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Nuclear Processing of Viral High-Molecular-Weight RNA in Cells Infected with Herpes Simplex Virus Type 1

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Nuclear RNA isolated from cells infected by herpes simplex virus type 1, strain F, was fractionated on formamide-sucrose gradients into two major classes, greater and less than 45S. These two classes of labeled nuclear RNA were hybridized to viral DNA fragments generated by digestion with the restriction enzymes *Hin*dIII and *Bgl*II. Early in infection, only a few DNA fragments hybridized to RNA, with slight differences between the two classes. Late in infection, all DNA fragments hybridized, showing that all viral RNA was present in large precursor molecules greater than 14 kilobases. The fragments that correspond to late gene products hybridized more of the small RNA than the large RNA. This suggests that the mRNA corresponding to late genes accumulated after the large precursors have been cleaved. Large (≥45S) and small (<45S) nuclear RNA and cytoplasmic RNA from cells late in infection were hybridized in excess to in vitro-labeled *Hin*dIII M and L fragments. More than 50% of the *Hin*dIII M fragment annealed with the large nuclear RNA, but only 36% of it annealed with the cytoplasmic RNA. The *Hin*dIII L fragment hybridized large nuclear RNA and cytoplasmic RNA to the same extent (30% and 26%). These results suggest that RNA complementary to the *Hin*dIII M fragment, which is the template for immediate early polypeptides, was regulated in the nuclei at the posttranscriptional level. This seems to suggest that temporal regulation of RNA cleavage occurs in the nucleus.

Several lines of evidence indicate that in mammalian cells, the RNA transcript of cellular or viral DNA is processed in the nucleus before becoming competent to function as a messenger. This processing includes capping and methylation of the 5′ end, polyadenylation of the 3′ end, specific internal methylation, splicing, and exclusion of complementary sequences. However, little is known concerning the regulation of these posttranscriptional modifications. Viruses may provide a good model to study this process.

We investigated the sequence homology of viral high-molecular-weight nuclear RNA in cells infected with herpes simplex type 1 (HSV-1). This was made possible by the mapping of early and late viral polypeptides (11, 12, 14). Previous results suggested that nuclear RNA consists of large primary transcripts (6, 19). More precisely, we have shown that more than 50% of the viral genome is transcribed into high-molecular-weight nuclear RNA and that these large molecules contain symmetrical transcripts (6).

In the study reported here, we compared two pools of viral nuclear RNA, fractionated under denaturing conditions, according to size, designating them as "large" and "small" nuclear RNA. We investigated their sequence homology to viral DNA fragments obtained by digestion with restriction enzymes.

This paper concludes the following. First, late after infection, herpes simplex is transcribed in the nucleus into RNA molecules greater than 14 kilobases. Second, the cleavages of RNA corresponding either to immediate early or to late polypeptides are differently regulated, suggesting the existence of a temporal mechanism.

MATERIALS AND METHODS

Cells and virus. Human epidermoid carcinoma no. 2 (HEp-2) cells were maintained in minimal essential medium supplemented with 5% newborn calf serum. HSV-1, F strain [HSV-1(F)], was obtained from B. Roizman (University of Chicago).

Preparation of viral DNA. The procedures for preparation and purification of viral DNA from purified nucleocapsids were as previously described (8) except that the DNA was treated with RNase. For in vitro labeling, viral DNA was purified by isopycnic banding in cesium chloride.

Isolation of nuclei from infected cells. Confluent HEp-2 cell monolayers were infected with HSV-1 at 100 PFU/cell. After 1 h at 37°C with agitation, the monolayers were rinsed, replenished with maintenance medium, and reincubated at 37°C. For ^{32}P labeling, cells were depleted of phosphate 2 h before addition of the radioisotope. At the end of the labeling period, the cells were rinsed three times with ice-cold

phosphate-buffered saline and mechanically scraped down. Nuclei were isolated by a modification of a previously described method (1). Briefly, cells were washed with 0.01 M Tris-hydrochloride (pH 8.0), centrifuged at 1,500 rpm, suspended in 0.01 M Tris (pH 8.0) containing 0.5% gum arabic (Merck) for 10 min, and disrupted with five strokes in a Dounce homogenizer. Cell disruption was monitored by phase-contrast microscopy. Two additional volumes of RSB buffer (containing 0.01 M sodium chloride, 2.5 mM magnesium chloride, and 0.01 M Tris-hydrochloride, pH 7.5) were immediately added, and the suspension was centrifuged for 5 min at 800 rpm. Nuclei were washed with RSB buffer.

Labeling of isolated nuclei. Purified nuclei were suspended at a concentration of 3×10^8 nuclei per ml in buffer containing 25 mM dithiothreitol, 5 mM magnesium chloride, 90 mM potassium chloride, 0.01 M Tris-hydrochloride (pH 7.8), 25% glycerol, 0.25 mM ATP, 0.125 mM GTP and CTP, and either [^{3}H]-UTP or [α-^{32}P]UTP (New England Nuclear Corp.). After 3 min of labeling at 37°C, the reaction was stopped by the addition of 4 μg of actinomycin D (Merck) per ml and a 50-fold excess of cold UTP (20).

Preparation of high-molecular-weight nuclear RNA. Purified nuclei were lysed by the addition of 0.5% sodium deoxycholate in RSB buffer and digested briefly with RNase-free DNase pretreated with 0.1 M sodium acetate–0.01 M sodium iodoacetate (pH 5.3) buffer at 55°C for 40 min as described by Zimmerman and Sandeen (21). RNA was then extracted with a mixture of sodium dodecyl sulfate and phenol-chloroform (4:1, vol/vol) as described elsewhere (13). The dry ethanol pellet was suspended in 98% formamide containing 5 mM Tris-hydrochloride (pH 7.0) and 5 mM EDTA, heated at 65°C for 2 min, and then chilled in ice. After dilution to 70% formamide, RNA was layered onto a preformed 5 to 20% (wt/wt) sucrose density gradient prepared in 70% formamide containing 5 mM Tris-hydrochloride (pH 7.5) and 5 mM EDTA and centrifuged for 15 h at 40,000 rpm in a Beckman SW41 rotor at 20°C. Fractions were monitored for RNA by UV adsorption at 260 nm, and fractions containing RNA were pooled and precipitated with ethanol. Before hybridization, RNA was treated twice with DNase followed by sodium dodecyl sulfate, phenol, and chloroform as described above.

Purification and assay of ds RNA. Double-stranded RNA (ds RNA) was isolated from purified RNA after self-annealing as described previously (5). The percentage of ds RNA in total or viral RNA was determined by treatment with 50 μg of RNase A and 10 U of RNase T_1 per ml in 2× SSC (1× SSC, 0.15 M sodium chloride and 0.015 M sodium citrate) at 37°C for 30 min.

Endonuclease digestions and separation of fragments on agarose gels. The *Hin*dIII (Biolabs) incubation mixture (100 μl) contained 1 μg of viral DNA, 20 mM Tris-hydrochloride (pH 7.6), 20 mM magnesium chloride, 30 mM sodium chloride, and 1 μl of enzyme. The *Bgl*II (Biolabs) incubation mixture (100 μl) contained 1 μg of viral DNA, 20 mM Tris-hydrochloride (pH 7.6), 10 mM magnesium chloride, 10 mM sodium chloride, and 1 μl of enzyme. Reactions were stopped after 2.5 h at 37°C by addition of 10 μl of 0.2 M EDTA (pH 8.5) and 10 μl of 60% (wt/wt) sucrose containing 0.1% bromophenol blue. DNA fragments were layered onto tube or slab gels of 0.4% (wt/wt) agarose (Seakem) and electrophoresed in buffer containing 0.036 M Tris-hydrochloride, 0.03 M NaH_2PO_4 (pH 7.7), and 0.05 M EDTA for 18 h at 4°C at 60 V. The length of migration was 20 cm for blotting experiments and 30 cm for the purification of fragments. DNA was located by staining with ethidium bromide.

Blot hybridization procedure. Alkaline-denatured DNA was transferred from the agarose gel to nitrocellulose membranes using the method described by Southern (17). Strips (0.3 cm) were cut lengthwise from nitrocellulose sheets (approximately 0.5 μg of DNA per strip). The strips were saturated with hybridization buffer containing 0.1 M Tris-hydrochloride (pH 7.5), 1 M sodium chloride, 25 mM EDTA, and 30% formamide. RNA samples in 5 mM Tris-hydrochloride (pH 7.5) were heated to 115°C for 5 min and chilled in ice, and hybridization was performed in 0.5 ml at 63°C for 20 h. After hybridization the blot strips were washed twice with 2× SSC at room temperature and treated with RNase A for 30 min at room temperature (10 μg/ml) in 2× SSC. After two washings in 2× SSC, the blot strips were exposed on preflashed (10) X-ray film (Curix RP2 Agfa Gevaert) at −70°C with an intensifying screen (Cronex Lightning-Plus, E. I. du Pont de Nemours and Co., Wilmington, Del.) (18). Autoradiographs were scanned using a Vernon densitometer. Designation of restriction endonuclease fragments follows the agreement reached at the Third Herpesvirus Workshop, Cold Spring Harbor, 1976, described by Jones et al. (6a) and shown in Fig. 1.

Radioactive labeling of HSV-1 DNA fragments. Slices containing individual fragments were cut from the gel. The agarose was dissolved in 5 volumes of saturated KI solution at 37°C for 20 min with shaking. The solution was mixed with 1 ml of hydroxyapatite (Biogel HTP, BioRad), which had been boiled and pre-equilibrated with 0.01 M potassium phosphate buffer (pH 7.6). Hydroxyapatite was washed with 0.01 M and 0.1 M potassium phosphate buffers (pH 6.7). DNA fragments were eluted with 1 ml of 0.4 M potassium phosphate buffer (pH 6.7), dialyzed against 1 M NaCl–0.05 M Tris (pH 7.5)–0.01 M EDTA in the presence of Dowex 50 (H^+ form) to remove ethidium bromide, and then concentrated and dialyzed against glass-distilled water.

Purified viral DNA fragments (0.1 μg) were labeled in vitro to a specific activity of 5×10^7 cpm/μg with [α-^{32}P]dCTP (300 Ci/mmol; Amersham) by using the technique of repair synthesis with *Escherichia coli* polymerase I (Boehringer-Mannheim) described by Rigby et al. (15) and modified by Given and Kieff (3). The reaction volume was 100 μl, the ratio of unlabeled to labeled triphosphate was 5 with 50 μCi of [^{32}P]-dCTP, and the reaction mixture was incubated at 14°C. The kinetics of synthesis were followed, and the reaction was stopped at the beginning of the plateau value. Total viral DNA was labeled by the same technique.

Hybridization of labeled RNA to DNA on filters. The RNA was denatured in 0.1× SSC at 115°C for 5 min and hybridized to HSV-1 DNA fixed to nitrocellulose filters. DNA was fixed to 25 mM Schleicher and Schuell B6 filters as previously de-

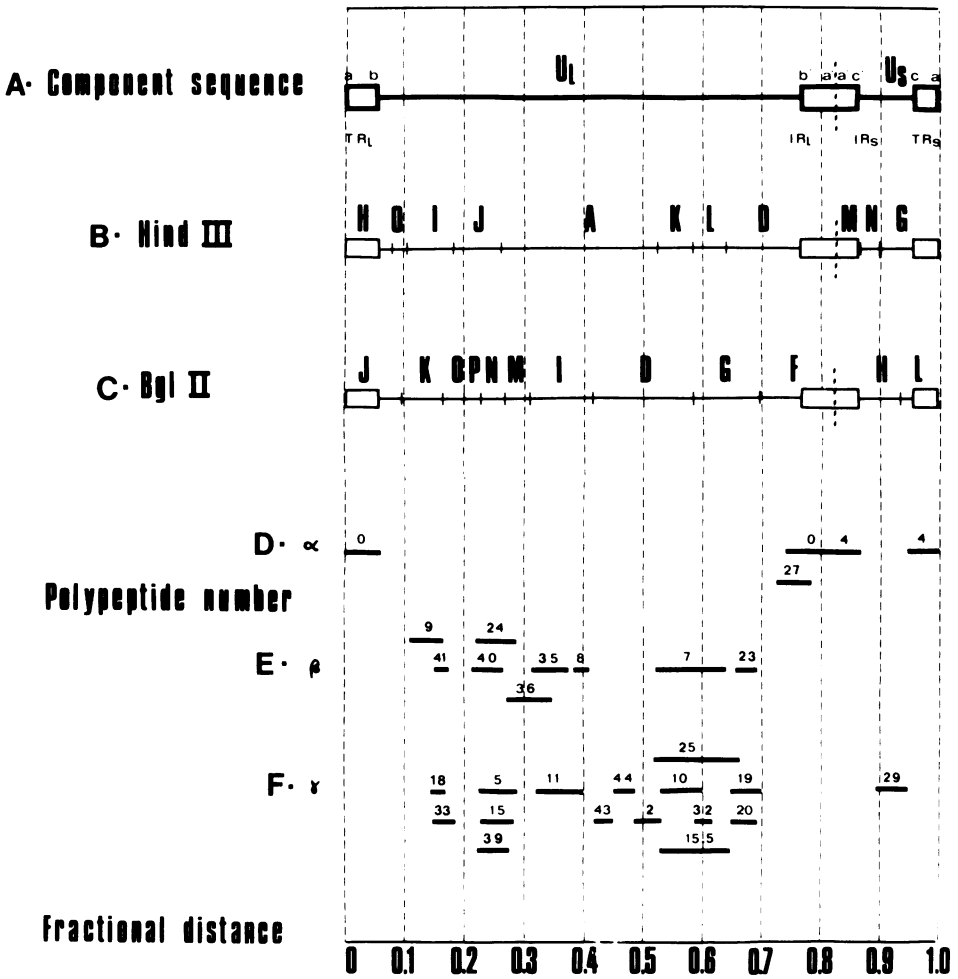

FIG. 1. *Schematic representation of restriction endonuclease and polypeptide maps of HSV-1(F) DNA. Line A is a diagrammatic representation of F DNA consisting of two covalently linked components, each containing a unique sequence (U_L and U_S) limited by two reiterated sequences (ab, b'a' and a'c', ca [12] or TR_L, IR_L and IR_S, TR_S [16]). These two components can invert, generating four isomers designated P (prototype), IS (inversion of S), I_L (inversion of L), and I_{SL} (inversion of both S and L). Lines B and C show the size and position of the DNA fragments, generated by the cleavage of P arrangement by HindIII and BglII restriction enzymes according to the Third Herpesvirus Workshop, Cold Spring Harbor, 1976, described by Jones et al. (6a). Lines D, E, and F show polypeptide mapping according to references 11, 12, and 14 and B. Roizman (personal communication), using the designations of Honess and Roizman (5): immediate early polypeptides (α), early polypeptides (β), and late polypeptides (γ).*

scribed (9). Small disks (6-mm diameter) were then punched out. A set of disks containing HSV-1 DNA and a blank were placed in a Beem capsule containing the denatured RNA in 200 μl of hybridization buffer (1 M NaCl, 25 mM EDTA, 0.1 M Tris, pH 7.5, 30% formamide). After incubation for 20 h at 63°C, the disks were removed, washed twice for 30 min each in 2× SSC, and digested with RNase A (10 μg/ml) for 1 h at room temperature.

To elute viral RNA, disks were rinsed at 63°C for 4 h in 2× SSC and incubated at 70°C for 10 min in 98% formamide, 5 mM Tris-hydrochloride, 5 mM EDTA, and 20 μg of yeast RNA per ml as a carrier.

Hybridization of unlabeled RNA to labeled DNA fragments in solution. Excess unlabeled selected RNA and in vitro-labeled DNA fragments were precipitated with ethanol. Washed pellets were dissolved in hybridization buffer containing 0.3 M Na^+ and 0.04 M phosphate buffer (pH 7.5), divided into 10-μl micropipettes, sealed, heated to 104°C for 7 min, and incubated at 75°C for intervals of up to 24 h. The amount of DNA driven into DNA-RNA hybrid was

monitored by digestion with S1 nuclease (Miles). In these experiments, the amount of denatured nonannealed DNA resistant to enzyme digestion, tested with the same concentration of yeast RNA, did not exceed 7% of the input DNA counts.

RESULTS

Sizing of viral nuclear RNA. To ascertain the size distribution of viral nuclear RNA, either cells were labeled in vivo for 20 min with [^{3}H]-uridine (50 μCi/ml, 54 Ci/mmol) and RNA was isolated 8 h postinfection, or isolated nuclei from 8-h-infected cells were labeled in vitro for 3 min with [^{3}H]UTP (500 μCi, 35 Ci/mmol). Most of the RNA had a small molecular weight when monitored by ^{3}H radioactivity (Fig. 2). However, when the RNA was hybridized to viral DNA on filters, two size classes were observed, 18S and 45S, for both samples. The population of large molecules was more abundant in RNA from labeled nuclei (Fig. 2B) than from labeled cells (Fig. 2A). RNA fractionated on formamide-sucrose gradients that were pooled according to the position of the 45S optical density marker was utilized in subsequent experiments.

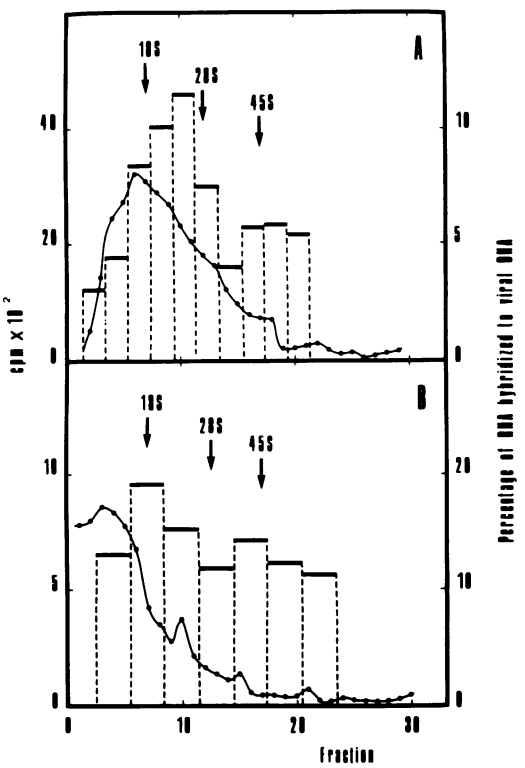

FIG. 2. *Formamide-sucrose gradient of labeled nuclear RNA from cells infected for 8 h. Curves represent radioactivity, and bars indicate specific viral RNA determined by hybridization with viral DNA. (A) Cells labeled for 20 min; (B) nuclei prepared at 8 h and labeled for 3 min. Arrows indicate rRNA sedimentation determined by optical density at 260 nm.*

Mapping of 8-h-infected viral RNA. Either infected cells were labeled with 1 mCi of ^{32}P per ml between 1 and 8 h postinfection and RNA was extracted from nuclei and cytoplasm, or 8-h-infected nuclei were isolated and labeled in vitro with [α-^{32}P]UTP for 3 min (1 mCi at 342 Ci/mmol). RNA was separated on formamide-sucrose gradients as described above, and RNA fractions of <45S (small nuclear RNA) and ≥45S (large nuclear RNA) from labeled nuclei were pooled and hybridized to an HSV-1 *Hin*dIII restriction digest of viral DNA which had been electrophoretically separated and transferred to nitrocellulose strips by the method of Southern (17). In both cases, large and small nuclear RNA hybridized to all DNA fragments (Fig. 3). No appreciable differences were observed between small nuclear and cytoplasmic RNA from labeled cells (Fig. 3A) or between large and small RNA from labeled nuclei (Fig. 3B). When the amount of hybridized RNA (large and small nuclear and cytoplasmic) was estimated by densitometric tracing (Fig. 4), large RNA hybridized more efficiently with DNA fragments D, F (H + M), G, and M than did small RNA. On the other hand, small RNA hybridized better with fragments K and L than did large RNA (Fig. 4A). These differences appeared less pronounced when RNA was labeled in vitro (Fig. 4B).

Mapping of viral nuclear RNA at various times after infection. Hybridization of nuclear RNA at early and late times after infection was compared. Cells were infected and labeled, respectively, between 0 to 2 h and 8 to 10 h postinfection with 1 mCi of ^{32}P per ml and RNA isolated as above. Nuclear RNA of <45S and ≥45S in size was hybridized to *Hin*dIII and *Bgl*II restriction endonuclease DNA fragments.

Early in the experiment (2 h), only slight differences in hybridization were observed (Fig. 5). Later (10 h), all DNA fragments hybridized; the differences between large and small RNA were more pronounced. Small nuclear RNA hybridized more efficiently with *Bgl*II D, I, K, O, and P fragments and with *Hin*dIII I, J, K, and L fragments than did large RNA.

Distribution of ds nuclear RNA. The proportion of ds sequences in viral RNA was determined. Cells were infected as described previously and labeled for 20 min with 50 μCi of [^{3}H]uridine (54 Ci/mmol). Nuclear RNA was isolated 8 h postinfection and separated on form-

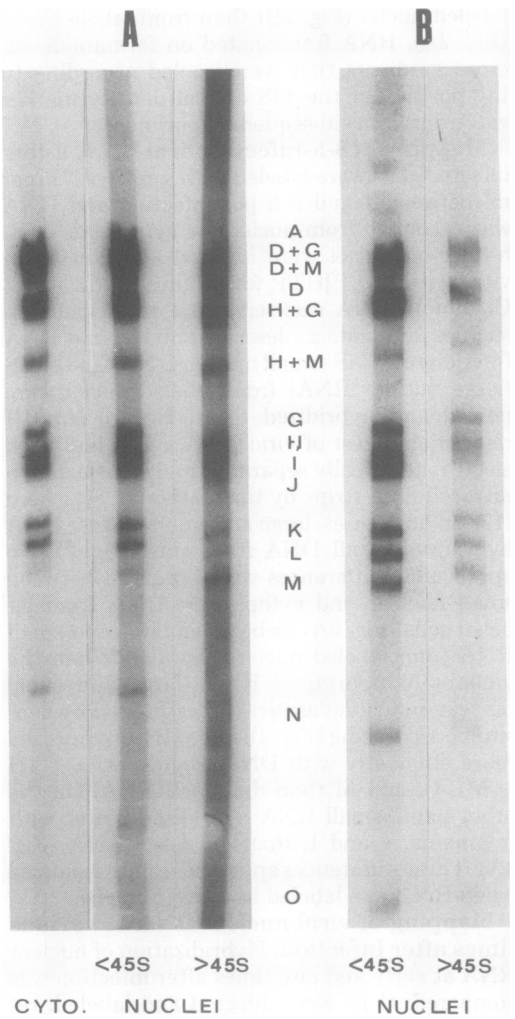

FIG. 3. *Autoradiographs of hybrids between HindIII viral DNA fragments and labeled large nuclear, small nuclear, and cytoplasmic RNAs prepared from 8-h-infected cells. (A) Cells were ^{32}P-labeled from 1 to 8 h postinfection. (B) Isolated nuclei were labeled in vitro with [^{32}P]UTP for 3 min.*

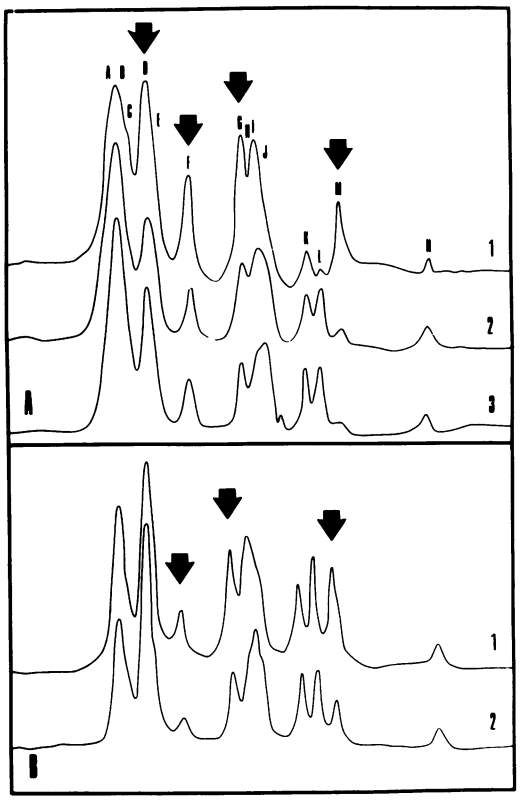

FIG. 4. *Densitometric traces of autoradiographs from Fig. 3 of hybrids between HindIII viral DNA fragments and large or small nuclear RNA from 8-h-infected cells. (A) Cell-labeled RNA: 1, large nuclear RNA; 2, small nuclear RNA; 3, cytoplasmic RNA. (B) Nuclei-labeled RNA. Arrows show the fragments which hybridized more efficiently with large than with small nuclear RNA.*

amide-sucrose gradients. In the first experiment, ds RNA was purified from large and small nuclear RNA after reannealing as described elsewhere (5). In the second experiment, large and small nuclear RNA were hybridized to viral DNA on filters. Viral RNA was eluted from the hybrid, and the percentage of ds RNA was determined after self-reannealing. The results (Table 1) indicated that the amounts of viral ds RNA in large and small viral RNA were of the same order of magnitude, and the ratio of ds RNA (large to small) was similar for the two experiments (1.4 and 1.3).

Fraction of *Hin*dIII M and L fragments represented in RNA transcripts accumulating in different compartments of cells. The purpose of these experiments was to analyze the transcriptions of two DNA fragments which hybridized differentially to the two categories of nuclear RNA by the blotting technique. We used the *Hin*dIII M and L fragments previously described (7, 12, 14), which are shown in Fig. 1 as corresponding, respectively, to early and mostly late functions. These fragments were labeled in vitro by nick translation with [α-^{32}P]dCTP and hybridized in solution to excess RNA. RNA was extracted from the nuclei and cytoplasm of 8-h-infected cells. Nuclear RNA was selected as described above in formamide-sucrose gradients. The results (Fig. 6) indicated that late in infection 30% and 16% of the *Hin*dIII L fragment, respectively, annealed to large and small nuclear RNA, whereas 26% annealed to cytoplasmic RNA. However, >50% of the *Hin*dIII M frag-

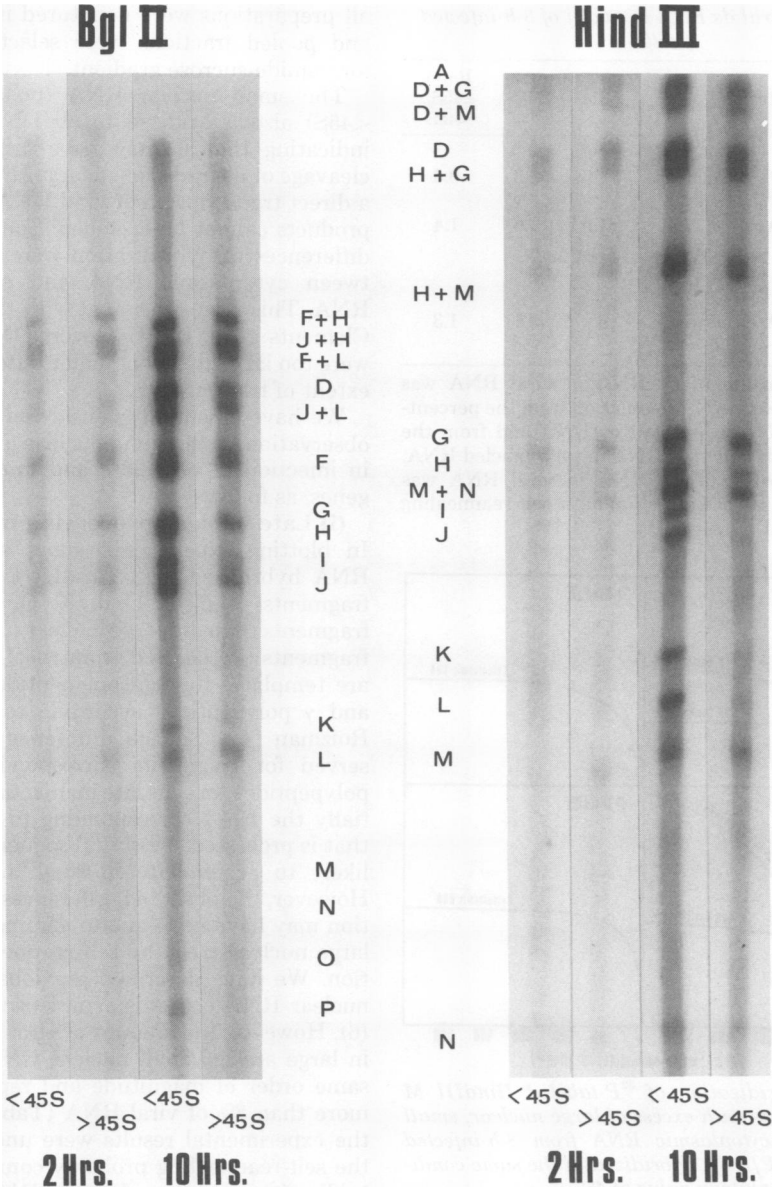

FIG. 5. *Autoradiographs of hybrids between HindIII and BglII viral DNA fragments and labeled large and small nuclear RNA prepared from 2-h- and 10-h-infected cells.*

ment annealed to large nuclear RNA (plateau value was not obtained), 33% annealed to small nuclear RNA, and 36% annealed to cytoplasmic RNA. Total in vitro-labeled viral DNA hybridized 45% with cytoplasmic RNA under the same conditions. These experiments showed that the *Hin*dIII M fragment was transcribed to a greater extent than the *Hin*dIII L fragment. However, the corresponding RNA was present only in the large nuclear RNA and seemed to be lost during the processing.

DISCUSSION

In this study, we have analyzed nuclear RNA prepared by labeling either whole HSV-1-infected cells or isolated nuclei. In vitro RNA synthesis by isolated nuclei represents mainly the elongation of chains initiated in intact cells, as described by Weber et al. (20). Viral RNA was distributed on gradients between two peaks at around 18S and 45S. Our results concerning viral nuclear RNA found late in infection led to the following conclusions.

TABLE 1. *Viral ds RNA in nuclei of 8-h-infected cells*

Size of RNA	<45S	≥45S	Ratio, ≥45S/<45S
Expt. 1[a]			
Viral RNA (% of nuclear RNA)	3.8	2.3	0.6
ds viral RNA (% of viral RNA)	1.9	2.6	1.4
Expt. 2[b]			
ds viral RNA (% of viral RNA)	6.2	8.1	1.3

[a] The proportion of ds RNA in viral RNA was calculated for each RNA population from the percentage of viral RNA in purified ds RNA and from the percentage of ds and viral RNA in total labeled RNA.

[b] The proportion of ds RNA in viral RNA was determined by RNase digestion after self-reannealing of purified viral RNA.

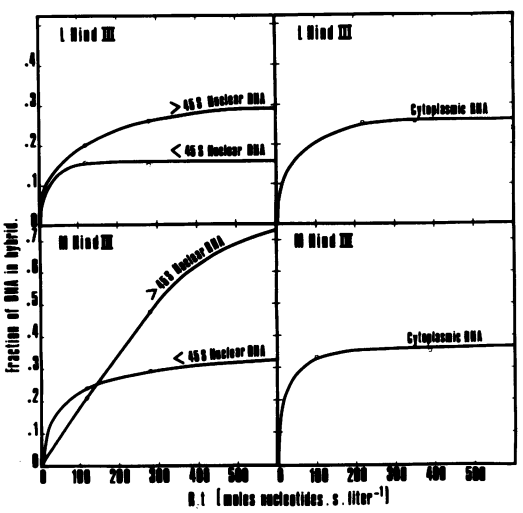

FIG. 6. *Hybridization of ^{32}P-labeled HindIII M and L fragments with excess of large nuclear, small nuclear, and cytoplasmic RNA from 8-h-infected cells. Viral [^{32}P]DNA hybridized in the same conditions, giving a plateau value of 45%.*

Herpesvirus DNA is transcribed mainly into RNA molecules of 45S or more than 14 kilobases. These precursors are cleaved into smaller mRNA molecules. We have already reached this conclusion from previous experiments using liquid hybridization of viral DNA with ≥45S nuclear RNA (6). In the study reported here, we confirmed this by using the Southern method. Large nuclear RNAs, especially those labeled for 3 min in isolated nuclei, hybridized to all DNA fragments. The large nuclear RNA could not have been an aggregate of small molecules, since all preparations were denatured in formamide, and pooled fractions were selected from the formamide-sucrose gradient.

The small nuclear RNA (pooled fractions, <45S) also hybridized to all DNA fragments, indicating that it may have originated from cleavage or degradation of large RNA. However, a direct transcription of viral DNA into smaller products cannot be excluded. Some very small differences in hybridization were observed between cytoplasmic RNA and small nuclear RNA. This is in agreement with the findings of Clements et al. (2). However, DNA fragments were too large to detect major differences in the extent of transcription.

We have essentially considered two types of observations concerning nuclear processing late in infection of both late and immediate early genes, as follows.

(i) Late nuclear processing of late genes. In blotting experiments, more small nuclear RNA hybridized with *Hin*dIII I, J, K, and L fragments of *Bgl*II D, I, K, O, and P DNA fragments than did large nuclear RNA. All these fragments are derived from the U_L region and are templates for late polypeptides (11, 12) (β and γ polypeptides according to Honess and Roizman [4]). Such a difference was not observed for fragments corresponding to early polypeptides. In cells late in infection, it is essentially the RNA corresponding to late function that is processed; thus viral sequences are more likely to accumulate in small nuclear RNA. However, the observed differences in hybridization may have been due to elimination of some large nuclear RNA by self-reannealing in solution. We have described previously that large nuclear RNA contains symmetrical transcripts (6). However, the amount of viral ds RNA both in large and in small nuclear RNA was of the same order of magnitude and represented not more than 8% of viral RNA (Table 1). Even if the experimental results were underestimated, the self-reannealing probably competes for hybridization of large and small RNA to the same extent. Therefore the observed difference in hybridization between large and small RNA seems to be related to the difference in concentration of an RNA corresponding to a given DNA fragment. This conclusion is further supported by the following two observations. First, the proportion of large viral RNA was higher in RNA from labeled nuclei than in RNA from labeled cells (Fig. 2). Second, the blotting hybridizations of large and small RNAs from labeled nuclei were similar, whereas it was different for RNAs from labeled cells (Fig. 4). During the short labeling time for nuclei, the RNA chains were

elongated but not processed to a great extent.

(ii) Late nuclear processing of immediate early genes. *Hin*dIII D, F (H + M), G, and M fragments have a more efficient relative hybridization with large than with small nuclear RNA from intact cells (Fig. 4). Almost the same result was obtained with RNA labeled for 3 min by the method of Weber et al. (20). These DNA fragments originate from inverted reiterated sequences identified as template of immediate early polypeptides (12, 14). Liquid hybridization between labeled *Hin*dIII M and L DNA fragments and an excess of RNA showed that a large part of *Hin*dIII M was transcribed into large nuclear RNA which was not found in the cytoplasm. Large nuclear RNA hybridized to more than 50% of the M fragment; therefore, both strands of the *Hin*dIII M fragment, corresponding, respectively, to ca and c'a' regions (Fig. 1), were transcribed into large nuclear RNA. This was confirmed by the more efficient hybridization of large RNA to the *Hin*dIII G fragment containing also the same reiterated complementary sequences (Fig. 4). *Hin*dIII L fragment was transcribed to almost the same level into large nuclear and cytoplasmic RNA. The data obtained by Southern hybridization and by liquid hybridization show that, late in infection, the processing of RNA derived from inverted reiterated sequences was diminished or less efficient in the nucleus.

In conclusion, our observations suggest that, late in infection, RNA cleavage in the nucleus for genes corresponding, respectively, to immediate early and to late polypeptides is differently regulated. Nuclear processing seems to be subject to temporal regulation.

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