600 bases. These data are shown in Fig. 8. In addition to a major DNA band at 600 bases, other major bands approximately 120, 200, 225, and 300 bases could be seen, as well as smaller amounts of DNA migrating with sizes of about 140, 175, and 250 bases. The size of these bands was reproducible in several experiments, and we concluded that there are a number of spliced members of mRNA family 4 which can contain one or another splice between their 5' end and defined positions ranging as far as 475 bases to the right of the 5' start of the mRNA. The 5' sequence to the left of the introns must be quite short (50 to 100 bases) since neutral gel fractionation of S1 digests indicates the heterogeneous bands are no more than 100 bases longer than these bands on alkaline gels (Fig. 7D, track ix).

Whether one or several members of mRNA family 4 actually encode the 69,000-d polypeptide can only be established when nucleotide sequence data on this region is available. The fact that the 2.3-kb shoulder of the *Sal*I-*Bam*HI fragment T-I (0.621–0.643)-specific, 2.7-kb mRNA (Fig. 4B) does not translate the 69,000-d polypeptide (Fig. 3D, track xi) suggests that the smallest members of mRNA family 4 do not encode it. This shoulder appears to en-

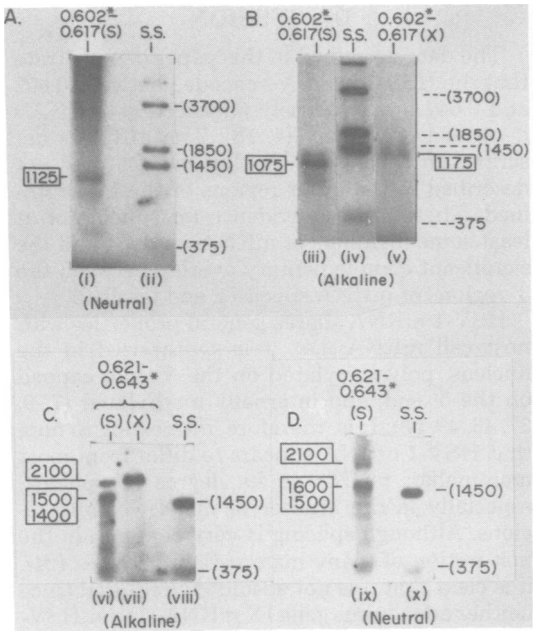


FIG. 7. *S1 nuclease and exonuclease VII mapping of the 5′ region of mRNA species encoded in the region 0.602 through 0.647. The size marker (S.S.) tracks (ii) and (iv) are as described in the legend to Fig. 6. DNA clones were labeled at their 5′ ends, indicated by the star, hybridized with late poly(A) mRNA, and digested with S1 nuclease or exonuclease VII, and the protected DNA fragments were fractionated by neutral or alkaline electrophoresis. (A) Neutral gel. Track (i) is the protected DNA after S1 digestion of hybrids formed with RNA and BamHI-SalI fragment I-P (0.602–0.617) DNA 5′ labeled at the BamHI site (0.602). The band is 1,125 bases long. (B) Alkaline gel. Track (iii) is as track (i). The band is 1,075 bases long. Track (v) is as tracks (i) and (iii), but it is after exonuclease VII (X) digestion. The band is 1,175 nucleotides long. (C) Alkaline gel. Track (viii) is a size standard derived by EcoRI digestion of SalI-BamHI fragment T-I (0.621–0.643) DNA cloned in pBR322 digested with BamHI 5′ end labeled with ^{32}P and then digested with EcoRI. The bands are the 1,450-base EcoRI-BamHI fragment I-I (0.633–0.643) and the 375-base band from pBR322. Track (vi) is the protected DNA after S1 nuclease digestion of hybrids between late polyribosomal poly(A) mRNA and SalI-BamHI fragment T-I (0.621–0.643) DNA 5′ labeled at the BamHI site (0.643). The major bands are 2,100 bases and 1,400 to 1,500 bases long. These sizes were confirmed by running similar digests versus the same size standards as shown in other panels. Track (vii) is the protected DNA after exonuclease VII digestion of the same material. The band is 2,100 bases long. Size of the major bands was confirmed by running similar digests versus the size standards seen in other panels. (D) Neutral gel. The details are as for panel C; track (x) is a size standard like track (vii) of panel C, and track (ix) is an S1 nuclease digestion like track (vi) of panel C. The bands are 2,100 and 1,500 to 1,600 bases. These sizes were also confirmed as described for panel C.*

code the 44,000-d polypeptide (Fig. 3D, track xi), so one or more of the spliced species may be biologically active.

Late polyribosomal poly(A) mRNA, when hybridized with the 1,750-base *Eco*RI-*Sal*I fragment I-T (0.633–0.645) DNA 5′ labeled at the *Sal*I site (0.645), protected a full-length 1,750-base band (data not shown). This indicates that there are no interruptions to the right of 0.633 in mRNA coding sequences for many members of mRNA family 4. A fainter band 500 bases in length could be seen, indicating that a small amount of mRNA in this family has its contiguous 5′ end 500 bases to the left of 0.645 or about 300 bases to the left of the *Bam*HI site at 0.643. A faint band of about this size could be seen in alkaline gels of S1 nuclease digests of hybrids between late polyribosomal poly(A) mRNA and *Sal*I-*Bam*HI fragment T-I (0.621–0.643) DNA 5′ labeled at the *Bam*HI site (0.643; Fig. 7G, track xvii). Exonuclease VII digests of such hybrids did not reveal such a band (Fig. 7C, track vii), which suggests that this very small species also has its 5′ end starting with the larger members of mRNA family 4; however, the small amounts of this mRNA species make such a conclusion only tentative.

The 3′ end of the bulk of the members of mRNA family 4 was measured to extend 340 bases to the right of the *Sal*I site at 0.645 by hybridizing late polyribosomal poly(A) mRNA with *Sal*I-*Hin*dIII fragment Q-L (0.645–0.647) DNA 3′ end labeled at the *Sal*I site (0.645; Fig. 6, track iii).

Other mRNA species mapping in the region 0.602 through 0.647. The four mRNA species or families of species described above comprise the ones that are fully characterizable by the techniques described in this report. Several minor mRNA species mapping around 0.602 were marginally detectable by S1 analysis (see above), and one of these probably corresponds to the low-abundance small (1-kb) mRNA detected in RNA transfer (Northern) blots of mRNA probes with cDNA to *Hin*dIII-*Bam*HI fragment L-O (0.592–0.602) (4).

One major mRNA species which is as yet not fully characterized has its 3′ end to the left of 0.647. This is seen when the 350-base *Sal*I-*Hin*dIII fragment Q-L (0.645–0.647) 3′ labeled at the *Hin*dIII site (0.647) was hybridized to late polyribosomal poly(A) mRNA and subjected to S1 nuclease analysis (Fig. 6, track iv). A major band 200 bases in length could be seen. This

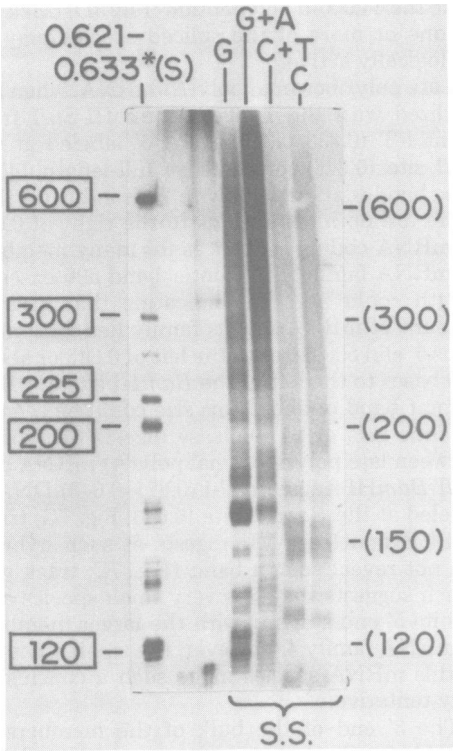


FIG. 8. *High-resolution fractionation of S1 nuclease-resistant DNA fragments generated by hybridization of SalI-EcoRI fragment T-A (0.621–0.633) DNA to late poly(A) mRNA. SalI-EcoRI fragment T-A (0.621–0.633) DNA 5′ labeled at the EcoRI site (0.633) was hybridized to late poly(A) mRNA. The hybrids were digested with S1 nuclease and then fractionated on a denaturing Maxam-Gilbert sequencing gel (34). A sequence ladder of chemically modified DNA was run as a size standard (S.S.). Sizes were determined by counting the ladder rungs upwards from the xylene cyanol dye front, which migrates with the 70-base position. Sizes beyond 250 bases were estimated. There are a number of bands; major ones ~600, ~300, 225, 200, and 120 bases in length are evident. No band smaller than 120 bases was seen.*

mRNA (species 5) is probably a 2.6-kb mRNA species seen when total *Hin*dIII fragment L (0.592–0.647) cDNA is used to hybridize to RNA transfer (Northern) blots of total late polyribosomal poly(A) RNA (data not shown).

We confirmed that no detectable mRNA's spanning the *Hin*dIII site at 0.647 have 5′ ends to the left by showing that no bands at all could be seen when late polyribosomal poly(A) mRNA was hybridized with *Eco*RI-*Hin*dIII fragment I-L (0.633–0.647) DNA 5′ end labeled at the *Hin*dIII site (0.647) and subjected to S1 nuclease digestion.

DISCUSSION

The data described in this paper demonstrate that the HSV-1 mRNA's encoded between 0.602 and 0.647 are as densely packaged as mRNA's described previously (4, 18). The mRNA's described in this report display two features not described in the other regions of the U_L examined. These are the evidence for splicing of at least some members of mRNA family 4 and the significant complementary overlap between the 3′ regions of mRNA species 2 and family 3.

HSV-1 mRNA shares general properties with host cell mRNA; i.e., it is synthesized in the nucleus, polyadenylated on the 3′ end, capped on the 5′ end, and internally methylated (7, 9, 37, 46, 49, 58). It is, therefore, interesting to note that HSV-1 mRNA appears to differ from most mammalian mRNA in its degree of splicing, especially in the interior of mRNA coding regions. Although splicing is very common in the maturation of many mammalian mRNA's (24), it is clear that it is not absolutely required since neither adenovirus gene IX mRNA (1) nor HSV-1 *tk* mRNA (35) is spliced.

In spite of the general lack of detectable splices in many HSV-1 mRNA species, the fact that at least some α HSV-1 mRNA's are spliced (62) means that HSV-1 DNA encodes splicing signals recognizable by the host cell. The data presented here for mRNA family 4 indicate that some sort of splicing signals can also be, at least occasionally, recognized in HSV-1-infected cells even late after infection. Future nucleotide sequence studies will allow us to identify signals and to establish which member(s) of this family of mRNA's has biological activity. Sequence studies will also indicate whether other viral mRNA's are spliced very near their ends.

Our studies to date allow us to make some generalizations concerning the packaging density of HSV-1 mRNA. The HSV-1 mRNA's so far mapped in the U_L have their 5′ or 3′ ends very close to ($\leq$100 bases) the 5′ or 3′ ends of mRNA's encoded off the same or opposite DNA strands (4, 19). We suggest then that, in the U_L region at least, there is only a small amount of viral DNA not directly involved in encoding mRNA. Thus, the patterns of rather dense packaging of polypeptide information seen in the smaller DNA viruses also holds for HSV-1.

This dense head-to-head, tail-to-tail, and head-to-tail packaging is manifest in complementary overlap between 5′ and 3′ ends of mRNA's mapping in the region of the U_L examined in this paper. The 3′ end of mRNA family 4 maps 340 bases into the 350 bases fragment *SalI-Hin*dIII fragment Q-L (0.645–0.647), whereas the 3′ end of mRNA species 5 maps 200 bases into this fragment from the other

side (Fig 5; Fig. 6, tracks iii and iv). Thus, these mRNA's overlap by nearly 200 bases. The 5′ end of mRNA family 3 extends 200 to 400 bases beyond the 5′ start of mRNA family 4 (Fig. 5). Thus, there must be complementary overlap at this end, too. Such overlap has been reported for several adenovirus mRNA families (22).

The complementary overlap between the 3′ end of mRNA 2 and mRNA family 3 is the most yet seen for mRNA's mapping in the U_L region of HSV-1 and is extensive enough so that it may involve actual amino acid coding sequences. The coding sequence for mRNA species 2 is only 1,440 bases, yet this mRNA encodes a polypeptide of nominal molecular mass of 64,000 d (Fig. 3B, track iii; Table 2). This requires that the full coding capacity of this mRNA be utilized. The exact length of the coding sequence for mRNA family 3 is not clear because of lack of good data on the length of its 3′ ends extending to the left of 0.617. Our data suggest this length is at least 400 bases. This means that the 3′ end of mRNA family 3 has a complementary overlap of as many as 800 bases with the 3′ end of mRNA species 2. Since mRNA family 3 encodes a very large polypeptide (85,000 d; Fig. 3B, track iv), it may well require more coding capacity than the 1,500 to 1,700 bases to the right of this complementary overlap. Again, nucleotide sequence analysis will allow a precise measure of any coding overlap.

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