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Isolation and Translation of mRNA Encoded by a Specific Region of the Herpes Simplex Virus Type 1 Genome

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We have examined in detail the major mRNA species encoded by the region of the herpes simplex virus type 1 genome encoded by *Hin*dIII fragment K (0.53-0.59 from the left end of the prototype arrangement of the genome) by using this restriction fragment bound to cellulose as a reagent for isolation of this mRNA. Before viral DNA replication in infected cells (early), a major species of viral mRNA 5.2 kilobases (kb) in length is abundant. After the onset of viral DNA replication (late), four mRNA species are abundant: 7, 5.2, 3.8, and 1.8 kb in size. We have used reverse transcriptase from avian myeloblastosis virus to make DNA complementary to these RNA species and their 3' ends. We have shown by hybridization of this complementary DNA to Southern blots of herpes simplex virus type 1 DNA that the 7-, 5.2-, and 1.8-kb mRNA species have their 3' ends to the right of 0.59 and are at least partially colinear. The 3.8-kb mRNA has a 3' end mapping to the left of the 3' ends of these other species. In vitro translation of *Hin*dIII fragment K-specific mRNA in a reticulocyte lysate system yielded three major polypeptide products: 140,000, 122,000, and 54,000 daltons (d). Less prominent species of 86,000 and 65,000 d also were produced. Translation of size-fractionated *Hin*dIII fragment K-specific mRNA showed that the 7-, 5.2-, and 3.8-kb mRNA's encoded the 54,000-, 140,000-, and 122,000-d polypeptides, respectively. The 140,000-d polypeptide was the major polypeptide translated using early *Hin*dIII fragment K-specific mRNA as a template. The 3.8-kb mRNA also encoded the 86,000-d polypeptide, whereas the 1.8-kb mRNA encoded a polypeptide that was indistinguishable from the 54,000-d polypeptide encoded by the 7-kb mRNA, in addition to the 65,000-d polypeptide. The implications of the data are discussed.

Study of the process of gene expression in herpes simplex virus type 1 (HSV-1) has been complicated by the large size and relative complexity of the viral genome. The characteristics of the viral DNA recently have been reviewed by Roizman (17). Briefly, the DNA has a molecular weight of 95×10^6 to 100×10^6 (6, 25). The KOS strain, which has been used in our laboratory, is 150 kilobase (kb) pairs in length, based on the ratio of its contour length to that of ϕX174 RFII (26). The linear HSV-1 genome is segmented into a long region (ca. 82%) and a short region (18%), each bounded by different inversely repeated sequences (18, 25). This arrangement results in four equimolar populations of the viral DNA which differ in the relative orientations of the long and short segments (7, 19, 28). One arrangement has, by convention, been chosen as the prototypical (P) configuration (17). All map coordinates and restriction fragment locations in this paper refer to the P configuration of the HSV genome.

In previous communications, we have described techniques for the isolation, localization, and in vitro translation of HSV-1 mRNA species. Isolation of viral mRNA homologous to individual restriction fragments of the HSV-1 genome and hybridization of ^{32}P-labeled, size-fractionated mRNA to "Southern blots" of HSV-1 DNA restriction digests allowed us to identify and localize a large number of viral mRNA species abundant in infected cells when viral DNA replication is proceeding (late after infection). The sizes of these mRNA species ranged from 1.5 to 9 kb (1). A subset of these mRNA's (16 in all) was identified, using Southern blot hybridization, as being abundant in infected cells before viral DNA replication (early after infection; 8). The increase in complexity of viral mRNA which accompanies the onset of viral DNA replication also is reflected by an increase in the complexity of the in vitro translation products of this mRNA (L. E. Holland, K. P. Anderson, C. Shipman, Jr., and E. K. Wagner, Virology, in press).

The studies mentioned above showed complex patterns of mRNA hybridizing to several regions

of the HSV-1 genome. One region of the long unique portion of the genome encompassed by *Hin*dIII fragment K (0.53–0.59 from the left end of the P configuration; see Fig. 1) is expressed as mRNA both before and after the onset of viral DNA replication. In addition, the number of mRNA species homologous to this region increases after the onset of viral DNA replication, reflecting the general increase in complexity of HSV-1 mRNA during the replication cycle of the virus. For this reason, we have chosen to examine in detail the mRNA species homologous to *Hin*dIII fragment K. Our present results show that the direction of transcription of individual viral mRNA species, as determined by localization of 3′ complementary DNA (3′-cDNA) made to these species, indicates that at least one major early and one major late species are partially colinear. Other species also must be rather densely packed within this region of the genome. Further, we find that each of these mRNA species encodes discrete polypeptides.

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 in all experiments. All infections were carried out at a multiplicity of infection of 10 PFU/cell, and time after infection was measured after a 30-min adsorption period.

Preparation of viral DNA and restriction fragments. Viral DNA was prepared from cytoplasmic and extracellular virions and purified by isopycnic centrifugation in CsCl as previously described (21, 22). Restriction endonucleases (*Hin*dIII, *Hpa*I, and *Bgl*II) were obtained from New England Biolabs. All digestions were carried out using 1 unit of enzyme per μg of DNA for 2 h at 37°C in 60 mM NaCl–7 mM $MgCl_2$–7 mM Tris (pH 7.4). Restriction fragments were separated by electrophoresis of 50- to 100-μg samples of restricted DNA on horizontal 0.5% agarose (Sigma) gels (42 by 20 by 1.2 cm) for 48 h at 1.2 V/cm. Individual bands were cut from the gel, and DNA was recovered by chromatography on hydroxylapatite as described (1). Restriction maps for our strain (KOS) of HSV-1 are based on published maps and have been confirmed as described previously (1, 8, 22).

Preparation and hybridization of RNA. T150 flasks of HeLa cells (2×10^7 cells per flask) were infected and labeled with either [^{3}H]uridine or [^{32}P]orthophosphate. For [^{3}H]uridine-labeled RNA, each T150 flask of infected cells was incubated either from 1 to 2 or from 5 to 6 h postinfection (hpi) with 180 μCi of [^{3}H]uridine (28 Ci/mmol, Schwarz/Mann) in 10 to 12 ml of medium 199 containing 5% fetal calf serum, which had been dialyzed against 0.15 M NaCl. For [^{32}P]orthophosphate-labeled RNA, flasks were incubated from 2 to 6 hpi with 1 mCi of carrier-free [^{32}P]orthophosphate (New England Nuclear Corp.) in 10 ml of Eagle minimal essential medium containing 10% the normal phosphate concentration and 5% dialyzed fetal calf serum.

Polyribosomal RNA was isolated from magnesium-precipitated polyribosomes (15) by proteinase K (Merck) digestion and phenol extraction as described previously in detail (21). Polyadenylated [poly(A)] RNA was isolated by chromatography on oligodeoxythymidylic acid [oligo(dT)]-cellulose. Criteria for the intactness of this poly(A) RNA have been discussed (1). A culture of 2×10^7 cells typically yields 4 to 5 μg of poly(A) polyribosomal RNA with a specific radioactivity of 120,000 cpm/μg for ^{3}H-labeled RNA and 50,000 cpm/μg for ^{32}P-labeled RNA. Radioactivity was measured as a 1% aqueous solution in Aquasol II (New England Nuclear Corp.) in a Beckman LS-230 scintillation counter.

RNA was hybridized with HSV-1 DNA restriction fragments covalently coupled to diazotized cellulose. Coupling of HSV-1 DNA to diazotized cellulose was as described previously (1). Briefly, the basic scheme of Noyes and Stark (14) was followed using 5 mM potassium phosphate (pH 6.5) as a buffer. Recently, we have found that reduction of the rinse time after activation of the ABM cellulose significantly increases binding. By using <1-min spins in an Eppendorf microcentrifuge to rinse the cellulose after activation, instead of 5-min spins in a Sorvall centrifuge, we have increased our coupling efficiency from 35 to 45% to 65 to 85% of input DNA.

RNA hybridization to DNA coupled to cellulose was carried out 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 (hybridization buffer) for 4 h at 57°C. After extensive rinsing as outlined previously (1), the hybrids were eluted with 98% recrystallized formamide containing 10 mM HEPES (pH 8.0) at 60°C, adjusted to 0.15 M sodium acetate and 20% formamide, and precipitated with ethanol at −20°C. To insure DNA excess in the hybridization, we used at least 5 to 7 μg of *Hin*dIII fragment K DNA bound to cellulose to hybridize an equivalent amount of infected-cell poly(A) RNA. Since *Hin*dIII fragment K is 9.6 kb pairs in length, this is equivalent to using 70 to 100 μg of total HSV-1 DNA. We have assumed that the average population of viral RNA species homologous to *Hin*dIII fragment K is present in amounts roughly equivalent to the size of the fragment.

Size fractionation of *Hin*dIII fragment K-specific mRNA. Two methods were employed to fractionate *Hin*dIII fragment K-specific mRNA after hybridization. For analytical sizing of mRNA and for preparation of individual messages for cDNA synthesis, hybridized RNA was electrophoresed on 1.2% agarose tube gels (15 by 0.6 cm) containing 10 mM methylmercury hydroxide (1, 2, 8). After electrophoresis, the gels were soaked in 50 mM β-mercaptoethanol for 20 min and then sliced at 2-mm intervals, and the radioactivity in individual slices was determined. Radioactive HeLa cell rRNA often was included as a size standard (9, 12, 27).

Preparative isolation of individual mRNA species for in vitro translation was performed by centrifugation through a 5 to 20% (wt/wt) linear sucrose gra-

dient. The ethanol pellet of the RNA sample to be fractionated was dissolved in 250 μl of 25 mM boric acid–2.5 mM $Na_2B_4O_7 \cdot 10H_2O$–5 mM Na_2SO_4–1 mM EDTA containing 10 mM methylmercury hydroxide. A 200-μl sample was layered on a 12-ml gradient containing 0.1 M NaCl–0.01 M Tris-hydrochloride (pH 7.4)–7 mM β-mercaptoethanol (gradient buffer). The remaining 50 μl was precipitated as a control after addition of 350 μl of 20% sucrose in gradient buffer. After centrifugation at 40,000 rpm for 6 h in a Beckman SW41 Ti rotor, 0.38-ml fractions were collected from the bottom of the centrifuge tube, samples were removed for determining radioactivity, and 1.0 ml of 95% ethanol was added to each fraction. After precipitation overnight at −20°C and collection by centrifugation, desired fractions were redissolved in 0.15 M potassium acetate, pooled, reprecipitated, and finally dissolved in 5.0 μl of water for translation.

Southern blots of restricted HSV-1 DNA. Restricted DNA was fractionated on horizontal 0.5% agarose gels (42 by 20 by 1.2 cm). Restriction fragments were transferred onto nitrocellulose paper (BA85, Schleicher and Schuell), and the paper was dried by the procedure of Southern (20). The paper then was divided into 4-mm strips for individual hybridizations.

Synthesis of 3′ and total cDNA. Specific viral mRNA species were partially degraded with alkali and eluted from agarose as described (8). The fragmented RNA had an average size of 400 to 500 nucleotides as determined by denaturing gel electrophoresis. The RNA was used in toto for total cDNA probe, or the 3′ ends were isolated by chromatography on oligo(dT)-cellulose for 3′-cDNA.

Total cDNA was synthesized using random oligodeoxynucleotides prepared from calf thymus DNA as a primer for reverse transcriptase, as described by Taylor et al. (24). RNA (5 to 10 ng) was coprecipitated with 5 to 10 μg of random primer and suspended in 0.1 ml of a buffer containing, per ml, 50 μmol each of KCl and Tris (pH 8.1), 8 μmol of $MgCl_2$, 0.4 μmol each of dATP, dGTP, and dCTP, 4 μmol of dithiothreitol, and 10 μg of actinomycin. This mixture was added to 50 μCi of [^{32}P]TTP (350 Ci/mmol, Amersham), and 10 U of reverse transcriptase from avian myeloblastosis virus (supplied by Joseph Beard) then was added. The sample was incubated for 60 min at 37°C, after which the reaction was stopped by the addition of sodium dodecyl sulfate to 0.5% and EDTA to 0.005 M. The mixture was digested with 10 μg of proteinase K for 20 min at 45°C, and the polymerized material was isolated by chromatography on G-50 Sephadex. The excluded radioactivity was phenol-chloroform extracted, and RNA was hydrolyzed overnight in 0.3 M NaOH. The solution was neutralized with acetic acid, and the cDNA was ethanol precipitated. The cDNA to the 3′ end of RNA is made in the same way using 0.1 μg of oligo(dT)$_{12-18}$ (Collaborative Research, Inc.) per ng of RNA as a primer, instead of random calf thymus DNA oligonucleotides (8). Under the described conditions, we routinely isolate about 5×10^6 cpm of total or 3′-cDNA for each μg of RNA or fragment of RNA used as a template.

Hybridization of cDNA to Southern blots was carried out for 72 h at 37°C in hybridization buffer containing 65% formamide and Denhardt solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin; 4). After hybridization, the DNA blots were rinsed in 65% formamide containing 2× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) for 4 to 5 h at 37°C and subjected to autoradiography for 24 to 72 h as needed using X-Omat R Film (Kodak) and B Cronex Lightning Plus (Dupont) intensifying screens (23).

In vitro translation of RNA and analysis of polypeptide products. RNA was translated in vitro using a micrococcal nuclease-treated rabbit reticulocyte lysate system obtained from New England Nuclear Corp. [^{35}S]methionine was used to label polypeptide translation products. RNA samples to be translated were ethanol precipitated twice, and the final pellet was rinsed with ice-cold 70% ethanol. Assay conditions were essentially as recommended by the supplier. Each 25-μl assay contained 10 μl of reticulocyte lysate, 40 to 50 μCi of [^{35}S]methionine (675 Ci/mmol), 80 mM potassium acetate, and 0.5 mM magnesium acetate, in addition to 2 μl of translation cocktail supplied with the lysate. The magnesium concentration used was optimized for translation of HSV-1-specific mRNA in a separate experiment. After incubation of the assay at 37°C for 60 min, 5 μl of 100-μg/ml pancreatic RNase and 20 U of T_1 RNase per ml in 25 mM EDTA were added, and the mixture was incubated for an additional 10 min at 37°C.

Polypeptide products of in vitro translation were fractionated by electrophoresis on discontinuous sodium dodecyl sulfate-polyacrylamide slab gels using the system of Laemmli (10). Polyacrylamide separating gels (9%; 10 cm by 14 cm by 1.5 mm) were used with 3% polyacrylamide stacking gels. Dried gels were autoradiographed using Kodak NS-2T X-ray film. Molecular weights of in vitro translation products were determined from their migration relative to unlabeled molecular weight markers (Pharmacia) and labeled translation products of RNA from adenovirus type 2-infected HeLa cells supplied with the translation kit.

RESULTS

Time and direction of synthesis of HSV-1 mRNA homologous to the region 0.53–0.59 on the HSV-1 genome. The location of *Hin*dIII fragment K on the HSV-1 genome (0.53–0.59 from the left end of the P arrangement), as well as cleavage sites for *Hin*dIII, *Bgl*II, and *Hpa*I restriction endonucleases, is shown in Fig. 1. To examine in detail the relative amounts and size distributions of viral mRNA homologous to this region of the DNA at both early and late times after infection, polyribosomal poly(A) RNA, pulse-labeled from 1 to 2 hpi (early) or from 5 to 6 hpi (late), was hybridized to excess *Hin*dIII fragment K DNA covalently coupled to cellulose. Our criteria for DNA excess are described in Materials and Methods.

Poly(A) polyribosomal RNA labeled from 1 to 2 hpi hybridized significantly to excess *Hin*dIII fragment K bound to cellulose (0.8% of input

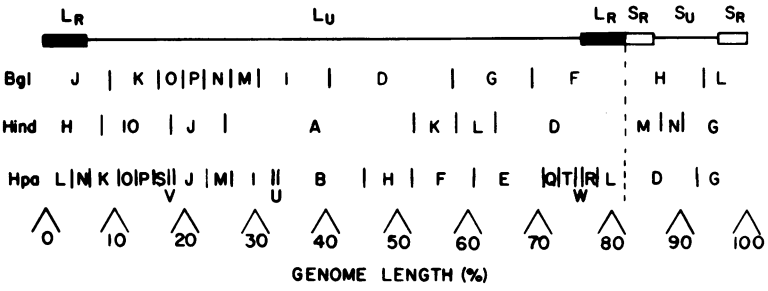

FIG. 1. *Map of restriction endonuclease cleavage sites for the KOS strain of HSV-1. The fragments generated by cleavage using restriction endonucleases BglII, HindIII, and HpaI are shown for the prototypical arrangement (P) of our strain of HSV-1 (1). The solid boxes represent the long repeat sequences (L_R), and the open boxes represent the short repeat sequences (S_R). Three other arrangements of the genome occur (7, 28). These are I_L, where the long segment is inverted relative to the short segment; I_S, where the short segment is inverted relative to the long segment; and I_{LS}, where both the short and long segments are inverted relative to the P arrangement. Terminal DNA sequences in one arrangement of the genome may be internalized in other arrangements, thereby generating additional fragments. For BglII, these fragments are A(F + H), B(J + H), C(F + L), and E(J + L). For HindIII, these fragments are B(D + G), C(D + M), E(G + H), and F(H + M). For HpaI, these fragments are A(D + L) and C(G + L).*

radioactivity), compared to a background level of <0.1% for uninfected cell RNA. When the hybridized RNA was fractionated by electrophoresis in denaturing agarose gels, a major mRNA species 5.2 kb in size was observed (Fig. 2A). Significant mRNA migrating with a size of 1.8 kb also could be seen.

Hybridization of mRNA labeled from 5 to 6 hpi to *Hin*dIII fragment K was more efficient (3.0% of input radioactivity). The 5.2- and 1.8-kb mRNA species were seen in increased amounts, and two new species of viral mRNA, 7.0 and 3.8 kb in size, were observed (Fig. 2B). The 7.0-, 5.2-, 3.8-, and 1.8-kb mRNA species also were the major species seen in long-term labeling experiments (Fig. 2C).

To determine the direction of transcription of these discrete mRNA species encoded by *Hin*dIII fragment K, we did the following experiments. Infected cells were labeled from 2 to 6 hpi with [^{32}P]orthophosphate, polyribosomal poly(A) RNA was isolated, and the RNA was hybridized to *Hin*dIII fragment K bound to cellulose. The homologous mRNA was fractionated on a denaturing agarose gel (Fig. 2C), and mRNA from the 7-, 5.2-, 3.8-, and 1.8-kb bands (peaks 1, 2, 3, and 4, respectively) was recovered (Materials and Methods). A portion (ca. 20%) of each of the 7-, 5.2-, and 3.8-kb RNA samples was used as a template for the synthesis of cDNA complementary to the total mRNA (total cDNA). After partial degradation in alkali, the remaining RNA from these peaks was passed through an oligo(dT)-cellulose column, and the poly(A)-containing fragments were used as templates for the synthesis of cDNA complementary to the 3′ ends of the mRNA (3′-cDNA). In the case of the 1.8-kb mRNA, the total sample was used as a template for 3′-cDNA since the amount of RNA found of this size was significantly less than for the other species.

The average size of this cDNA is quite small. Denaturing agarose gel electrophoresis of this cDNA (Fig. 3) shows that >99% migrates faster than 18S rRNA (2 kb), and >90% migrates faster than the 4S + 5S RNA (75 to 200 nucleotides). The weight average size of our cDNA does not exceed 100 nucleotides. This size distribution is important in our determination of location of 3′ ends (see below).

Both total and 3′-cDNA of each mRNA species were hybridized to Southern blots of restriction digests of HSV-1 DNA. Total cDNA to the 7-kb mRNA encoded by *Hin*dIII fragment K (peak K1, Fig. 2C) hybridized mainly to this fragment with some hybridization to the neighboring *Hin*dIII fragment L (0.59–0.64; Fig. 4A). This also was true for the 5.2-kb mRNA (peak K2; Fig. 2C; data not shown). In contrast, 3′-cDNA to 7-kb mRNA hybridized mainly to *Hin*dIII fragment L (Fig. 4A). These results indicate that this mRNA is transcribed from left to right with its 3′ end somewhat to the right of 0.59. The 3′-cDNA's to 5.2-kb and 1.8-kb mRNA homologous to *Hin*dIII fragment K (peaks K2 and K4, Fig. 2C) show similar hybridization patterns, indicating that these mRNA species also have 3′ ends to the right of 0.59 (Fig. 4B and 4D). Their direction of synthesis also must be left to right. These results confirm our earlier conclusions concerning the direction of transcription of the 5.2-kb mRNA based on the use of pooled size-fractionated early infected cell mRNA as a template for 3′-cDNA (8).

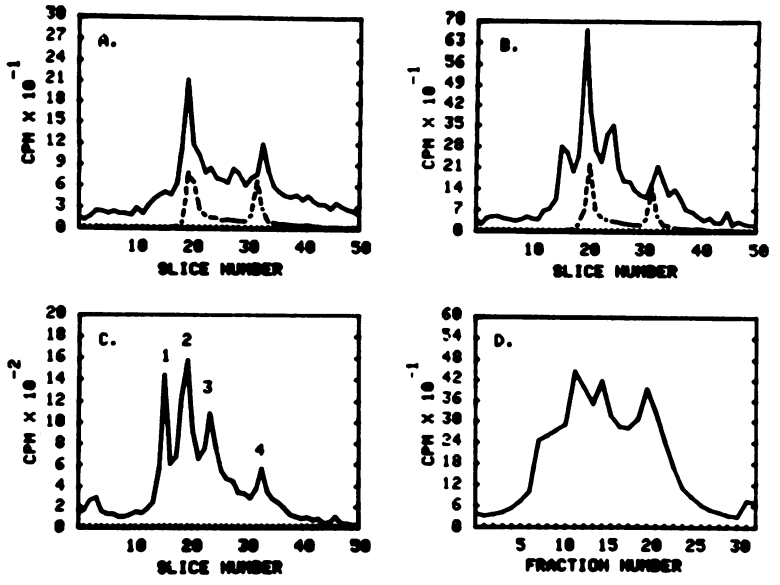

FIG. 2. *Size distribution of HSV-1 mRNA homologous to HindIII fragment K (0.53–0.59). Poly(A) polyribosomal RNA isolated from cells at various times after infection was hybridized to 5 μg of HindIII fragment K DNA (corresponding to 80 μg of total HSV-1 DNA) coupled to cellulose.The hybrids then were fractionated either by electrophoresis in denaturing agarose gels or sedimentation in a sucrose gradient. Radioactivity of individual slices or fractions was determined. Migration in gels was from left to right; sedimentation through the sucrose gradient was from right to left. Dotted lines indicate the positions of ^{32}P-labeled 28S and 18S HeLa rRNA included as internal size standards (5.2 kb and 2.0 kb, respectively; 12, 27). (A) Denaturing agarose gel profile of HindIII fragment K-specific mRNA isolated from infected cells at 2 hpi after a 1-h pulse-label with 15 μCi of [^{3}H]uridine per ml. (B) Denaturing agarose gel profile of HindIII fragment K-specific mRNA isolated from infected cells at 6 hpi after a 1-h pulse-label with 15 μCi of [^{3}H]uridine per ml. (C) Denaturing agarose gel profile of HindIII fragment K-specific mRNA isolated from infected cells at 6 hpi after a 4-h label with 100 μCi of [^{32}P]orthophosphate per ml. The numbers indicate the peaks used for cDNA synthesis. The size in kilobases of each peak was assigned based on migration of analogous ^{3}H-labeled RNA of (B). Peak 1 was 7 kb, peak 2 was 5.2 kb, peak 3 was 3.8 kb, and peak 4 was 1.8 kb. (D) Sucrose gradient profile of HindIII fragment K-specific mRNA isolated from infected cells at 6 hpi after a 2-h pulse-label with 15 μCi of [^{3}H]uridine per ml. Fractions from the gradient were pooled and designated as indicated below for translation studies. Fractions 1 to 5, >7 kb; fractions 6 to 9, 7 kb; fractions 10 to 12, 5.2 kb; fractions 13 to 15, 3.8 kb; fractions 16 and 17, ~2.5 kb; fractions 18 to 21, 1.8 kb; fractions 22 to 29, <1.8 kb. The sizes of each pool were determined from sedimentation of HeLa rRNA in a parallel sucrose gradient.*

The 3′ ends of these three mRNA species cannot extend very far to the right of 0.59 because there is little hybridization of total cDNA made from these mRNA's to *Hin*dIII fragment L. The fact that 3′-cDNA of these mRNA's hybridizes in very small amounts to *Hin*dIII fragment K substantiates the claim that the 3′ ends of these RNA molecules are very near to 0.59, since cDNA made under our conditions is quite small (see Fig. 3). We can state conclusively that the 3′ ends of these mRNA's do not extend beyond 0.61 because 3′-cDNA to total *Hin*dIII fragment K-specific mRNA does not hybridize to *Hpa*I fragment E (0.61–0.71, Fig. 5D).

When 3.8-kb mRNA seen late (peak K3, Fig. 2C) was used as a template for cDNA synthesis, a different pattern of hybridization was observed. Whereas total cDNA to this RNA hybridized mainly to *Hin*dIII fragment K, 3′-cDNA hybridized equivalently to *Hin*dIII fragment K and *Hin*dIII fragment L (Fig. 4C). The 3′-cDNA to this RNA also hybridized efficiently to *Bgl*II fragment D (0.41–0.58), as well as to *Bgl*II fragment G (0.58–0.70, Fig. 5B). The 3′-cDNA to the 7-kb mRNA hybridized almost exclusively to the latter fragment (Fig. 5A).

Several possible explanations exist for these results. One possible explanation is that the 3.8-kb mRNA has a 3′ end to the left of 0.58, but contaminating material in the 3.8-kb peak derived from partial degradation of the 7- and 5.2-kb mRNA's yields 3′-cDNA hybridizing to the right of 0.59. Our earlier finding, that considerable material smaller than 5.2 kb yields 3′-cDNA mapping in *Hin*dIII fragment L at 2 hpi (8), is

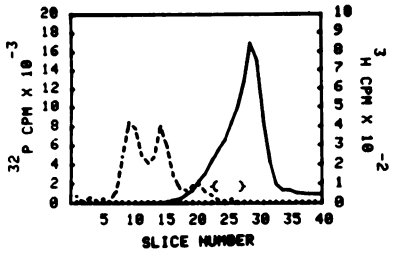

FIG. 3. *Size distribution of 3'-cDNA probes. Samples of 3'-cDNA synthesized from poly(A) RNA according to the protocol in the text was fractionated on a 1.2% agarose gel containing 10 mM methylmercury hydroxide. The gel was sliced in 3-mm intervals, and the radioactivity of each slice was determined. The solid line (corresponding to the left axis) shows the size distribution of the ^{32}P-labeled 3'-cDNA. The dotted line (corresponding to the right axis) indicates the position of ^{3}H-labeled HeLa cell rRNA species included as internal size standards. The brackets indicate the migration of bromophenol blue marker dye.*

consistent with this interpretation. A second possibility is that there are two mRNA species of this size range, one colinear with the 7-kb and 5.2-kb mRNA species, and one with a 3' end to the left of 0.58. The fact that two polypeptides are encoded by mRNA of this size (see below) is significant. An interruption in the DNA-encoded sequence of the 3' end of this RNA (due to splicing, for example) also could explain these results. The possibility that the structure of the 3' end of the 3.8-kb mRNA allows more extensive synthesis of cDNA leading to greater hybridization to the left of 0.58 is considered unlikely since we have never seen any significant variation in the size of the cDNA synthesized under our conditions (see Fig. 3). More extensive fragmentation of the HSV-1 genome in this region using other restriction endonucleases may lead to more concrete conclusions concerning the number and precise location of 3.8-kb mRNA within *Hin*dIII fragment K.

We cannot precisely localize the 5' ends of the mRNA species homologous to *Hin*dIII fragment K since we have not used any 5'-specific probes. The body of the mRNA species described, however, must be located primarily within this fragment (0.53–0.59) since we see little hybridization of total cDNA to the right of 0.59 (*Hin*dIII fragment L) and none to the left of 0.53 (*Hin*dIII fragment A, 0.26–0.53). However, we cannot exclude the possibility that short or interrupted leader sequences map outside of this region.

Translation of HSV-1 mRNA homologous to *Hin*dIII fragment K. Poly(A) polyribosomal RNA from 8×10^7 infected cells was isolated at 6 hpi and hybridized to *Hin*dIII fragment K DNA coupled to cellulose as outlined in Materials and Methods. After rinsing, elution, and precipitation, the hybrids were either translated in vitro directly or reselected on oligo(dT)-cellulose before translation in a micrococcal nuclease-treated reticulocyte lysate system using [^{35}S]methionine as a label. An autoradiograph of a sodium dodecyl sulfate-polyacrylamide gel of the translation products is shown in Fig. 6A. Comparison of the translation products of the *Hin*dIII fragment K selected mRNA (tracks 2

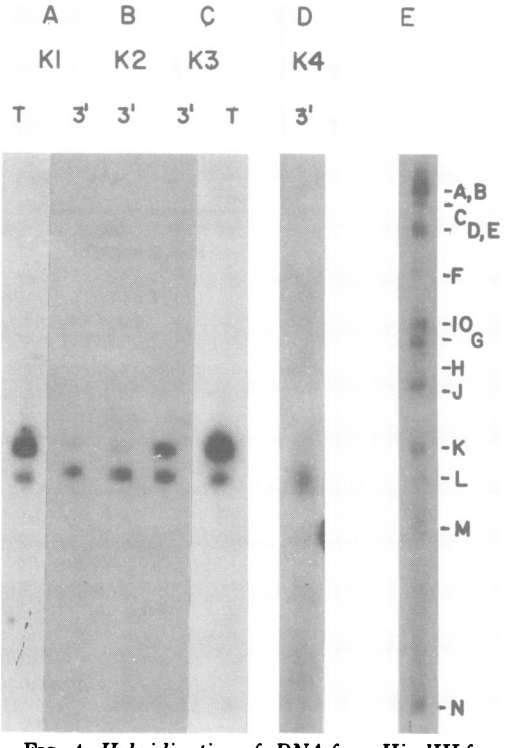

FIG. 4. *Hybridization of cDNA from HindIII fragment K-specific mRNA size classes to HindIII restriction fragments of HSV-1 DNA. Specific viral mRNA size classes homologous to HindIII fragment K (0.53–0.59), described in the text and shown in Fig. 2C, were eluted from denaturing agarose gels and partially degraded, and poly(A)-containing fragments were isolated by chromatography on oligo(dT)-cellulose. The cDNA to the 3' ends was made using oligo(dT) as primer, and cDNA to the total RNA was made using random calf thymus DNA oligonucleotides as primer. The cDNA was hybridized to Southern blots of a HindIII restriction digest of HSV-1 DNA. All procedures were as described in the text. (A) Total (T) and 3'-cDNA to the 7-kb mRNA (peak 1, Fig. 2C). (B) 3'-cDNA to the 5.2-kb mRNA (peak 2, Fig. 2C). (C) Total (T) and 3'-cDNA to the 3.8-kb mRNA (peak 3, Fig. 2C). (D) 3'-cDNA to the 1.8-kb mRNA (peak 4, Fig. 2C). (E) Total ^{32}P-labeled poly(A) RNA from infected cells labeled 2 to 6 hpi, hybridized to a similar HindIII blot to indicate band resolution.*

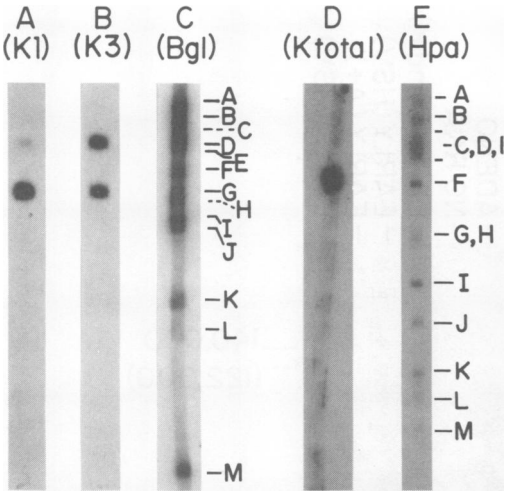

FIG. 5. *Hybridization of 3'-cDNA from HindIII fragment K-specific mRNA to BglII and HpaI restriction fragments of HSV-1 DNA. (A) 3'-cDNA synthesized from 7-kb mRNA (peak 1, Fig. 2C) hybridized to a Southern blot of a BglII digest of HSV-1 DNA. (B) 3'-cDNA from 3.8-kb mRNA (peak 3, Fig. 2C) hybridized to a Southern blot of a BglII digest of HSV-1 DNA. (C) ^{32}P-labeled infected cell poly(A) mRNA hybridized to a Southern blot of a BglII digest of HSV-1 DNA to indicate relative positions and resolution of bands. BglII bands N, O, and P are not shown. (D) 3'-cDNA synthesized from total HindIII fragment K-specific mRNA hybridized to a Southern blot of HpaI-digested HSV-1 DNA. (E) cDNA synthesized from HSV-1 DNA hybridized to a Southern blot of HpaI-digested HSV-1 DNA. The smaller fragments (N through T), which do not hybridize cDNA synthesized from HindIII fragment K-specific mRNA, are not shown here.*

and 3) with the translation products of total late infected cell poly(A) polyribosomal RNA (track 1) clearly demonstrated the specificity of the hybridization reaction. Total poly(A) polyribosomal RNA isolated from infected cells at 6 hpi encodes a complex mixture of at least 30 resolvable polypeptide products ranging in size from >155,000 d to <20,000 d. This is essentially the same pattern observed when this RNA is hybridized to total HSV-1 DNA coupled to cellulose prior to translation (see track 6, Fig. 6B; also, Holland et al., Virology, in press). In contrast, in vitro translation of late poly(A) polyribosomal RNA hybridized to *Hin*dIII fragment K DNA coupled to cellulose resulted in the synthesis of only three major polypeptide products with molecular weights of 140,000, 122,000, and 54,000 (Fig. 6A, tracks 2 and 3). Other bands of reduced intensity on autoradiographs (one at 86,000 d and a doublet at 64,000 to 65,000 d) also were consistently observed. The 140,000- and 54,000-d translation products consistently migrated as diffuse bands in polyacrylamide gels. No specific sized bands of radioactivity less than 50,000 d were consistently observed when *Hin*dIII fragment K-specific mRNA was used as a template, although radioactive bands in this size range were occasionally seen in some gels. The band of radioactivity migrating with a size of 50,000 d occurred even when no RNA was used for translation and is an endogenous product of the in vitro system. Reselection of *Hin*dIII fragment K mRNA on oligo(dT)-cellulose after hybridization (track 3) had no effect on the number, sizes, or relative amounts of polypeptides synthesized, although overall incorporation of [^{35}S]methionine into polypeptides was greater for the reselected mRNA. We concluded from this that our hybridization method yields viral mRNA of sufficient integrity to be utilized directly for in vitro translation.

In vitro translation of size fractions of *Hin*dIII fragment K-specific mRNA separated by sedimentation through a sucrose gradient allowed us to determine the template specificity of each size class of mRNA homologous to this region. [^{3}H]uridine-labeled poly(A) polyribosomal RNA isolated from 1.6×10^8 infected cells at 6 hpi and hybridized to *Hin*dIII fragment K DNA coupled to cellulose was sedimented through a 12-ml 5 to 20% sucrose gradient after denaturation with methylmercury hydroxide. A count profile of this gradient is shown in Fig. 2D. Fractions corresponding to the 7.0-, 5.2-, 3.8-, and 1.8-kb mRNA species were pooled as described in the legend to Fig. 2 and translated individually. An autoradiograph of a polyacrylamide gel of the in vitro translation products of each of these pools is shown in Fig. 7.

Each of the polypeptides identified in Fig. 6 was found to be transcribed by specific size fractions of *Hin*dIII fragment K-specific mRNA. The 7-kb mRNA encoded the 54,000-d polypeptide exclusively (track 4), and the 5.2-kb mRNA encoded the 140,000-d polypeptide (track 5). RNA sedimenting with a size corresponding to 3.8-kb mRNA stimulated synthesis of two polypeptides in vitro (Fig. 7, track 6). The major translation product of this mRNA was the 122,000-d polypeptide, but a significant amount of the 85,000-d polypeptide was also visible. This second polypeptide could be due to the presence of a second mRNA species of this size range, or it could be an artifact resulting from translation of this specific mRNA in our in vitro system.

The 1.8-kb mRNA also encoded more than one polypeptide in vitro (Fig. 7, track 8). The major translation product was a 54,000-d polypeptide indistinguishable in size or diffuseness

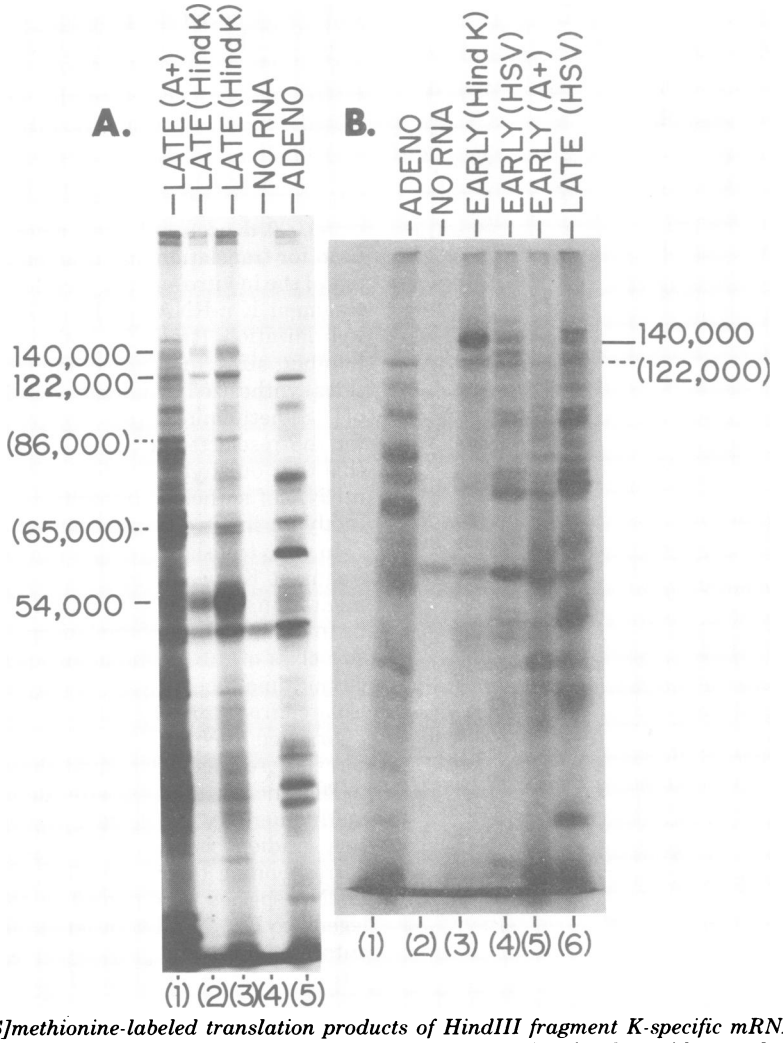

FIG. 6. *[^{35}S]methionine-labeled translation products of HindIII fragment K-specific mRNA. Shown are autoradiographs of 9% sodium dodecyl sulfate-polyacrylamide gels of polypeptides synthesized in vitro according to the procedure in the text. Captions at the tops of the gels indicate the RNA used for each translation. Numbers to the left and right of the gels indicate the molecular weights of polypeptide products encoded by HindIII fragment K-specific RNA. (A) Approximately equivalent amounts of HindIII fragment K-specific mRNA isolated from HSV-1-infected cells at 6 hpi either were translated directly after hybridization (track 2) or were repurified on oligo(dT)-cellulose before translation (track 3). The translation products of total infected-cell poly(A) polyribosomal RNA isolated at the same time after infection are shown in track 1. Control translations with no added RNA and with adenovirus-infected cell mRNA are shown in tracks 4 and 5, respectively. (B) Track 3 shows the translation products of HindIII fragment K-specific mRNA isolated from infected cells at 2 hpi. Track 4 shows the translation products of HSV-1-specific mRNA isolated from cells at this time after infection, and track 5 shows the translation products of total infected-cell poly(A) polyribosomal RNA isolated at 2 hpi. For comparison purposes, track 6 shows the translation products of HSV-1-specific mRNA isolated at 6 hpi. Control translations are shown in tracks 1 and 2.*

from that encoded by the 7-kb mRNA. It is unlikely that this polypeptide is the translation product of degraded 7-kb mRNA, since reselection of poly(A) RNA from the 1.8-kb fraction of the gradient encoded the same polypeptide product in a second translation(not shown). The implications of this are presented in the Discussion. The 1.8-kb mRNA also encoded a 64,000- to 65,000-d doublet not seen using any other size of *Hin*dIII fragment K-specific mRNA. This suggests that another mRNA species of this size range is present.

The longer exposure time necessary to detect the major polypeptide products of size-fraction-

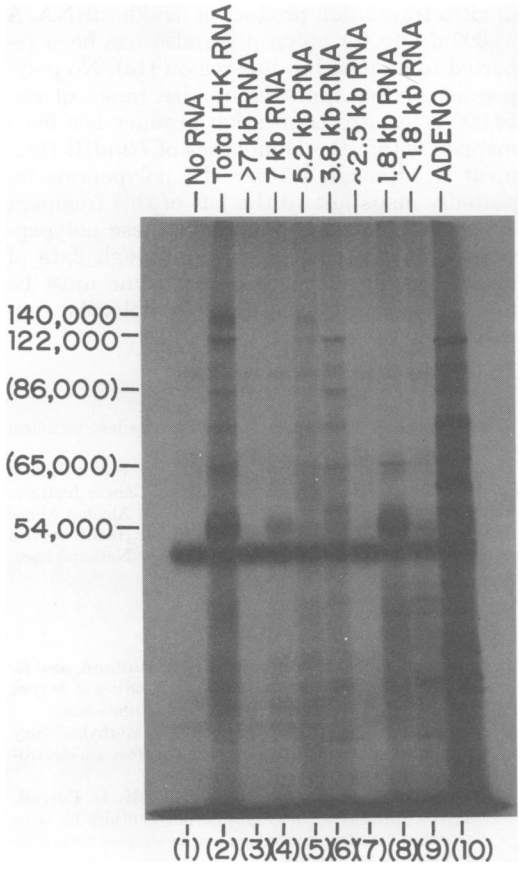

FIG. 7. *[^{35}S]methionine-labeled in vitro translation products of size-fractionated HindIII fragment K-specific mRNA. HindIII fragment K-specific mRNA isolated from infected cells at 6 hpi was sedimented through a 12-ml 5 to 20% sucrose gradient as discussed in the text. Fractions from the gradient (Fig. 2D) were pooled as described in the legend to Fig. 2 and translated in vitro. Track 2 shows the translation products of this mRNA before fractionation; tracks 3 to 9 show the translation products of each of the designated size classes. Control translations with no RNA and with adenovirus-infected HeLa cell mRNA are shown in tracks 1 and 10.*

ated RNA discussed above showed several other bands of radioactivity migrating in the gels. Some of these (95,000, 65,000, and 50,000 d and smaller) are seen in control translations when no exogenous RNA is added. These are endogenous products of the translation system. Other minor bands (e.g., 75,000 d in tracks 2 and 6 and <50,000 d in tracks 7 and 8) are products of specific size classes of mRNA and could be encoded by minor mRNA species or could be artifacts of in vitro translation of specific mRNA species. Partial degradation of larger mRNA species and lack of resolution of the sucrose gradient can explain identical bands synthesized from RNA of adjoining fractions of the gradient. An example of this is seen in tracks 5, 6, and 7 of Fig. 7, where reduced amounts of the 54,000-d polypeptide encoded by the 7-kb mRNA can be seen.

Our assignment of the 140,000-d polypeptide as the translation product of the 5.2-kb mRNA predicted that this polypeptide is encoded by early RNA. We tested this by translating in vitro *Hin*dIII fragment K-specific mRNA isolated from cells at 2 hpi. As shown in Fig. 6B, early poly(A) polyribosomal RNA, which hybridized to total HSV-1 DNA coupled to cellulose, encoded at least 16 major polypeptides ranging from 170,000 to <20,000 d in size (track 4). Some of these polypeptides are difficult to detect when total early poly(A) polyribosomal RNA from infected cells is translated (track 5). When early *Hin*dIII fragment K-specific mRNA is translated in vitro, only one major polypeptide, 140,000 d in size, is produced (track 3). This corresponds to the 140,000-d polypeptide translated from 5.2-kb mRNA late after infection. A small amount of the 122,000-d polypeptide also is produced. This polypeptide does not comigrate with any major polypeptides translated from total early HSV-1-specific mRNA and is probably the translation product of small amounts of 3.8-kb mRNA present at this time after infection (see Fig. 2A) and enriched by the preparative hybridization. No polypeptide products corresponding to 1.8-kb mRNA were observed when early *Hin*dIII fragment K-specific mRNA was translated, even though a significant amount of 1.8-kb mRNA was observed in gel profiles of this RNA (Fig. 2A).

DISCUSSION

It is clear from the studies reported here that the complex processes of RNA biogenesis described for smaller DNA viruses also must apply to HSV-1. No less than four abundant mRNA species totaling 18 kb in length map within a small region of the HSV-1 genome not much larger than 9.6 kb pairs (the size of *Hin*dIII fragment K), and definitely no larger than 12 kb pairs (between the left boundary of *Hin*dIII fragment K and the right boundary of *Hpa*I fragment F [Fig. 1]). At least two of these mRNA species (the 7-kb and 5.2-kb mRNA), and possibly some of the 1.8-kb mRNA, share sequences since they are transcribed in the same direction and their 3′ ends map similarly. These mRNA's become abundant at different times after infection and encode different polypeptides when translated in vitro. At least one other mRNA (3.8 kb) encoding yet another polypeptide also

may share some sequences with one or both of these mRNA's, although the possibility that it is transcribed from the opposite strand of the DNA cannot be excluded. We cannot, at this time, speculate about whether actual polypeptide coding sequences of any of these mRNA species overlap.

Although analysis of the positions of the mRNA species mapping within *Hin*dIII fragment K is complex, the polypeptide products encoded by individual mRNA species have been determined by in vitro translation of size-fractionated mRNA homologous to this region of the viral genome. The 5.2-kb mRNA, which is abundant in infected cells before viral DNA replication, encodes a 140,000-d polypeptide, whereas the 7-kb and 3.8-kb mRNA species, which are abundant in infected cells only after the onset of viral DNA replication, encode 54,000-d and 122,000-d polypeptides, respectively. The possible origins of a second minor polypeptide (86,000 d) translated from 3.8-kb mRNA have been discussed thoroughly in the Results section. The 1.8-kb mRNA appears to encode a polypeptide doublet of 64,000 to 65,000 d, in addition to the 54,000-d polypeptide, suggesting heterogeneity in the mRNA population of this size.

The translation of the 1.8-kb mRNA hybridizing to *Hin*dIII fragment K is intriguing. This mRNA, whose 3′ end also appears to map in *Hin*dIII fragment L, encodes a 54,000-d polypeptide, which migrates identically with the 54,000-d polypeptide encoded by the 7-kb mRNA. Translation of this 1.8-kb mRNA after repurification on oligo(dT)-cellulose resulted in synthesis of the same polypeptide, demonstrating that this RNA is not a degradation product of the 7-kb mRNA. If the polypeptides encoded by these mRNA's are in fact identical (as is most probably the case), this would suggest that sequences from the 5′ end of the 7-kb mRNA can be processed to yield a functional 1.8-kb mRNA. Experiments are in progress to further investigate this problem.

Correlation between polypeptides translated in vitro from *Hin*dIII fragment K-specific mRNA and infected-cell polypeptides mapped to this region of the genome by intertypic recombination experiments is very good. Morse et al. (13) identified a 139,000-d early (β) polypeptide mapping in this region of the genome which correlates very well with the 140,000-d polypeptide translated in vitro from the 5.2-kb mRNA. Marsden et al. (11) also reported a polypeptide of 136,000 to 143,000 d mapping in this region. A 117,000-d polypeptide, which appears later in infection than the 139,000-d β polypeptide (13), correlates well with the 122,000-d in vitro translation product of 3.8-kb mRNA. A 64,000-d late (γ) polypeptide also has been reported to map within this region (13). No polypeptide of the approximate size range of our 54,000-d in vitro translation product has been mapped within the boundaries of *Hin*dIII fragment K, although a 51,500-d γ polypeptide reportedly maps just to the left of this fragment (13). The biological function of these polypeptides is unknown at present, although data of Fenwick et al. (5) suggest that some must be involved in shut-off of host macromolecular synthesis.

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