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RNA Synthesis in Cells Infected with Herpes Simplex Virus

XII. Sequence Complexity and Properties of RNA Differing in Extent of Adenylation

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Fractionation of polyadenylated RNA from cells infected with herpes simplex virus by affinity chromatography on columns of poly(U) immobilized on glass-fiber filters yielded three major classes of RNA-containing poly(A) chains with average lengths of 30, 50, and 155 adenylate residues [poly(A)$_{30}$, poly(A)$_{50}$, poly(A)$_{155}$]. In contrast, nitrocellulose membranes bound predominantly a fraction of RNA containing poly(A)$_{155}$. The distribution of cytoplasmic RNA in the three classes was found to be independent of the labeling interval, ranging from 10 min to 6 h. Cytoplasmic poly(A) RNA consisted mainly (57 to 68%) of the poly(A)$_{155}$ class; this was also the major class (68%) of polyadenylated RNA found in polyribosomes. Nuclear poly(A) RNA consisted largely (42 to 50%) of poly(A)$_{30}$ class, whereas high-molecular-weight nuclear RNA sedimenting at >45*S* contained almost exclusively the poly(A)$_{30}$ tracts. Hybridization experiments involving unlabeled RNA and labeled viral DNA demonstrated the presence of viral RNA sequences complementary to approximately 40% of viral DNA in all polyadenylated RNA classes. Inasmuch as unfractionated cytoplasmic RNA arises from approximately 40% of the viral DNA, we conclude that most, if not all, viral RNA species present in the cytoplasm are adenylated. In contrast to these results, only a fraction of poly(A)$_{155}$ RNA, complementary to 21% of viral DNA, bound to nitrocellulose filters. The selective binding of poly(A)$_{155}$ sequences to nitrocellulose filters might be related to its secondary structure, since transcripts homologous to 40% of viral DNA bind to nitrocellulose membranes, provided the RNA is denatured prior to filtration. The data suggest that poly(A) tracts arise by at least two separate steps. The first involves the appearance of poly(A)$_{30}$ tracts in the high-molecular-weight nuclear transcripts. The second involves polyadenylation to poly(A)$_{50}$ and poly(A)$_{155}$ RNA classes concomitant with processing and transport to the cytoplasm.

An earlier report from this laboratory (1) described the utilization of the nitrocellulose (Millipore) filter binding technique for analyses of polyadenylated RNA in human cells (HEp-2) infected with human herpesvirus 1 (herpes simplex virus type 1, HSV-1). These analyses demonstrated the presence of polyadenylic acid sequences, estimated to be about 165 nucleotides long, covalently linked to herpesvirus-specific RNA and suggested that they were added post-transcriptionally.

Subsequent studies showed that polyribosomal RNA may be fractionated into two classes by the nitrocellulose filter binding technique. The RNA that binds to the filters was shown to be highly adenylated and complementary to 21% of viral DNA (19). Analysis of the kinetics of hybridization indicated that RNA selected in this manner was of one abudance class, i.e., roughly homogeneous with respect to molar concentrations (19), and corresponded to the abundant RNA class found late in HSV infection (6). RNA passing through the filters contained a different set of transcripts and was assumed to be either minimally adenylated or nonadenylated.

In this paper, we report evidence that polyadenylated RNA from both infected and uninfected cells, as well as virus-specific RNA, form several classes differing in the length of the poly(A) chain and furnish data on some properties of these classes.

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MATERIALS AND METHODS

Cells and virus. The procedures for the propagation of HEp-2 (human epidermoid carcinoma no. 2) cells and the assay, production, and pertinent properties of the F strain of HSV-1 were described elsewhere (5, 16, 20). In the studies described in this paper, confluent monolayers of HEp-2 cells were infected with the F strain of HSV-1 at multiplicities of 10 to 50 PFU per cell.

Labeling of RNA. Infected and uninfected cells were labeled at times specified in the text with 10 to 50 μCi of [2,8-^{3}H]adenosine (New England Nuclear Corp., Boston, Mass.) per ml of Eagle minimal essential medium (EMEM) supplemented with 1% dialyzed calf serum. For ^{32}P labeling of RNA, cell monolayers were rinsed three times and overlayed at 8 h preinfection with low-phosphate medium (EMEM-P_i) containing 1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.1) and 1% dialyzed calf serum that had been redialyzed against either EMEM-P_i or 0.12 M NaCl-10 mM Tris, pH 7.1, to remove P_i. Cells were exposed to virus for 1 h, rinsed three times with EMEM-P_i, overlayed with low-phosphate medium containing 100 to 150 μCi of $^{32}P_i$ (New England Nuclear Corp.) per ml, and harvested at 8 h postinfection.

Cell fractionation. Cells were scraped and harvested in phosphate-buffered saline (4), washed once in phosphate-buffered saline, suspended in Iso-Hi buffer (10 mM Tris-hydrochloride, pH 8.4; 0.14 M NaCl; and 1.5 mM $MgCl_2$) and lysed by the addition of Nonidet P-40 (Shell Oil Co., New York, N.Y.) to a final concentration of 0.5%. After 20 min on wet ice, the nuclei were separted from the cytoplasmic extract by centrifugation at 800 $\times$ *g* for 10 min. The cytoplasmic extract was then centrifuged at 10,000 rpm for 10 min in the Sorvall SS34 rotor to remove mitochondria. Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5% to the supernatant fraction. For some experiments, polyribosomal and free cytoplasmic fractions were prepared as described by Silverstein et al. (19). The nuclei and cytoplasmic fractions were frequently stored at -70 C for 1 to 3 days before extraction of RNA.

Extraction of RNA. Briefly, as described by Brawerman et al. (3), RNA was extracted from cytoplasmic extracts containing 3 mM EDTA by the hot phenol-SDS method, including a reextraction of the nonaqueous residue with Tris buffer, pH 9.0. Following two to three extractions with chloroform-2% isoamyl alcohol, the RNA was precipitated with ethanol and stored at -20 C. For extraction of nuclear RNA, nuclei were first suspended in about 4 volumes of reticulocyte standard buffer (10 mM NaCl-1.5 mM $MgCl_2$-10 mM Tris-hydrochloride, pH 7.4), lysed by the addition of sodium deoxycholate to a final concentration at 0.5%, and treated briefly at 37 C with 50 μg of DNase I per ml (RNase free, Worthington Biochemical Corp., Freehold, N.J.), and the RNA was extracted as above. The DNase had been pretreated with iodoacetate to inactivate residual RNase (23). This RNA was then dissolved in 1 ml of 10 mM Tris-hydrochloride (pH 7.4)-1 mM $MgCl_2$ and again treated with DNase I (RNase free) at 20 μg/ml for 1 h at 37 C. The DNase activity was stopped by addition of EDTA to a final concentration of 2 mM. The RNA was reextracted first with phenol and then with chloroform-isoamyl alcohol and finally precipitated with ethanol.

Fractionation of polyadenylated RNAs. Polyadenylated RNA was separated from the nonadenylated RNA by its ability to bind to nitrocellulose filters (12) or to poly(U) immbolized on glass-fiber filters (18). The procedure used in this laboratory for binding adenylated RNA to nitrocellulose filters in 0.5 M KCl was described elsewhere (1). For separation of polyadenylated RNA by affinity chromatography on columns of poly(U), 750 μg of poly(U) (Sigma Chemical Co., St. Louis, Mo.) was bound to each GF/C glass-fiber filter (Whatman) by the method of Sheldon et al. (18). Specifically, two poly(U) filters cut into 1- to 2-mm pieces were suspended in NETS buffer (0.12 M NaCl, 1 mM EDTA, 10 mM Tris-hydrochloride, 0.2% SDS, pH 7.4) and packed into a Pasteur pipette or a 5-ml disposable pipette to form a column with a bed volume of about 0.75 ml. The columns were washed with 25 to 35 ml of 70% (vol/vol) formamide in ETS buffer (1 mM EDTA, 10 mM Tris-hydrochloride, 0.2% SDS, pH 7.4) to remove unbound poly(U) and then equilibrated with 30 ml of NETS buffer. RNA samples were heated 5 min at 75 C in 1 ml of ETS buffer and chilled on ice, and NaCl was added to a final concentration of 0.2 M. The RNA was slowly loaded onto the poly(U) column at a flow rate of about 0.1 ml/min, and the adenylated RNA was allowed to bind for 10 to 15 min at room temperature. The RNA solution was passed through the column two more times, at a somewhat faster rate, to ensure exhaustive binding. Nonspecifically bound RNA was removed by washing the column with 25 ml of NETS buffer. Polyadenylated RNA was subsequently eluted from the column by one of two methods. The first procedure involved eluting bound RNA with 60% (vol/vol) formamide (Matheson Coleman and Bell, Elk Grove Village, Ill.) in ETS buffer. The second procedure involved the elution of RNA of higher affinity with a 100-ml 0 to 99% (vol/vol) linear gradient of formamide in ETS buffer at a flow rate of 0.75 ml/2 min per fraction. Formamide concentration was determined from the refractive index. It should be noted that, whereas the elution profile of adenylated RNA was invariant, the elution position of different classes of adenylated RNA varied with the batch of formamide used.

Analysis of poly(A) tracts. Polyadenylated RNA selected by filtration through nitrocellulose filters or by affinity chromatography on poly(U) columns was precipitated twice with 2 volumes of ethanol, dried under vacuum, and suspended in 0.25 to 1.0 ml of ET buffer (1 mM EDTA, 10 mM Tris, pH 7.4). A portion of this sample was removed and brought to 1.0 ml by the addition of ET buffer. A 10- to 20-μl sample was removed and precipitated with 10% trichloroacetic acid to determine input radioactivity. The rest of the sample was heated at 75 C for 5 min and then chilled on wet ice. NaCl (0.2 M final concentration), 4 μg of RNase A (Worthington), and 10 U of RNase T_1 (Calbiochem, San Diego, Calif.) were

added, and the RNA was digested for 1 h at 37 C. A 50-μl sample was removed and precipitated with 10% trichloroacetic acid to determine the fraction resistant to these RNases. Then 50 to 70 μg of carrier yeast RNA and an equal volume of cold 10% trichloroacetic acid were added, and the mixture was allowed to stand on ice for 10 min. The precipitated poly(A) tracts were collected by centrifugation (15 min, 8,000 × g), washed successively with 80% ethanol in 0.1 M Tris (pH 9) and 80% ethanol in 0.1 M Tris (pH 7.4), dried under vacuum, and suspended in 50 μl of sample buffer (0.2% SDS, 5 mM EDTA, 5 mM Tris, pH 7.4). Samples were heated to 75 C for 5 min, chilled on ice, and mixed with 10 μl of 50% (wt/vol) sucrose in sample buffer containing bromophenol blue. The samples were layered onto 10-cm 10% polyacrylamide-SDS gels (acrylamide/methylenebisacrylamide ratio was 40:1) prepared according to Bishop et al. (2) and subjected to electrophoresis for 2.75 h at 90 V. Gels were cut into 2-mm slices and allowed to swell overnight at 37 C in 0.6 ml of a 1:1 mixture of Soluene 100 (Packard Instrument Co., Downers Grove, Ill.) and toluene. Ten milliliters of toluene-based scintillation liquid was added, and the radioactivity was measured in a liquid scintillation spectrometer. Either a [^{3}H]tRNA or a [^{14}C]-tRNA sample was run in a parallel gel as a marker.

Size distribution of polyadenylated RNA. The size distribution of RNA was determined by electrophoresis in polyacrylamide gels made with 2.4% acrylamide and either 0.5 or 0.7% agarose (SeaKem). The gels were prepared and used according to Bishop et al. (2) but with the addition of agarose. The gels were cut into 1- or 2-mm slices, and radioactivity was measured as described above. The molecular weight of RNA was calculated from the linear relationship between the log of molecular weight and electrophoretic mobility. HEp-2 cell ribosomal RNAs with assigned molecular weights of 0.68×10^6 and 1.70×10^6 were used as markers.

Analysis of poly(A) chain length. RNA bound and eluted from poly(U) columns was digested with RNases A and T_1 and subjected to electrophoresis through 10% acrylamide-0.2% SDS gels as described above. Gel slices from the poly(A) peak regions were each chopped into small pieces; the poly(A) tracts were eluted from each gel slice by shaking first for several hours at 37 C in 0.75 ml of 0.3 M NaCl and then twice for 2 h at 37 C with 0.5 ml of water. The combined extracts were filtered through GF/C glass-fiber filters. After addition of 75 μg of yeast RNA, the poly(A) was precipitated at 0 C with 10% trichloroacetic acid. The precipitates were sedimented by centrifugation (15 min, 12,000 × g), washed once with 80% ethanol in 0.1 M Tris (pH 9) and once with 80% ethanol in 50 mM Tris (pH 7.4), centrifuged, drained, and dried. The RNA was dissolved in 25 μl of 0.3 M NaOH, hydrolyzed for 16 h at 37 C, neutralized with 1 μl of 5 N H_3PO_4 and 1 μl of 6 N HCl, and spotted in 2-cm streaks on PEI cellulose thin-layer chromatography plates (Brinkmann Instruments, Inc., Westbury, N.Y.). The plates were developed for 1.75 h with 0.07 M sodium phosphate (pH 3.4) and then dried at 60 C. The areas corresponding to adenosine and 3'-AMP were cut out, and radioactivity was determined in toluene scintillation fluid. The number, average-chain length, was calculated from the ratio of radioactivity in 3'-AMP to adenosine.

Base composition of poly(A) tracts. ^{32}P-labeled RNA was fractionated by poly(U) chromatography, precipitated twice with ethanol in the presence of 100 μg of carrier yeast RNA, dissolved in 0.5 ml of 10 mM Tris (pH 7.4)-1 mM $MgCl_2$, and digested with DNase (Worthington Biochemical Corp., RNase free, iodoacetate treated) at 20 μg/ml for 30 min at 37 C. After the addition of EDTA to give a final concentration at 2 mM and of 0.5 ml of ET buffer, the samples were heated to 90 C for 3 min and chilled. NaCl and RNases A and T_1 were added to give final concentrations of 0.2 M, 10 μg/ml, and 20 U/ml, respectively, and the RNA was digested for 75 min at 37 C. After the addition of 75 μg of carrier yeast RNA, the poly(A) was precipitated with ethanol, dissolved in 1 ml of ETS buffer, heated 4 min at 75 C, bound to poly(U), and eluted with 60% formamide as described above. After ethanol precipitation, the poly(A) tracts were subjected to electrophoresis, eluted from gel slices, and reprecipitated as described above. Poly(A) tracts were dissolved in 20 to 25 μl of 0.3 N NaOH, hydrolyzed 16 h at 37 C, spotted on Whatman 3MM paper, and subjected to electrophoresis for 3.5 h at 55 V/cm in pyridine-acetate buffer, pH 3.5 (8% acetate, 0.8% pyridine). Radioactivity in the nucleotide spots was measured in toluene-based scintillation liquid.

Viral DNA. Viral DNA was extracted by detergent lysis of nucleocapsids and checked for contamination with host cell DNA by isopycnic banding in the Beckman model E centrifuge. After pancreatic RNase and Pronase digestion and phenol extraction, the DNA was labeled in vitro with [^{3}H]dTTP by repair synthesis using *Escherichia coli* DNA polymerase I as previously described (11), reextracted with phenol, and dialyzed against 40 mM sodium phosphate buffer, pH 6.8. A constant fraction (5%) of denatured DNA that had been labeled in vitro was resistant to digestion by *Neurospora crassa* nuclease, and all data were adjusted to take into account this resistant fraction.

DNA-RNA hybridization. The hybridization tests were done in liquid with trace amounts of in vitro-labeled DNA and excess unlabeled RNA, such that the hybridization reaction was driven by RNA (6). Details of the hybridization reaction were the same as described by Kozak and Roizman (11). Specifically, denatured labeled DNA and excess unlabeled RNA in 0.23 M NaCl and 0.04 M sodium phosphate buffer (pH 6.8) were sealed in a glass capillary pipette and incubated at 75 C for various lengths of time. After the incubation, the fraction of DNA converted to hybrid form was assayed by digesting with the single-strand-specific nuclease purified from *N. crassa*. DNA reassociation during the hybridization, as measured by incubating viral DNA in the presence of uninfected HEp-2 RNA, was determined for each incubation time period, and the value was subtracted from each experimental point. In most instances the extent of DNA reassociation did not exceed 5%.

RESULTS

Presence of poly(A) in RNA that does not bind to nitrocellulose filters. In previous studies (1, 19) on mRNA in cells infected with HSV-1, we used the nitrocellulose filter binding technique (3) for the isolation of poly(A) RNA. Adenylated viral RNA selected by this method was transcribed from a limited region of the HSV-1 DNA (19). In light of the possibility that nitrocellulose filters select only a portion of the adenylated RNA species (7), we analyzed the poly(A) tract distribution in RNA fractionated by this method. Cytoplasmic RNA extracted from cells labeled with [^{3}H]adenosine was passed through nitrocellulose filters to yield bound and flow-through fractions. Each fraction was examined for the presence of poly(A) tracts by digesting with RNases T_1 and A and subsequent electrophoresis of the trichloroacetic acid-insoluble, RNase-resistant RNA.

As shown in Fig. 1A, the poly(A) tracts present in RNA that bound to the filters were relatively homogeneous in size and had a mean electrophoretic mobility about one-half that of tRNA. These were previously described by Bachenheimer and Roizman (1) and were estimated to be about 165 bases long. Figure 1b demonstrates that RNA which does not bind to the filters contains several size classes of poly(A) tracts. Most of these showed a greater mobility than those present in the bound RNA, with one portion migrating faster than the bromophenol blue marker.

Isolation of polyadenylated RNA by binding to poly(U) columns. Polyadenylated RNAs were further analyzed by affinity chromatography on columns of poly(U) immobilized on glass-fiber filters (18). Data on the RNA-binding properties of these columns are given in Table 1. Each column quantitatively bound 50 μg and retained up to 168 μg of commerical [^{3}H]poly(A). Experiments with RNA from infected cells showed that the same fraction (10%) of adenosine-labeled RNA was found irrespective of the amount (0.1 to 1.0 mg) of RNA applied to the columns. The bound RNA contained essentially all of the RNase-resistant material, indicating that the columns bound virtually all of the polyadenylated RNA present in the cytoplasm.

RNA that did not bind to nitrocellulose filters in the previous experiment was passed through a poly(U) column. The bound RNA was eluted with 60% formamide, precipitated, and analyzed for poly(A) tracts by RNase digestion followed by gel electrophoresis. Figure 1C shows that this RNA contained all of the size classes of poly(A) found in the nitrocellulose membrane flow-through material. As shown in Fig. 1D, total cytoplasmic RNA that bound to a poly(U) column contained all size classes of poly(A) tracts.

Size distribution of RNA fractionated by nitrocellulose filters and poly(U) columns. RNA fractionated by the two methods was analyzed by polyacrylamide gel electrophoresis. An electropherogram of RNA not retained by poly(U) is shown in Fig. 2A. This and other electrophoretic analyses for shorter time periods have shown that this RNA consisted almost entirely of 28, 18, 5, and 4S RNA. RNA bound to poly(U) (Fig. 2B) had a heterogeneous distribution characteristic of mRNA; none of the structural RNA species found in the flow-through fraction was detected in the bound RNA. Analyses of RNA fractionated by binding to nitrocellulose filters (Fig. 2C) showed that the unbound RNA consisted of structural RNA species and of RNA with a heterogeneous size distribution ranging from 10 to 28S. RNA retained by the filters showed a size distribution similar to RNA selected by the poly(U) affinity columns but contained, in addition, two prominent peaks of RNA with molecular weights of about 0.92×10^6 and 2.7×10^6, respectively (Fig. 2D). Although the origin of these species is unknown, they might arise from abortive rRNA processing in infected cells (21). These results show that the poly(U) glass-fiber filter column efficiently separates messenger-like RNA from structural RNA components.

Fractionation of polyadenylated RNA by poly(U) affinity chromatography. Inasmuch as RNA binding to poly(U) columns contains a wide range of classes of poly(A), it should be possible to fractionate these molecules according to their differential affinity for poly(U). To test this possibility, cytoplasmic RNA labeled from 2 to 8 h postinfection with [^{3}H]adenosine was adsorbed to a poly(U) column. The column was thoroughly washed with NETS buffer, and the bound RNA was eluted with ETS buffer, followed by a linear concentration gradient of formamide. The elution profile, displayed in Fig. 3, demonstrates the existence of RNA classes differing in affinity for poly(U).

The column fractions denoted as a, b, and c were pooled and analyzed by RNase digestion for poly(A) content. The RNase resistance of these fractions, 8.7, 19.8, and 34.7%, respectively, indicated a separation according to poly(A) content. Gel electropherograms of the released poly(A) tracts are shown in Fig. 4. The weakly bound RNA eluted by the low-ionic-strength buffer (fraction a) contained short

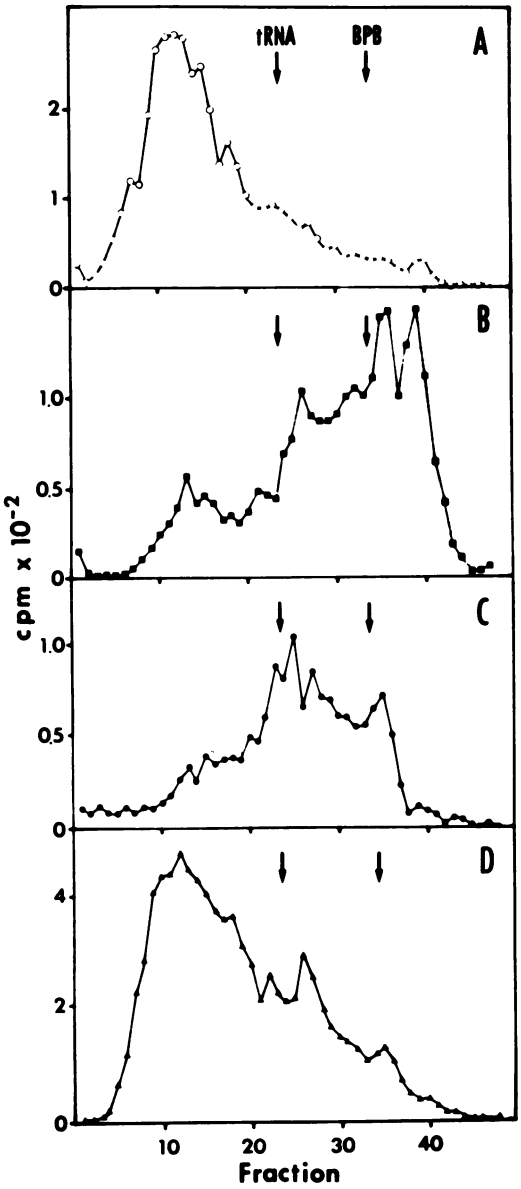

FIG. 1. *Poly(A) tract analysis of RNA fractionated by nitrocellulose filters and poly(U) columns. Cytoplasmic RNA was isolated (without DNase treatment) from cells labeled with [³H]adenosine from 2 to 8 h postinfection. Part was passed through nitrocellulose filters to separate it into bound and flow-through fractions, which were precipitated with ethanol. Two-thirds of the flow-through fraction was dissolved in NETS buffer and bound to a poly(U) column. The bound RNA was eluted with 60% formamide and precipitated with ethanol. The RNA samples were dissolved in 0.2 ml of 1 mM $MgCl_2$-10 mM Tris-hydrochloride, pH 7.5, digested for 20 min at 37 C with DNase I (RNase free) at 25 μg/ml, and then mixed with 10 μl of 0.1 M EDTA and 255 μl of 10 mM Tris-hydrochloride, pH 7.5. Poly(A) tracts were released by digestion with RNases A and T_1 and analyzed by electrophoresis through 10% polyacrylamide-SDS gels. Procedures are detailed in the text. Poly(A) tracts analyzed were from (A) nitrocellulose filter-bound RNA, (B) nitrocellulose filter flow-through RNA, (C) nitrocellulose flow-through RNA bound to Poly(U), and (D) whole cytoplasmic RNA bound to Poly(U). In this and subsequent figures, the direction of electrophoresis is from left to right. BPB, Bromophenol blue dye.*

poly(A) tracts with electrophoretic mobility greater than that of bromophenol blue (Fig. 4A). RNA eluted with intermediate formamide concentrations (fraction b) contained poly(A) tracts of intermediate size whose main peak migrated with a slightly greater mobility than that of tRNA (Fig. 4B). The RNA most avidly bound by the affinity column (fraction c) contained mainly the large poly(A) sequences of lower mobility than tRNA (Fig. 4C). This poly(A) class had a size distribution similar to that found in RNA binding to nitrocellulose filters (see Fig. 1A).

Chain length and heterogeneity of poly(A) tracts. Assuming that the poly(A) in all classes is located at the 3′ position on the RNA molecules, the numbers, average poly(A) chain lengths, can then be determined from the ratio of [³H]adenosine found internally to that found at the 3′ end of the poly(A) chains. The results of such analyses on the poly(A) classes found in infected-cell cytoplasmic RNA (Table 2) show that the lengths of the medium and large tracts are approximately what would be predicted from their electrophoretic mobilities relative to the tRNA marker. The size calculated for the short poly(A), however, is slightly larger than expected from its mobility. From the mobility of the short poly(A) relative to the markers and from a comparison with reported poly(A) chains of similar mobility (9, 14), we would estimate its size to be closer to 25 nucleotides. Therefore, in the subsequent discussion, the three classes of poly(A) tracts will be designated as $poly(A)_{30}$, $(A)_{50}$, and $(A)_{155}$. The fraction of adenosine liberated from each size class of poly(A) indicates that the poly(A) chains of all the adenylated RNA classes are primarily located at the 3′ end of the molecules. These results further demonstrate that the cytoplasmic RNA of HSV-1-infected cells can be separated into three major classes according to poly(A) chain length.

As summarized in Table 3, the base composition of the three classes of poly(A) tracts is in accord with the data that the RNase-resistant chains designated in this paper as $poly(A)_{50}$ and $poly(A)_{155}$, from the nucleus and cytoplasm,

TABLE 1. *Binding of RNA to poly(U)-GF/C columns*[a]

RNA applied	% RNA bound		% RNA not bound	
	Total	RNase resistant	Total	RNase resistant
[^{3}H]poly(A), 50 μg	97.5		2.4	
[^{3}H]poly(A), 250 μg	67.2		32.8	
[^{3}H]A cytoplasmic RNA, 100 μg	9.6	36.7	90.1	0.78
[^{3}H]A cytoplasmic RNA, 1.1 mg	10.6	25.5	82.3	0.87

[a] Columns, each containing two GF/C glass-fiber filters, were loaded with a total of 1.5 mg of poly(U) and were then prepared and used as described in the text. Bound RNA was eluted with 60% (vol/vol) formamide in ETS buffer. The RNA was extracted from the cytoplasm of HEp-2 cells labeled with [^{3}H]adenosine from 4 to 8 h after infection with HSV-1.

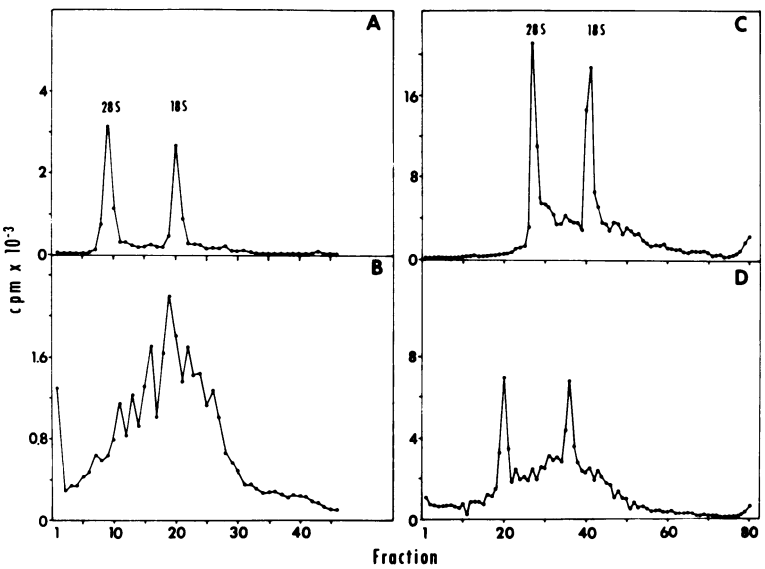

FIG. 2. *Polyacrylamide gel electrophoresis of RNA fractionated by nitrocellulose filters and poly(U) columns. Cytoplasmic RNA from cells labeled with [^{3}H]adenosine from 2 to 8 h postinfection was separated by the nitrocellulose filter or poly(U) column methods into bound and unbound fractions. RNA samples (1.4 × 10^4 to 20 × 10^4 counts/min) in 50 μl of sample buffer (5 mM Tris-acetate[pH 7.4]-5 mM EDTA-0.2% SDS) were heated for 5 min at 65 C and chilled. Then 10 μl of 50% (wt/vol) sucrose in sample buffer was added and the samples were layered onto polyacrylamide gels (0.6 by 10 cm), made with 2.4% acrylamide, 0.12% bis-acrylamide, 0.5% agarose, and 0.2% SDS. Electrophoresis was at 90 V for 2.5 h (samples A and B) or 3.5 h (samples C and D). Gels were cut into 1.25-mm slices, and the radioactivity was measured as described in Materials and Methods. (A) Poly(U) flow-through RNA; (B) poly(U)-bound RNA; (C) nitrocellulose filter flow-through RNA; (D) nitrocellulose filter-bound RNA.*

were essentially pure poly(A). The poly(A)$_{30}$ tracts consisted mainly (86 to 87%) of adenosine but also contained significant amounts of cytosine and guanine. This cannot be accounted for by contamination with oligonucleotides, because the tracts were repurified on poly(U) prior to these analyses.

Comparison of poly(A) RNA labeled for different intervals. The different size classes of poly(A) in the cytoplasmic RNA of infected cells could be explained by two possible precursor-product relationships: (i) elongation of short poly(A) chains and/or (ii) shortening of long poly(A) chains by cleavage or degradation. We therefore compared the poly(U) elution profiles of RNA labeled for 6 h with the profiles of RNA labeled for a period of 10 or 45 min at 5 h postinfection. Figure 5A and B show that the poly(U) elution profile of RNA labeled during a 10-min pulse was not significantly different from that of the 6-h labeling period shown in Fig. 3. Analysis of poly(A) tracts from the 10-min-labeled RNA (Fig. 4D–F) showed that it contained the same three major poly(A) size

classes as did the RNA labeled for 6 h. The results, summarized in Table 4, show that the distribution of radioactivity among the small, medium, and large poly(A) RNA classes [poly(U) fractions a, b, and c] was not appreciably affected by the duration of time of labeling within the ranges tested and thus do not support the possible precursor-product relationships listed above.

Distribution of poly(A) tracts in polyribosomal and free cytoplasmic RNA. The cytoplasmic extract of cells labeled from 2 to 8 h postinfection was fractionated into polyribosomal and free cytoplasmic fractions. RNA extracted from each fraction was then chromatographed on poly(U) columns. The results (Table 4b) indicate that 68% of the adenosine-labeled polyribosomal RNA fractionated with the $poly(A)_{155}$ class, i.e., that the RNA present on polyribosomes consisted mainly of molecules terminated by the long poly(A) tracts. Conversely, the free cytoplasmic RNA was enriched for RNA sequences containing shorter poly(A) tracts.

Figure 6 shows the poly(U) column elution profile of labeled RNA isolated from the nuclei of infected cells. The results and subsequent poly(A) tract analyses indicated that the nuclear RNA fractions contained the same three poly(A) size classes as those found in the cytoplasm. However, the nuclei contained a larger fraction of RNA containing the $poly(A)_{30}$ chains. Moreover, in both a 10-min and 6-h labeling period, more than 40% of the total [^{3}H]adenosine label in poly(A) RNA was found incorporated into RNA containing small poly(A) tracts (Table 4c).

Poly(A) tracts in high-molecular-weight nuclear RNA. Previous data from this laboratory (22) suggested that high-molecular-weight nuclear RNA could be a precursor to viral mRNA. More recently, Jacquemont and Roizman (10) showed that infected-cell nuclear RNA sedimenting faster than 45S contains sequences complementary to more than 50% of HSV DNA. In all experiments designed to measure the extent of adenylation of this RNA, cells were labeled with [^{3}H]adenosine from 2 to 8 h postinfection. Nuclei were isolated, and the RNA was extracted and centrifuged through a sucrose gradient; RNA sedimenting faster than 45 S was pooled and analyzed for poly(A) tracts. Figure 7 shows that this RNA contained almost exclusively the short poly(A) sequences.

Size distribution of RNA in the three adenylated RNA classes. To investigate whether the different polyadenylated classes contain different size classes of RNA, we analyzed RNA

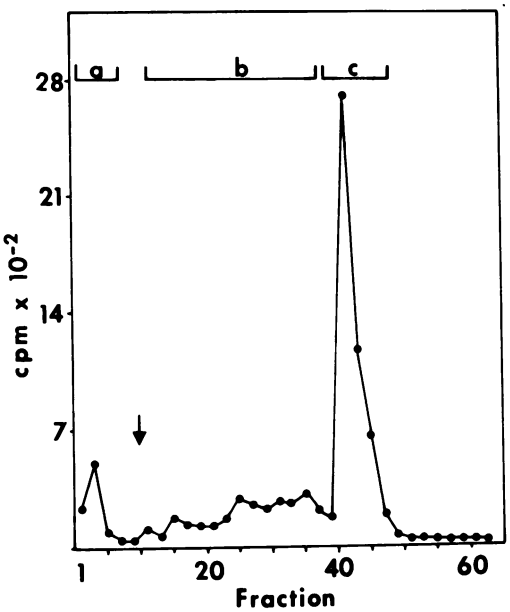

FIG. 3. *Poly(U) affinity chromatography of cytoplasmic RNA from HSV-infected cells. Cytoplasmic RNA from cells labeled with [^{3}H]adenosine from 2 to 8 h postinfection was fractionated on poly(U)-GF/C columns as described in the text. Arrow points to the start of the formamide gradient. Samples (25 μl) of the column fractions were dried on GF/C filters, and radioactivity was measured in toluene scintillation liquid. Fractions denoted by a, b, and c were pooled for subsequent analysis.*

from each poly(A) class by electrophoresis on 2.4% polyacrylamide gels. As shown in Fig. 8, all three populations showed a broad heterogeneous distribution. However, we have noted in several experiments that RNAs characterized by the shorter poly(A) tracts exhibited a lower average electrophoretic mobility. Assuming that mobility is proportional to the log molecular weight, the data suggest that the $poly(A)_{30}$ RNAs have about a 1.5-fold greater average molecular weight than the other polyadenylated fractions.

Distribution of polyadenylated RNA in uninfected cells. The elution profiles of uninfected HEp-2 cell cytoplasmic and nuclear RNAs from poly(U) columns are shown in Fig. 9. The percentage of adenosine-labeled RNA in the three polyadenylated classes is given in Table 4. Further analysis has shown that these fractions contain the same poly(A) size classes found in infected cells (data not shown). Compared with infected cells, the uninfected nuclei appear to contain a lower percentage of RNA containing the large poly(A) class. However,

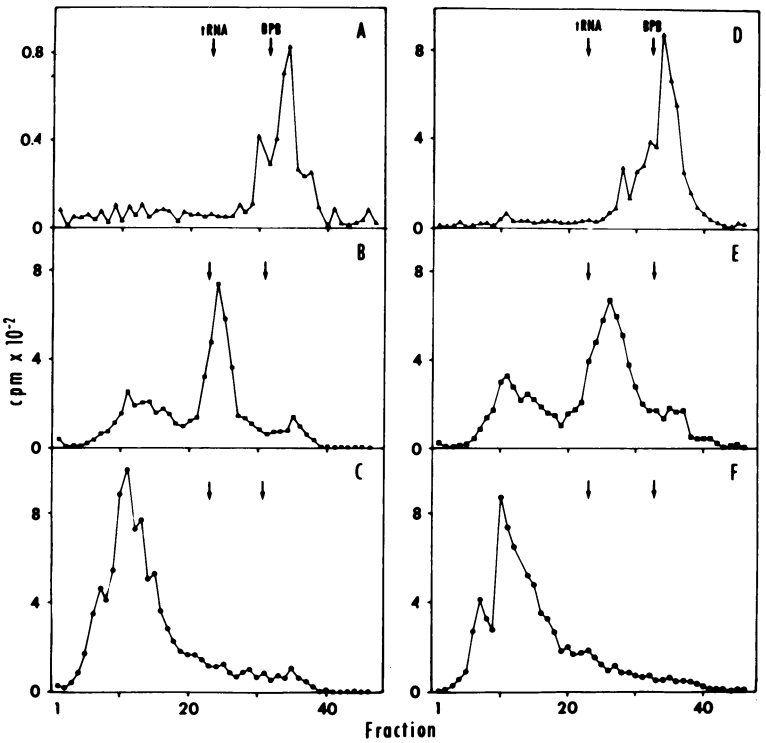

FIG. 4. *Poly(A) tract analysis of RNA fractionated by poly(U) affinity chromatography. Pooled RNA fractions a, b, and c from the poly(U) chromatography runs shown in Fig. 3 and 5A were digested with RNases A and T_1, precipitated with trichloroacetic acid, and subjected to electrophoresis for 2.75 h at 90 V through polyacrylamide gels (10% acrylamide, 0.25% bisacrylamide, 0.2% SDS). [^{3}H]tRNA from HEp-2 cells was run in a parallel gel. (A) (B) and (C) poly(A) tracts from poly(U) column fractions a, b, and c, respectively, of 6-h-labeled RNA (Fig. 3); (D) (E) and (F) poly(A) tracts from poly(U) fractions a, b, and c, respectively, of RNA labeled for 10 min (Fig. 5A).*

these data indicate that the general pattern of polyadenylation found in HSV-infected cells also holds for uninfected HEp-2 cells.

Complexity of adenylated viral RNA. The preceding sections dealt with the properties of total infected- and uninfected-cell polyadenylated RNA. Experiments described in this section deal specifically with virus-specific RNA sequences and were designed to determine whether (i) viral RNA sequences differ in the size of the poly(A) chain and (ii) whether the length of the poly(A) chain is genetically determined by the virus.

Two series of experiments were done. In the first, cytoplasmic RNA from 8-h infected cells was fractionated on nitrocelluose membranes or by chromatography on immobilized poly(U); these RNAs were then hybridized in liquid to labeled viral DNA as described in Materials and Methods. The results of these analyses (Table 5) showed the following: unfractionated cytoplasmic RNA hybridized to 43% of HSV-1 DNA; the RNA fractions bound by nitrocellulose filters and by immobilized poly(U) were complementary to 21 and 36 to 40% of viral DNA, respectively.

The data summarized in Table 5 agree with our previous observations concerning the total amount of viral DNA serving as template for the RNA accumulating in the cytoplasm at 8 h postinfection (11, 15) and the fraction bound to nitrocellulose filters (19). Moreover, in light of the observation that nuclei of 8-h infected cells contain transcripts arising from at least 60% of viral DNA (11, 15; N. Frenkel and B. Roizman, manuscript in preparation), whereas the cytoplasm contains non-self-complementary transcripts arising from 43% of the DNA, these data suggest that most, if not all, species of viral RNA accumulating in the cytoplasm are adenylated. At first glance, these data suggest that the RNA sequences arising from 21% of the viral DNA are the sole recipients of long poly(A) chains and are therefore capable of binding to nitrocellulose filters, whereas the remainder of the RNA has shorter poly(A)

tracts and binds to poly(U) but not to nitrocellulose filters. To test this hypothesis, a second series of experiments was designed to measure the sequence complexity of each of the three adenylated RNA classes isolated by poly(U) chromatography. Two experiments were done. In the first, unlabeled RNA from the cytoplasm of 8-h infected cells was chromatographed on a poly(U) column. A companion column of the same dimensions was loaded with an equivalent amount of unlabeled cytoplasmic RNA mixed with a small amount of [^{3}H]adenosine-labeled cytoplasmic RNA extracted from 8-h infected cells. The RNA from each column was then eluted with a linear formamide gradient. The elution of the labeled RNA from the second column was monitored by radioactivity and served as a check on the elution of the unlabeled RNA from the first column whose elution profile could be monitored only by measuring absorbance at 270 nm (Fig. 10). To ensure that no residual viral DNA eluted with the RNA, the fractions containing poly(A)$_{30}$, poly(A)$_{50}$, and poly(A)$_{155}$ RNA classes were pooled, ethanol precipitated, digested with DNase I, phenol extracted, and exhaustively dialyzed against 0.04 M sodium phosphate buffer, pH 6.8. Each class of adenylated viral RNA was then hybridized to trace amounts of labeled viral DNA as described in Materials and Methods. In the second, trace amounts of [^{32}P]RNA from infected cells were added to unlabeled RNA from 8-h infected cells to aid in monitoring the elution. The results of these hybridizations with RNA eluted from the first experiment (Fig. 11 a-c) show the following. (i) Each of the polyadenylated RNA fractions contains viral sequences complementary to 35 to 40% of viral DNA. Inasmuch as unfractionated cytoplasmic RNA hybridizes to 43% of HSV DNA, this indicates that all three poly(A) RNA fractions contain essentially the same viral RNA sequences. (ii) From analyses of the kinetics of the hybridization (6), we estimated that viral sequences are most abundant in poly(A)$_{155}$ RNA and least abundant in poly(A)$_{30}$ RNA. The results of the second experiment were essentially identical.

TABLE 2. *Determination of poly(A) chain lengths*

Determinant[a]	Poly(U) column fraction		
	a	b	c
Radioactivity in Ap	18,704	35,760	62,022
Radioactivity in A	498	714	412
No. avg chain length	38	50	151

[a] Radioactivity represents net ^{3}H counts per 20 min. Adenosine (A) and 3'-AMP (Ap) were separated by thin-layer chromatography as described in the text.

TABLE 3. *Base composition of poly(A) tracts*[a]

Source of poly(A)	% ^{32}P in			
	5'-UMP	5'-GMP	5'-AMP	5'-CMP
Cytoplasmic				
poly(A)$_{30}$	0	12.7	87.3	0
poly(A)$_{50}$	0.4	2.1	95.5	2.0
poly(A)$_{155}$	0.1	1.2	97.3	1.4
Nuclear				
poly(A)$_{30}$	0.4	4.3	86.2	9.1
poly(A)$_{50}$	0.7	1.8	95.0	2.5
poly(A)$_{155}$	0.1	1.1	98.0	0.8

[a] [^{32}P]RNA from infected cells was fractionated on poly(U) columns. Poly(A) tracts of three size classes were isolated and subjected to base composition analysis as detailed in Materials and Methods.

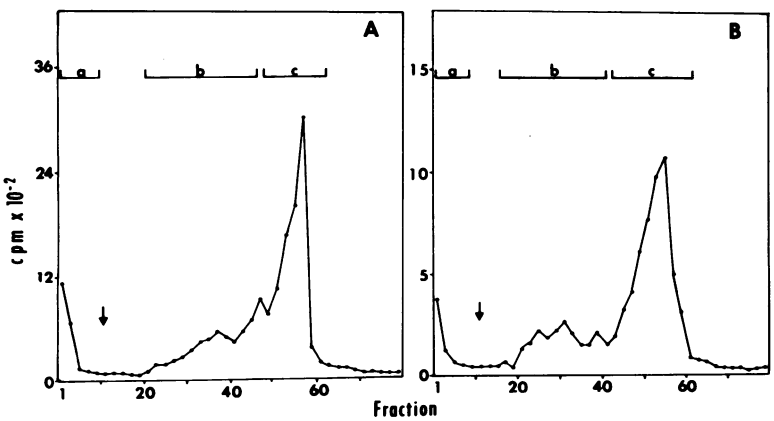

FIG. 5. *Poly(U) affinity chromatography of short-pulse-labeled RNA from HSV-infected cells. Cytoplasmic RNA was fractionated on poly(U) columns as described in the legend to Fig. 3 and the text. Cells were labeled with [^{3}H]adenosine for (A) 10 min (5.0 to 5.17 h postinfection) and (B) 45 min (4.0 to 4.75 h postinfection).*

TABLE 4. *Distribution of poly(A) adenylated RNA eluted from poly(U)-GF/C columns*[a]

Source of RNA	Duration of labeling	Time of labeling postinfection (h)	% Adenylated [^{3}H]A-RNA eluted from poly(U)		
			a	b	c
Cytoplasm[b]	10 min	5-5.2	12.2	31.3	56.5
Cytoplasm	45 min	4-4.75	8.8	23.3	67.9
Cytoplasm	6 h	2-8	9.6	25.0	65.4
Polyribosomes	6 h	2-8	8.1	24.3	67.6
Free cytoplasmic fraction	6 h	2-8	20.4	31.7	47.8
Nuclei[c]	10 min	5-5.2	41.5	20.2	38.3
Nuclei	6 h	2-8	49.3	19.9	30.6
Uninfected cell cytoplasm	6 h		10.2	29.4	60.4
Uninfected cell nuclei	6 h		46.2	39.0	17.8

[a] [^{3}H]adenosine-labeled RNA was fractionated into three polyadenylated classes (a, b, and c) by poly(U) affinity chromatography as described in the text. The table shows the distribution of polyadenylated RNA normalized to 100%.

[b] The [^{3}H]adenosine-labeled RNA not binding to poly(U) in the 10-min and 6-h labeling samples were, respectively, 79, 85, and 88% of total radioactivity in RNA.

[c] The [^{3}H]adenosine-labeled RNA not binding to poly(U) in the 10-min and 6-h labeled samples was, respectively, 90 and 93% of total [^{3}H]adenosine.

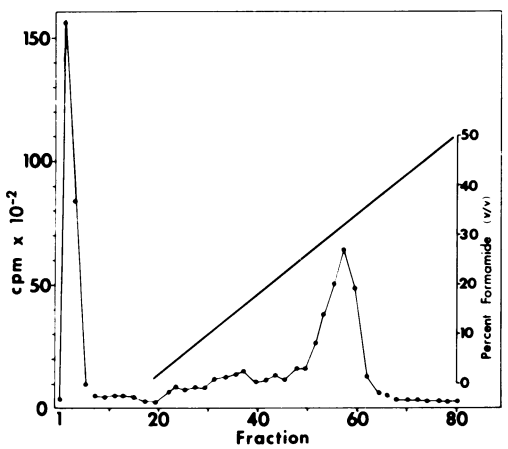

FIG. 6. *Poly(U) affinity chromatography of nuclear RNA from HSV-infected cells. Nuclear RNA from cells labeled with [^{3}H]adenosine for 10 min (from 5 to 5.17 h postinfection) was analyzed by chromatography on a poly(U)-GF/C column as described in the legend to Fig. 3 and in the text. Figure also shows the elution of RNA as a function of formamide gradient in the elution buffer.*

Selective partitioning of poly(A)$_{155}$ RNA. Viral polyadenylated RNAs present in the cytoplasm late in infection form two populations. One population selectively binds to nitrocellulose filters and is homologous to 21% of viral DNA; this population is a component of a larger population that can be isolated by poly(U) chromatography and is transcribed from 40% of the viral DNA. Therefore, the length of the poly(A) chain cannot be the only factor responsible for binding to nitrocellulose membranes. The following experiment was designed to explore the contribution of secondary structure in the selective binding of the poly(A)$_{155}$ RNA class to nitrocellulose filters. Poly(A)$_{155}$ RNA isolated by formamide gradient elution from a poly(U) column was concentrated by ethanol precipitation and heat denatured prior to filtration through a nitrocellulose membrane. Absorbance measurements indicated that more than 90% of the poly(A)$_{155}$ RNA was bound to the filter. Hybridization of this RNA to labeled viral DNA showed that it was complementary to about 40% of viral DNA, whereas the undenatured, unfractionated RNA bound to nitrocellulose filters hybridized to only 21% of viral DNA (Fig. 12).

DISCUSSION

Several observations presented in this paper merit discussion. These concern the apparent segregation of RNA into classes differing in size of the poly(A) chain and some aspects of poly(A) metabolism in both uninfected and HSV-infected cells.

Fractionation of the RNA according to the size of the poly(A) chains. The salient features of the data are as follows. (i) Nitrocellulose filters bound predominantly to RNA containing long poly(A) chains. This observation is not novel (7), but we shall return to it later in the text in connection with the observation that not

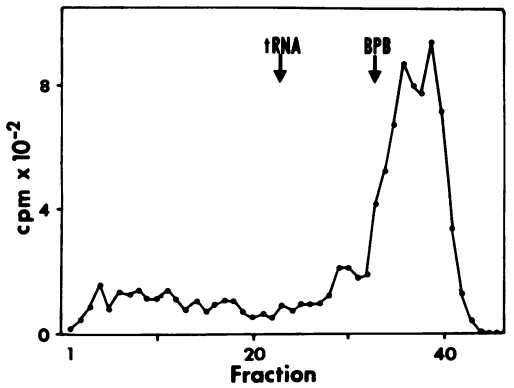

FIG. 7. *Poly(A) tract analysis of high-molecular-weight nuclear RNA. Nuclear RNA from cells labeled with [³H]adenosine from 2 to 8 h postinfection was centrifuged in an SW27 rotor for 12 h at 28,000 rpm through a linear gradient at 20 to 45% (wt/vol) sucrose in 0.1 M NaCl-10 mM Tris-hydrochloride (pH 7.5)-1mM EDTA-0.5% SDS-0.5% Sarkosyl. RNA sedimenting at >45S was concentrated by vacuum dialysis, precipitated two times with ethanol, and resuspended in reticulocyte standard buffer. After treatment with DNase I, extraction with phenol and chloroform-isoamyl alcohol (described in text), and precipitation with ethanol, a sample was treated with RNases A and T_1. The poly(A) tracts were analyzed by electrophoresis through 10% polyacrylamide gels.*

all RNA with long poly(A) chains bound to nitrocellulose filters.

(ii) Immobilized poly(U) bound RNA with poly(A) chains ranging in size from less than 30 to more than 155 nucleotides in length.

(iii) The chain length of poly(A) linked to RNA was nonrandomly distributed. Thus, analyses of the RNA eluted with a linear formamide gradient from poly(U) columns showed that it contained three classes differing in the length of the poly(A) chain. Both the electrophoretic mobilities and chemical analyses of the poly(A) chains obtained by RNase digestion of the RNA showed that the average composition of the poly(A) chains in the three RNA classes is $poly(A)_{30}$, $poly(A)_{50}$, and $poly(A)_{155}$, respectively. The RNA bound to the nitrocellulose filters was a subset of the $poly(A)_{155}$ RNA class.

(iv) The distribution of the three classes of poly(A) RNA was nonrandom. The $poly(A)_{155}$ class was the most abundant polyadenylated RNA in both cytoplasm and polyribosomes. Conversely, the free cytoplasmic poly(A) RNA was enriched in the $poly(A)_{30}$ and $poly(A)_{50}$ classes. The $poly(A)_{30}$ class was the most abundant adenylated RNA in the nucleus and was the only polyadenylated species present in high-molecular-weight (>45S) nuclear RNA. However, in view of the observations that the distribution of poly(A) sizes in infected and uninfected cells is similar, the questions arise as to whether viral RNA sequences are similarly distributed and whether the distribution of the

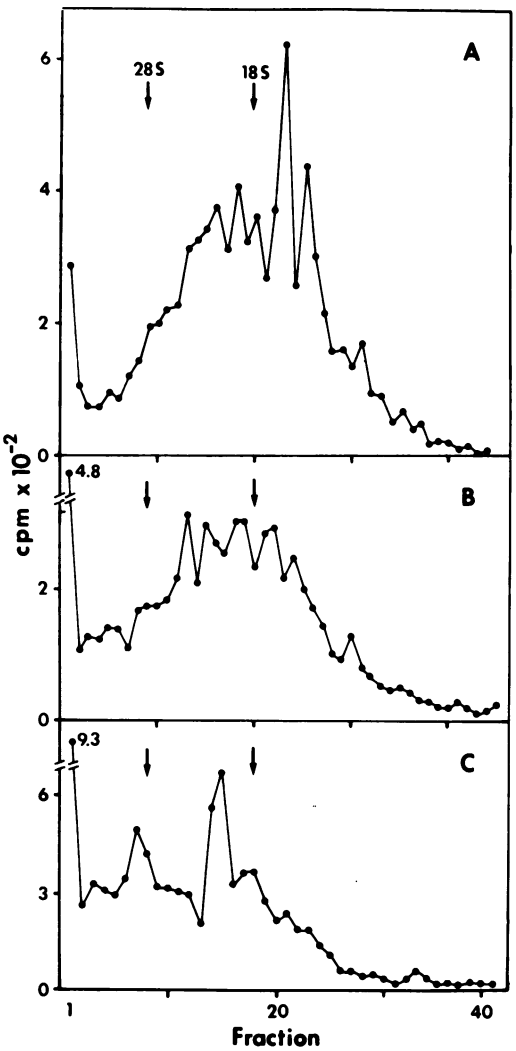

FIG. 8. *Size distribution of poly(A) RNA classes. Cytoplasmic RNA from cells labeled from 2 to 8 h postinfection was eluted from poly(U) columns into three fractions, a, b, and c. The RNA in each fraction was subjected to electrophoresis for 2.5 h at 90 V through polyacrylamide gels (2.4% acrylamide, 0.75% agarose). A poly(U) flow-through fraction, containing rRNA, was run in a parallel gel as marker. (A) $Poly(A)_{155}$ RNA; (B) $poly(A)_{50}$ RNA; (C) $poly(A)_{30}$ RNA.*

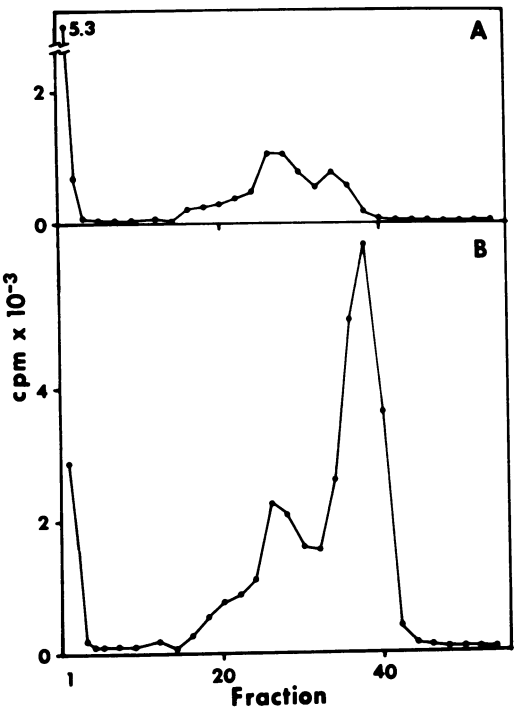

FIG. 9. *Poly(U) chromatography of RNA from uninfected HEp-2 cells. Mock-infected HEp-2 cells were labeled for 6 h with [³H]adenosine. RNAs were isolated and subjected to affinity chromatography on poly(U)-GF/C columns, as described in the text. (A) nuclear RNA; (B) cytoplasmic RNA.*

poly(A) chain lengths reflects metabolism of poly(A).

Fractionation and properties of adenylated cytoplasmic viral RNA sequences. Analyses of the hybridization of RNA eluted from immobilized poly(U) columns and from nitrocellulose filters made apparent several interesting features of viral RNA.

(i) The 8-h cytoplasmic RNA eluted from poly(U) columns hybridized with 36 to 40% of viral DNA. Inasmuch as the total cytoplasmic RNA hybridized with 43% of viral DNA, we concluded that most, if not all, viral RNA sequences in the cytoplasm are adenylated.

(ii) The RNA fractionated according to the length of the poly(A) chain contained viral RNA sequences. Based on their abundance, the bulk of viral RNA sequences are in the class containing the poly(A)$_{155}$ chains.

(iii) In accord with the results published previously (19), the RNA bound and eluted from nitrocellulose filters hybridized with only 21% of viral DNA. These results are thus in striking contrast with the observations that RNA in the poly(A)$_{155}$ class as eluted from the poly(U) column hybridize with approximately 40% of viral DNA. Implicit in these results is the conclusion that the presence of poly(A) is not, per se, the

TABLE 5. *Hybridization of adenylated RNA to HSV-1 DNA*

Source of RNA[a]	R_ot[b]	% DNA hybridized[c]
Nitrocellulose bound	50	21
Poly(U) bound	40	36-40
Whole cytoplasm	300	43

[a] Nitrocellulose-bound RNA is polyribosomal RNA extracted from HSV-1(F)-infected HEp-2 cells 8 h postinfection, selected for polyadenylated species by nitrocellulose filter binding, and then eluted from filters as described in Materials and Methods. Poly(U)-bound RNA is total cytoplasmic RNA extracted from HSV-1(F)-infected HEp-2 cells 8 h postinfection and selected for polyadenylated species by affinity chromatography on poly(U) columns as described in Materials and Methods. Whole cytoplasm RNA is total cytoplasmic RNA extracted from HSV-1(F)-infected HEp-2 cells 8 h postinfection and without subsequent selection for polyadenylated species. This preparation of RNA, in contrast to the others, contains ribosomal RNA species.

[b] R_ot = moles of nucleotides × seconds × liter^{-1}. The R_ot values chosen in each case yield the maximum amount of hybridization detected with each RNA species.

[c] These values have been corrected for (i) the fraction of denatured DNA resistant to *Neurospora crassa* nuclease digestion and (ii) DNA reassociation.

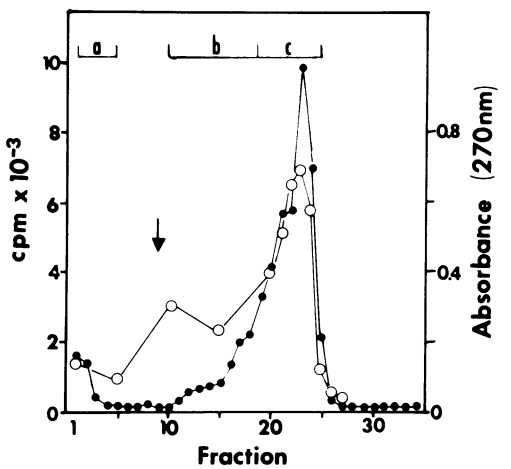

FIG. 10. *Preparative poly(U) chromatography of unlabeled cytoplasmic RNA. Cytoplasmic RNA from 8-h infected cells was fractionated by chromatography on poly(U) columns. Symbols: (○) elution profile of unlabeled infected-cell RNA monitored by absorbance at 270 nm; (●) elution profile from a companion column of labeled infected-cell RNA mixed with carrier uninfected-cell RNA and monitored by radioactivity. Arrow points to the start of the linear formamide gradient. Fractions indicated by letters a, b, and c, corresponding to poly(A)$_{30}$, poly(A)$_{50}$, and poly(A)$_{155}$ RNA, respectively, were pooled for hybridization assays shown in Fig. 11.*

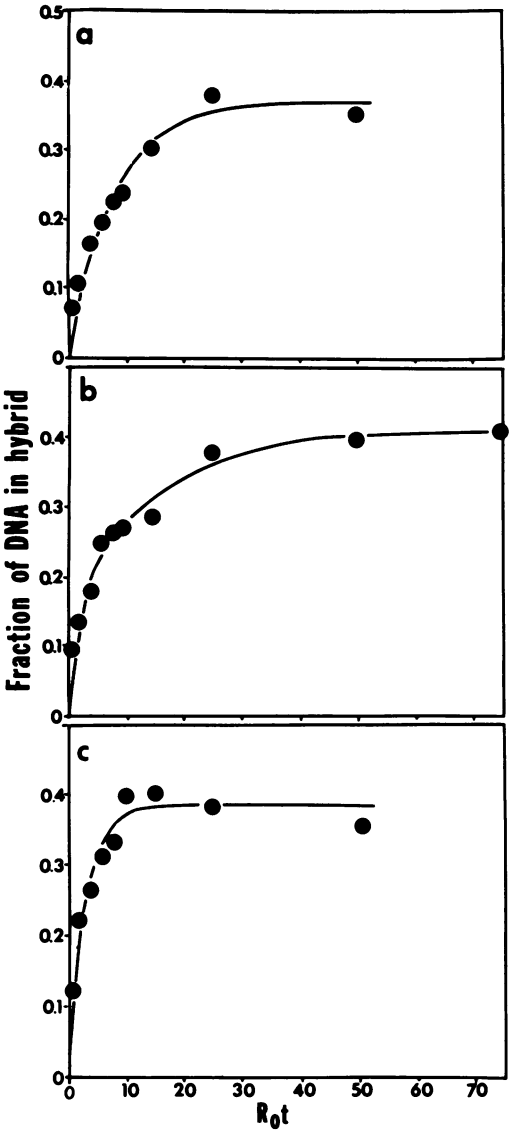

FIG. 11. *Sequence complexity of adenylated RNAs fractionated by poly(U) chromatography. The pooled fractions from the preparative poly(U) column fractionation of unlabeled 8-h cytoplasmic RNA (Fig. 10) were hybridized to trace amounts of labeled HSV-1 F strain DNA. The lines are computer plots calculated as previously described (6) and fitted by the nonlinear least-squares regression method. (a) Poly(A)$_{30}$ RNA; (b) poly(A)$_{50}$ RNA; (c) poly(A)$_{155}$ RNA.*

determinant for binding to nitrocellulose filters. One possible explanation suggested by the experiment shown in Fig. 12 is that binding of the adenylated RNA to nitrocellulose filters might be affected by the secondary structure of the RNA. Further support for this hypothesis comes from our observation that elimination of the heat denaturation step prior to binding of RNA poly(U) causes an appreciable decrease in the amount of RNA bound and in particular in the amount of RNA eluting in the poly(A)$_{155}$ class. We have, however, not excluded alternative hypotheses and these are being tested. Although the precise feature of adenylated RNA responsible for binding to nitrocellulose membranes is not known, it must be shared by the abundant viral RNA sequences and not by the scarce species and, in consequence, it might conceivably play a role in regulating RNA abundance.

Adenylation of viral RNA. Several observations made in this study concern poly(A) metabolism in both infected and uninfected cells. Specifically, (i) there is no apparent difference in the size and distribution of poly(A) chains of infected and uninfected cells. As indicated earlier in the text, the size of the poly(A) chains on viral RNA sequences coincides with the distribution of the poly(A) chains in uninfected cells. We have no evidence, therefore, that the basic features of poly(A) metabolism are altered after infection. The possibility that specific features of adenylation are altered remains open and cannot be resolved by the information currently available.

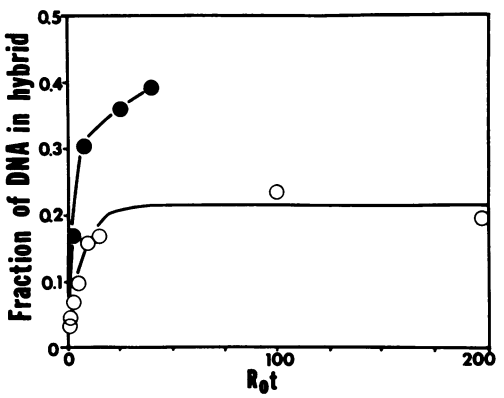

FIG. 12. *Complexity of viral denatured and nondenatured A$_{155}$ RNA bound to nitrocellulose filters. Eight-hour-infected-cell cytoplasmic RNA (●) was fractionated on poly(U) column. The poly(A)$_{155}$ RNA was heat denatured and passed through a nitrocellulose filter, which retained more than 90% of the RNA. The RNA eluted from the nitrocellulose filter was hybridized with trace amounts of labeled DNA. Eight-hour infected-cell polyribosomal RNA (○) that had been eluted from a nitrocellulose filter as described in Materials and Methods was hybridized with trace amounts of labeled viral DNA.*

(ii) Comparison of the cytoplasmic products of long and short labeling intervals failed to show a precursor-product relationship between the RNA containing short and long poly(A) chains. However, this finding should be viewed with caution. Such a relationship could well be obscured by leakage of RNA with short poly(A) chains from the nucleus during extraction. Along with the observation that the RNA containing $poly(A)_{30}$ chains is 1.5 times the size of the $poly(A)_{155}$ RNA, the evidence favoring this possibility is that the amount of this RNA in the cytoplasmic fraction increases with the number of washings of the isolated nuclei. It is conceivable that the $poly(A)_{50}$ and some of the $poly(A)_{30}$ class resulted from aging degradation of the long poly(A) chains, as has been observed in mammalian cells (17), in HSV-1 infected cells (S. L. Bachenheimer and B. Roizman, unpublished data), and in reticulocytes (8). However, to account for the distribution we have observed even in a 10-min labeling period, this aging process would have to occur rapidly and by cleavage of poly(A) from longer chains in segments of discrete size.

(iii) Of particular significance is the observation that the high-molecular-weight RNA contains exclusively $poly(A)_{30}$ tracts. This observation is inconsistent with the hypothesis that RNA is polyadenylated by a single enzyme or in a single step and that the fully adenylated RNA is then translocated into the cytoplasm. Rather, the more consistent model is that the adenylation takes place in two steps, i.e., on high-molecular-weight RNA with the appearance of $poly(A)_{30}$ chains and on processed RNA to $poly(A)_{155}$ chain lengths. We do not think that the available data exclude the possibility that in viral RNA $poly(A)_{30}$ chains, unlike the $poly(A)_{155}$ chains, might arise by transcription in a manner analogous to that proposed for *Dictyostelium discoideum* (13) and HeLa cell heterogeneous nuclear RNA (14). Since the approximately 50 polypeptides currently identified as virus specific account for most of the DNA represented in polyribosomes as mRNA (15), we could expect approximately 50 chains of dT_{30}, accounting for less than 1% of the total base pairs and for 5.5% of the dT content of the DNA. This amount would not have been detected in the hybridization analyses done previously (1) and is being investigated further.

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