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Quantitation of Herpes Simplex Virus Type 1 RNA in Infected HeLa Cells

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We have quantitatively analyzed the size and amount of herpes simplex virus (HSV)-specific RNA synthesized in HeLa cells using DNA and RNA excess hybridization. At 2 h after infection (early), transcripts from 20% of the total HSV DNA are present on polyribosomes as poly(A+) RNA. At this time, viral poly(A+) RNA comprises 60 to 75% of the newly synthesized poly(A+) mRNA on polyribosomes. By 6 h after infection (late), poly(A+) HSV RNA transcribed from 35 to 40% of the viral DNA is found on polyribosomes. These viral poly(A+) transcripts comprised as much as 90% of newly synthesized poly(A+) mRNA and are measurably larger than viral poly(A+) transcripts isolated early. Some but not all of this size difference is due to the fact that the poly(A) tails on early transcripts are shorter than those found on transcripts made late. Even late after infection, a small but readily measurable amount of cellular poly(A+) RNA is still being made and entering polyribosome complexes. In the nucleus, late after infection, poly(A+) HSV RNA is complementary to 50% of the total HSV DNA. Both early and late after infection, total nuclear viral transcripts are, on the average, larger than viral transcripts found on polyribosomes; however, nuclear HSV poly(A+) RNA is not measurably larger than the corresponding cytoplasmic viral poly(A+) sequences at either time. A major portion (30 to 40%) of the polyribosomal HSV RNA made either early or late after infection is not polyadenylated. This HSV poly(A−) RNA is transcribed from the same sequences as HSV poly(A+) RNA but, when labeled and isolated either early or late after infection, both nuclear and polyribosomal viral poly(A−) RNA molecules sediment faster in sucrose-formaldehyde gradients than their polyadenylated counterparts.

Herpes simplex virus (HSV) is a well-characterized representative of large nuclear replicating viruses. The expression of HSV DNA during productive infection affords an interesting model system for studying gene expression. HSV-specific mRNA is known to share characteristics with normal eucaryotic mRNA. Viral mRNA containing poly(A) sequences can be isolated (R. Swanstrom and E. Wagner, Fed. Proc., vol. 34, abstr. 1727, 1974; 2, 17, 18); this poly(A+) viral mRNA is methylated and has a capped 5′ end of the type standard for eucaryotic and nuclear replicating virus mRNA's (manuscript in preparation), and this viral poly(A+) RNA cannot appear on polyribosomes in the presence of the drug cordycepin (Swanstrom and Wagner, Fed. Proc.). Also, the weight average size of viral sequences isolated from polyribosomes is smaller than that of viral sequences found in the nucleus (25).

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The transcription program of HSV has been qualitatively well described. The replication cycle shows a distinct temporal restriction of transcription where, prior to viral DNA replication (early), 20% of the total viral DNA is expressed as a major concentration class of RNA. Transcription of at least a full single-strand equivalent of the viral genome takes place only with the onset of viral DNA synthesis (late) (4, 11, 20, 21, 27). Quantitatively, HSV transcription has been less well defined. The size of HSV transcripts has been estimated at low resolution by low-efficiency hybridization of sucrose gradient-fractionated total radioactive nuclear and cytoplasmic RNA from infected cells (25). The relative amounts of viral RNA present in different cellular fractions at different times after infection have been estimated, but the exact amount of viral RNA in these fractions has remained undetermined (4, 21). Finally, we have reported that there is a fraction of HSV-specific RNA on polyribosomes that does not

contain poly(A) sequences [poly(A−) RNA]; however, the relationship between this fraction and viral poly(A+) RNA has been unclear (Swanstrom and Wagner, Fed. Proc.; R. I. Swanstrom, Ph.D. thesis, University of California, Irvine, 1975).

In this paper we describe experiments carried out using low-temperature DNA excess hybridization to both quantitate and isolate HSV-specific RNA in the various fractions of infected cells. Notable among our findings are: (i) 60 to 70% of mRNA sequences on polyribosomes are viral by 2 to 3 h after infection; (ii) there is a persistent, small fraction of mRNA on polyribosomes that is cellular even late after infection; (iii) viral polyribosome-associated poly(A−) RNA shares the same sequences with, but is larger in average size than, viral poly(A+) RNA; and (iv) HSV-specific polyribosome-associated poly(A−) RNA appears in the cytoplasm in the presence of cordycepin and independently of viral poly(A+) RNA.

MATERIALS AND METHODS

Cells and virus. HeLa cells were grown in monolayer culture as described previously (23). Our standard plastic culture flasks (T-150; Falcon, Oxnard, Calif.) have a 150-cm^2 growing area and contain 4×10^7 cells. The origin and passage of our laboratory strain of HSV type 1 (L strain) has been described (26–28). All virus used in these experiments is routinely plaque purified after six passes as described (26). Unless noted, the standard infection was carried out at 30 PFU/cell using conditions previously described (20, 21). Time after infection was measured after virus adsorption (0.5 h).

Drugs. Cordycepin (3-deoxyadenosine) was purchased from Sigma Chemical Co. (St. Louis, Mo.). This drug was used at a concentration of 50 μg/ml. Cycloheximide was purchased from Nutritional Biochemicals (Cleveland, Ohio).

Radioactive RNA and DNA. Radioactive RNA was prepared by labeling infected cells at either 2 h (early) or 5 h (late) after infection for 1 h with 15 μCi of [^{3}H]uridine per ml (150 μCi/T-150 flask; 27 Ci/mmol; Schwarz Bio Research, Inc., Orangeburg, N.J.). ^{32}P-labeled marker 28S and 18S rRNA was prepared from uninfected cells labeled for 20 h with 250 μCi of [^{32}P]orthophosphate (Amersham-Searle, Arlington Heights, Ill.) in medium containing 0.1× normal phosphate concentration and 10% calf serum that had been dialyzed versus phosphate-free 0.15 M saline.

Radioactive labeling of HSV DNA has been described (21). Cells were labeled for 16 h with 15 μCi of [^{3}H]thymidine per ml (53 Ci/mmol; Schwarz/Mann). The ^{3}H-labeled HSV DNA used in these experiments had a specific activity of 300,000 cpm/μg. The procedures for purification and alkali fragmentation and renaturation of the HSV DNA have been described (26, 28). We have extensively shown that such alkali denatured and fragmented HSV DNA reanneals with normal kinetics to better than 90% under our conditions (26, 28).

Fractionation of cells into nuclei and cytoplasm and isolation of polyribosome-associated fraction. We used the method of Palmiter (12) to lyse cells, separate nuclei and cytoplasm, and prepare polyribosome-associated material. All glassware was acid washed in 6 N HNO_3 and then rinsed in glass-distilled water containing 0.1% diethyl pyrocarbonate (DEPC; Sigma Chemical Co., St. Louis, Mo.) prior to use. All buffers were treated with 0.1% DEPC and autoclaved prior to use.

Cells were chilled on ice, rinsed twice in ice-cold saline (0.15 M NaCl) containing 50 μg of cycloheximide per ml, and then rinsed once with 20 mM ice-cold HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4) containing 50 μg of cycloheximide per ml. The HEPES buffer was aspirated, and the cells were overlaid with polysome buffer (25 mM Tris, 25 mM NaCl, 5 mM $MgCl_2$, pH 7.5, and 200 μg of heparin per ml [PPB]) containing 2% Triton X-100 (Calbiochem, San Diego, Calif.). After 5 min on ice, the cells were scraped, the lysate was homogenized with five strokes of a tight-fitting Dounce homogenizer, and the nuclei were deposited by centrifugation in a Sorval SS-34 rotor for 5 min at 3000 rpm.

The cytoplasm was further clarified by centrifugation at 10,000 rpm for 5 min to remove mitochondria. Polyribosome-associated material was precipitated with Mg^{2+} by adjusting the cytoplasmic fraction to 120 mM Mg^{2+} by addition of sufficient 2 M $Mg(Ac)_2$. After 75 min on ice, the polyribosomes were deposited by centrifugation at 15,000 rpm for 10 min in an SS34 rotor through a 3- to 5-ml pad of 1 M sucrose containing PPB without Triton X-100. This pelleted material contains ribosomes, polyribosomes and other ribonucleoprotein (12), and RNA isolated from it is referred to hereafter as polyribosome-associated RNA.

Sucrose gradient sedimentation profiles of polyribosome-associated RNA were obtained by the method of Palmiter (12). The polyribosome pellet was resuspended in 0.5 ml 20 mM HEPES, gently homogenized on ice, and layered over a 12-ml 15 to 40% sucrose gradient containing 25 mM NaCl, 25 mM Tris, 5 mM $MgCl_2$ (pH 7.5), and 200 μg of heparin per ml. Centrifugation was at 4°C for 75 min at 40,000 rpm in a Spinco SW40 rotor. The gradient was pumped through a Gilford recording spectrophotometer, and 0.5-ml fractions were collected. Radioactivity was determined by counting 100-μl portions of each fraction in Aquasol scintillator (New England Nuclear Corp., Boston, Mass.) with a Beckman LS-230 liquid scintillation counter.

RNA purification. RNA was extracted from isolated nuclear, cytoplasmic, or polyribosome fractions using a modification of our standard phenol and chloroform method (24). The nuclear fraction, after DNase digestion in HSB (0.5 M NaCl-10 mM Tris-50 mM $MgCl_2$, pH 7.4), was adjusted to 25 mM EDTA-1% sodium dodecyl sulfate (SDS) and then digested for 20 min at 37°C with 200 μg of protease K (Merck Pharmaceuticals, Elmsford, N.Y.) per ml that had been self-digested for 30 min at 37°C at a

concentration of 10 mg/ml to remove any nuclease activity. Polyribosome-associated material was dissolved in extraction buffer (0.1 M NaCl-10 mM Tris-5 mM EDTA, pH 7.5) containing 0.5% SDS and similarly digested with protease K. After protease K digestion, the solutions were extracted using extraction buffer-saturated phenol containing 0.1% 8-hydroxyquinolin.

Gradient-purified unlabeled RNA was prepared as previously described (20). To assure the removal of any trace amounts of DNA remaining in unlabeled nuclear RNA after DNase digestion, the gradient-purified RNA was phenol extracted, dialyzed against 0.01 M NaCl-0.01 M Tris-0.0015 M $MgCl_2$, pH 7.4, and digested again with 200 μg of electrophoretically purified DNase (Worthington Biochemical Corp., Freehold, N.J.). This RNA was then phenol extracted for a final time and ethanol precipitated twice.

We insured that our extraction procedure resulted in quantitative yield of poly(A) containing RNA [poly(A+) RNA] by two types of control experiments. In one, [^{3}H]uridine-labeled poly(A+) RNA isolated from uninfected cell polyribosomes by the procedure described below was mixed with the nuclear fraction or polyribosome fraction of unlabeled test cells and subjected to redigestion and reextraction. This reextracted poly(A+) RNA binds quantitatively to poly(U) glass-fiber columns (see below), indicating that poly(A−) RNA is not being generated by the loss of poly(A) tails from RNA molecules during extraction. Second, radioactive poly(A) (Miles Laboratories, Inc., Elkhart, Ind.) was quantitatively recovered with RNA in similar tests, also indicating no specific loss of poly(A+) RNA during isolation.

Separation of poly(A+) and poly(A−) RNA. We are currently using two methods of separating poly(A+) and poly(A−) in our laboratory; both give identical results. (i) Poly(U)- Sepharose (Pharmacia, Uppsala, Sweden) chromatography of purified RNA samples was carried out by the method of Lindberg and Persson (6). (ii) Poly(U)-glass-fiber filters containing 0.5 mg of poly(U) (Sigma Chemical Co., St. Louis, Mo.) were prepared by the method of Sheldon et al. (16). Columns (9 by 0.5 cm) were made from two filters using disposable 1-ml syringes. The RNA solution from no more than 8×10^7 cells was passed through such a column two or three times in a buffer containing 0.12 M NaCl, 0.01 M Tris, and 0.001 M EDTA, pH 7.5. RNA not binding to the column [poly(A−) RNA] was collected by ethanol precipitation. The column was then washed with 6 ml of the same buffer and then with 5 ml of the buffer warmed to 45°C. Poly(A+) RNA was eluted with 3 ml of a buffer containing 60% formamide, 0.01 M Tris, and 0.001 M EDTA, pH 7.5.

The poly(A+) RNA isolated by either method was then either dialyzed against extraction buffer or diluted to a final formamide concentration of 20% with 0.15 M NaAc and then precipitated with 3 volumes of ice-cold ethanol and collected by centrifugation. Although we routinely added 25 to 50 μg of purified uninfected cell rRNA as carrier to the poly(A+) fraction, we could attain quantitative yield of radioactive poly(A+) without it. We measured the yields of poly(A+) and poly(A−) RNA from typical preparations. For a T-150 culture (4×10^7 cells), we recovered 4 to 5 μg of poly(A+) RNA from the polyribosome-associated material and 15 to 20 μg of the nuclear poly(A+). We recovered 100 to 200 μg of poly(A−) RNA from the polyribosomes or the nuclear fraction; the vast majority of this latter material is rRNA and tRNA. On the basis of the yields of poly(A^+) RNA and the total amount of radioactivity recovered from these fractions (see Table 2) in infected cells under our labeling conditions, we estimated that the average specific radioactivity of poly(A+) RNA isolated after a 60-min pulse is 30,000 to 40,000 cpm/μg.

Control experiments using formaldehyde-sucrose density gradient centrifugation (see below) showed that the separation of poly(A+) and poly(A−) RNA results in no detectable degradation of RNA and that poly(A+) RNA can be isolated as high-molecular-weight molecules (see below). Both types of poly(U) columns retained 90% or more of radioactive poly(A) molecules of chain length greater than five or six nucleotides. This limit was determined by sizing the bound and unbound fractions of commercial [^{3}H]poly(A) by acrylamide gel electrophoresis. This efficiency of binding was nearly as high even after preparative amounts of poly(A+) RNA were bound to the column. Thus, the poly(A−) RNA referred to here contains at most three to five consecutive adenosine nucleotides at the 3′ terminus.

Sizing of poly(A) sequences. For sizing experiments, cells were labeled for 1 h with 500 μCi of [^{3}H]adenosine (42 Ci/mmol, New England Nuclear Corp.) per T-150 culture. Poly(A+) RNA (up to 5 μg) labeled with [^{3}H]adenosine was digested with 15 μg of RNase A and 15 U of RNase T_1 (Sigma) in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 37°C for 30 min. After the digestion, SDS and EDTA were added to concentrations of 0.5% and 0.005 M, respectively, along with carrier tRNA. The RNA was then phenol extracted, ethanol precipitated, and dialyzed against 0.15 M NaCl. The RNA was ethanol precipitated again and resuspended in 100 μl of electrophoresis buffer (0.03 M Na_2HPO_4-0.036 M Tris-0.001 M EDTA, pH 7.8) (7) with 15% glycerol. Control experiments show that poly(A) sequences of chain length five or more are precipitated with ethanol at −20°C. The poly(A) fragments were then applied to a 9.5-cm gel of 12% acrylamide, and electrophoresis was carried out at 4mA/gel by the method of Morrison et al. (10). The gels were scanned at 260 nm, frozen, and cut into 2-mm slices. The slices were treated with 0.25 ml of Protosol (New England Nuclear Corp.) and counted in a liquid scintillation counter with a toluene-base fluor. AMP, $(Ap)_5A$, $(Ap)_{10}A$, and tRNA were added as internal length markers. The poly(A) markers were a gift from W. M. Stanley, Jr.

Purification and assay of S_1 nuclease. The single-strand nuclease from *Aspergillus oryzae* (S_1) was purified through the DEAE-cellulose column step of the procedure of Vogt (22). An equal volume of glycerol was added to each fraction, and the fractions were stored at −20°C. The standard assay was with

25 μl of enzyme solution under conditions previously described (21, 26). Small amounts (less than 0.1 μg) of either native or denatured radioactive HSV DNA were added to the assay mixture and incubated at 45°C for 30 min. Undigested DNA was precipitated with 5% trichloroacetic acid and collected on glass-fiber filters, and its radioactivity was determined.

RNA excess solution hybridization. RNA excess hybridizations were carried out in 1× SSC at 72°C as described previously (21). Small amounts of radioactive alkali-fragmented, denatured HSV DNA (0.05 to 0.1 μg/ml) were incubated with RNA isolated from infected cells (up to 4 mg/ml). Denatured calf thymus DNA (5 μg/ml) was added to the hybridization reaction mixture as carrier. At various times, 50-μl portions were removed from the hybridization solution and digested with S_1 nuclease. Analysis of the data and corrections for small amounts of DNA self-annealing when needed have been described (20).

DNA excess hybridization. DNA excess hybridization and fractionation of hybrids on hydroxyapatite (HAP) were by the method of Lewis et al. (5) modified to accommodate the high guanine plus cytosine content of HSV DNA. Hybridization was carried out in 0.3 to 0.5 ml in conical Konteflex vials in a buffer containing 0.5 M NaCl, 0.1 M Tris, 10 mM EDTA, pH 7.5, and 50% formamide. Hybridization mixtures contained 50 μg of alkali-fragmented HSV DNA per ml and usually 6,000 to 10,000 cpm of radioactive RNA per ml. Twice this amount of RNA was used with cellular fractions where the proportion of HSV-specific RNA was low. This DNA-RNA ratio corresponds to a 200- to 300-fold weight excess of HSV DNA over newly synthesized RNA based on a specific activity of this RNA of 30,000 to 40,000 cpm/μg (see above).

Hybridization was carried out to a DNA C_0t of 20 to 25 (1 C_0t is 50 μg of DNA per ml incubated for 1 h) (26, 28) at 55°C for analytical determinations and at 47°C for preparative experiments; this lower temperature was used to obviate any possible thermal degradation of RNA at the analytical temperature. Preparative hybridization is 60 to 70% as efficient as analytical in driving viral RNA into hybrids. We tested renaturation of HSV DNA under both conditions by the addition of a small amount of radioactive HSV DNA and isolating hybrids on HAP (see below). We found quantitative reannealing of the viral DNA by a C_0t of 4 to 5 at both temperatures.

After incubation at the hybridization temperature, the mixtures were diluted 10-fold into 0.2 M phosphate buffer (PB—an equimolar mix of NaH_2PO_4 and Na_2HPO_4) containing 8 M urea previously treated with Chelex-100, (Bio-Rad Laboratories, Calif.) and 20 μg of yeast RNA per ml as carrier. The mixture was applied to jacketed HAP column (0.6 by 0.6 cm) at 45°C and rinsed with PB-8 M urea until no more radioactivity was eluted (usually three 3-ml rinses). Hybrids were eluted with 3 ml of 0.45 M PB and desalted by passage through G-25 Sephadex columns (30 by 0.9 cm) equilibrated in extraction buffer. Fractions (1 ml) were collected, and the hybrids were located by determining the radioactivity of portions and precipitated by the addition of 2 to 3 volumes of ice-cold ethanol. Hybrids were then either denatured by heating to 72°C in 90% formamide containing 0.01 M NaCl-0.01 M Tris, pH 7.5, or by heating to 72°C in 3% formaldehyde as described below. Denatured RNA was recovered from formamide in the same manner as described above for recovery of poly(A+) RNA.

Fractionation of RNA on formaldehyde sucrose gradients. Formaldehyde denaturation and sucrose gradient density fractionation were carried out by the methods of Boedtker (3) and Robberson et al. (14). Samples of RNA or DNA-RNA hybrids containing ^{32}P-labeled marker rRNA and unlabeled HeLa cell rRNA were adjusted to 3% formaldehyde by the addition of 0.1 volume of a solution of 30% formaldehyde in a buffer containing 90 mM Na_2HPO_4-10 mM NaH_2PO_4. The samples were heated at 72°C for 5 to 10 min, quenched on ice, and layered over an 18-ml gradient of 5 to 20% sucrose containing 0.1 M NaCl, 0.02 M PB, and 1% formaldehyde. Typically, centrifugation was for 20 h at 4°C at 24,000 rpm in a Spinco SW27 rotor. Gradients were fractionated into 0.7- to 0.8-ml fractions through a Gilford spectrophotometer, and radioactivity was determined by counting in Aquasol. Radioactivity counting was done with a Beckman LS-230 Scintillation counter with windows set so that no ^3H radioactivity was seen in the ^{32}P channel and 0.5% of the ^{32}P radioactivity was seen in the ^3H channel. All double-label experiments have been corrected for this spillover.

RESULTS

Quantitation of HSV-specific RNA using DNA excess hybridization. Radioactive HSV-specific RNA was isolated from infected cells by preparative hybridization with excess HSV DNA followed by fractionation on HAP as described above. Samples of such RNA were denatured and then rehybridized with viral DNA under analytical conditions. The data from a number of experiments are shown in Fig. 1. It can be seen that under the conditions used viral RNA can be quantitively driven into DNA-RNA hybrids and that one round of hybridization is sufficient to drive 60 to 75% of a pure sample of viral RNA into hybrids.

We used a single round of such analytical DNA excess hybridization to measure the absolute amount of HSV RNA in a given fraction from infected cells by comparing the efficiency of hybridization of an unknown sample with that of pure viral RNA in a parallel hybridization. Control experiments (Table 1) established that the amount of viral RNA in mixtures of viral and cellular RNAs can be accurately determined by this method. Also presented in Table 1 are control experiments that demonstrated that viral RNA hybridizes to viral DNA with equivalent efficiency through a 16-fold range of RNA-to-DNA concentration ratios. No decrease in the level of viral RNA hybridization

is seen even at the highest RNA to DNA ratio, which is five times that routinely used to obtain the data presented below. Further evidence that our analytical hybridizations are carried out in vast HSV DNA excess is that the measured efficiency of viral RNA hybridization in samples from infected cells is invariant when the input radioactivity is varied as much as threefold (data not shown).

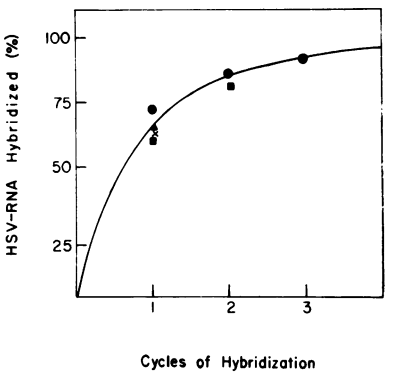

FIG. 1. *Extent of hybridization of HSV-specific RNA in the presence of excess viral DNA. [^{3}H]uridine-labeled viral RNA was isolated from infected cell polyribosomes late after infection by preparative DNA excess hybridization as described in Materials and Methods. A single cycle of analytical DNA excess hybridization consisted of a 20-h incubation of a radioactive sample of pure viral RNA with alkali-fragmented, denatured viral DNA at 55°C as described. RNA not hybridized during a 20-h incubation was separated from hybridized RNA as material not binding to HAP in 0.2 M PB-8 M urea. The unhybridized material was dialyzed against extraction buffer (0.15 M NaCl-0.01 M Tris-0.001 M EDTA), ethanol precipitated, and subjected to another round of analytical DNA excess hybridization. Data from four experiments carried out with different preparations of HSV DNA are indicated by different symbols.*

Distribution of HSV RNA synthesized in infected HeLa cells. Cultures of HeLa cells were infected and labeled with [^{3}H]uridine at 2 to 3 h (early) or 5 to 6 h (late) after infection as described above. The cells were fractionated, and nuclear and polyribosomal-associated RNA was extracted and fractionated into poly(A+) and poly(A−) fractions as described above. Portions of each fraction were subjected to DNA excess hybridization, and the amount of HSV-specific RNA in each was calculated by comparing the amount of radioactive RNA isolated as hybrid with a pure sample of HSV RNA run in parallel. The results of a number of such determinations were extrapolated to show the total amount of viral and cellular RNA synthesized during the labeling period in a standard culture of 4×10^7 cells. These data are shown in Table 2.

Early after infection, only about 5% of the total nuclear RNA synthesized is HSV specific; however, 25 to 30% of the poly(A+) RNA in the nucleus is viral at this time. The proportion of viral poly(A+) RNA associated with cytoplasmic polyribosomes is quite high (60 to 70% of the total) even at this time after infection. A similarly high proportion of viral RNA in the polyribosome-associated poly(A+) fraction is found when cells are infected at a multiplicity of infection of 10 PFU/cell instead of the normal 30 PFU/cell used in these experiments. The proportion of total polyribosome-associated poly(A−) RNA that is HSV specific is low (10%), but this corresponds to 45% of the total HSV polyribosome-associated RNA.

Late after infection, viral RNA accounts for about 20% of the newly made RNA found in the nucleus. A third of the newly made nuclear poly(A+) RNA and about 15% of the nuclear poly(A−) RNA made at this time are viral. About 70% of the total polyribosome-associated

TABLE 1. *Amount of HSV RNA in RNA mixtures using DNA excess hybridization*[a]

Amt of RNA incubated with 25 μg of HSV DNA						RNA recovered as hybrid			
HSV[b]		HeLa		Total		Hybrid[c]		Calculated viral RNA[d]	Actual % viral RNA
cpm	μg	cpm	μg	cpm	μg	cpm	%		
6,000	0.2	0	0	6,000	0.2	3,978	61	94	100
6,000	0.2	6,000	3	12,000	3.2	4,000	33	51	50
8,000	0.2	42,000	22	50,000	22	5,140	10	15	17
0	0	6,000	3	6,000	3	97	1.6	2	0
2,425	0.08	0	0	2,425	0.08	1,770	73	100	100
6,000	0.2	0	0	6,000	0.2	3,660	61	94	100
38,000	1.3	0	0	38,100	1.3	23,622	62	95	100

[a] Details of analytical DNA excess hybridization are given in Materials and Methods.
[b] Isolated by preparative DNA-excess hybridization of polyribosomal RNA from cells late after infection.
[c] Determined by HAP chromatography.
[d] Calculated from the percentage in hybrid; 65% hybrid was normalized to 100% viral RNA (see Fig. 1).

TABLE 2. *Distribution of viral and cellular RNA synthesized in infected HeLa cells*[a]

Labeling period (h after infection)	Nucleus (cpm × 10^{-5})						Polyribosomes (cpm × 10^{-4})[b]					
	Total RNA			HSV RNA[c]			Total RNA			HSV RNA[c]		
	(A+)	(A−)	Total	(A+)	(A−)	Total	(A+)	(A−)	Total	(A+)	(A−)	Total
Uninfected	5±1.2	92±4	97±5.2				6.0±0.8	96±4	102±5			
2–3 (early)	3.6±1.6	80±4	84±5.6	1.0±0.2	4.0±0.4	5.0±.6	20±8	92±8	108±16	12±2.8	9.6±1.6	21.6±4.4
5–6 (late)	6.4±0.4	40±0.8	46±1.2	2.8±0.8	6.0±0.4	8.8±1.2	14±1.6	16±1.6	30±3.2	12±2.6	8±3.6	20±7.2

[a] Cells were infected at a multiplicity of 30 PFU/cell. Data are an average of three separate experiments for each time point. Cells were labeled for 1 h with 140 μCi of [^{3}H]uridine/T-150 flask, and data are total radioactivity recovered per T-150 culture (4 × 10^7 cells).

[b] Polyribosomes were isolated by the method of Palmiter (12) as described in Materials and Methods.

[c] HSV-specific RNA was determined by analytical DNA excess hybridization of a 5,000- to 10,000-cpm portion of each RNA sample as described in Materials and Methods.

RNA made late after infection is HSV specific. Half of the poly(A−) polyribosome associated RNA is viral and, as is the case early after infection, this nonpolyadenylated viral RNA accounts for 30 to 45% of the total polyribosome-associated HSV-specific RNA. Nearly all (75 to 90%) of the poly(A+) RNA made late after infection and found on polyribosomes is HSV specific.

At no time after infection did viral sequences account for all the labeled poly(A+) found on polyribosomes. We tested early and late poly(A+) polyribosome-associated RNA for the presence of newly synthesized cellular RNA sequences by analytical hybridization in excess HeLa cell DNA. At both times after infection, labeled cell-specific RNA was found to be associated with polyribosomes in amounts consistent with the proportion of viral sequences in these fractions (data not shown).

Sequence specificity of HSV RNA containing poly(A) sequences. Unlabeled RNA was extracted from the polyribosomes of HSV infected-cells at 2 and 6 h after infection. Hybridization between fractions of the RNA and HSV DNA was carried out under conditions of excess RNA as described above. The data for the hybridization of poly(A+) from cells early and late after infection are shown in Fig. 2A. Poly(A+) HSV RNA present early after infection anneals rapidly to 20% of the total HSV DNA. Late after infection the amount of the HSV genome present as polyribosome-associated poly(A+) RNA increases to a value of between 35 and 40% of the total HSV DNA.

Unlabeled RNA isolated from the nucleus at 6 h after infection was also separated into poly(A+) and poly(A−) fractions. The annealing of poly(A+) nuclear RNA to viral DNA is shown in Fig. 2B. At least 50% of the total viral DNA can be driven into DNA-RNA hybrids by the end of the reaction.

Size distribution of HSV-specific poly(A+) RNA. Radioactive poly(A+) RNA from the nucleus and polyribosomes of infected cells was preparatively hybridized with excess HSV DNA as described bove. The hybrids were denatured by treatment with formaldehyde and fractionated on 5 to 20% sucrose gradients containing formaldehyde as described above and shown in Fig. 3. There is little obvious difference between the size of the nuclear and polyribosome-associated poly(A+) RNA either early or late. The broad peak of HSV-specific poly(A+) RNA early after infection sediments slower than that of the poly(A+) HSV RNA late after infection. This difference is small but quite reproducible and corresponds to a maximum difference of 10% in the apparent average *s* value of the poly(A+) RNA isolated at these times.

Size range of poly(A) sequences on polyribosomal RNA. Because of the small but reproducible difference between the average *s* value of early and late HSV poly(A+) RNA, we examined the length of the poly(A) tracts on polyribosome-associated RNA isolated at these times after infection. HSV-infected cells were labeled with [^{3}H]adenosine early (1 to 2 h) after infection. Poly(A+) RNA was isolated from the polyribosomes, and poly(A) sequences were recovered from RNase digestion as described above. The size range of the radioactive poly(A) sequences was determined by electrophoresis in 12% acrylamide gels. The size distribution of the radioactive poly(A) present on the polyribosomes at the end of the labeling period is shown in Fig. 4A. There is a heterogeneous size distribution that can be divided into three regions. We used AMP, $(Ap)_5A$, $(Ap)_{10}A$, and tRNA as internal markers and the gel calibration method of Morrison et al. (10) to estimate the size range of the poly(A) fragments. The size ranges in the three regions shown in Fig. 4

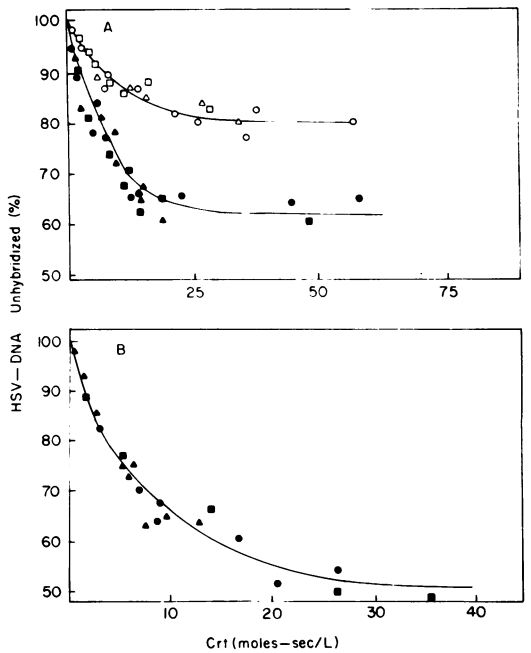

FIG. 2. *Sequence specificity of HSV-specific poly(A+) RNA. Hybridization solutions containing alkali-fragmented denatured HSV DNA, and unlabeled poly(A+) RNA isolated from polyribosomes either early (2 h) or late (6 h) after infection were incubated at 72°C in 1× SSC. At various times, portions were removed, and the amount of DNA-RNA hybrids formed was assayed by S_1 nuclease digestion as described in Materials and Methods and previously (21). Different symbols represent different experiments. (A) Annealing of polyribosomal poly(A+) RNA. The concentrations of early poly(A+) RNA (open symbols) in three experiments were 140, 100, and 45 μg/ml. Four experiments are shown for the annealing of late polyribosomal poly(A+) RNA (closed symbols). The concentrations of RNA used were 110, 50, 40, and 55 μg/ml. (B) RNA excess hybridization of HSV DNA with late nuclear poly(A+) HSV RNA. Poly(A+) RNA from three preparations (120, 75, and 35 μg/ml) was used. Corrections for the self-annealing of DNA were made as described in Materials and Methods and previously (21).*

correspond to poly(A) lengths of greater than 100, 100 to 30, and less than 30 nucleotides. We observed a similar pattern with uninfected cell cytoplasmic poly(A) labeled for the same period of time (data not shown). The size distribution of cytoplasmic poly(A) sequences labeled from 5 to 6 h (late) after infection is shown in Fig. 4B. Again, the distribution of radioactivity can be divided into three broad size ranges similar to those shown in Fig. 4A. In this case, however, the proportion of the largest poly(A) tracts is significantly higher than early after infection. We also examined the poly(A) sequences of polyribosome-associated. HSV-specific RNA isolated by preparative DNA excess hybridization. No significant alterations in the proportion of poly(A) tracts of different lengths were seen with this RNA as compared to total polyribosome-associated RNA.

Sequence specificity of HSV RNA lacking poly(A) sequences. We hybridized unlabeled poly(A−) RNA from the nuclei and polyribosomes of infected cells under conditions of RNA excess as described above for poly(A+) RNA. The results are shown in Fig. 5. Poly(A−) HSV RNA isolated from polyribosomes early after infection rapidly hybridizes to about 20% of the viral DNA. The poly(A−) RNA fraction from polyribosomes isolated late after infection hybridizes to the same fraction of the HSV DNA as found for the poly(A+) RNA isolated from polyribosomes at this time. It is seen in Fig. 5 that between 35 and 40% of the HSV DNA is represented as poly(A−) transcripts found on polyribosomes.

The hybridization of early and late total polyribosome-associated HSV RNA to viral DNA is also shown in Fig. 5. Total HSV RNA from polyribosomes isolated at 2 h after infection rapidly hybridizes to 20% of the viral DNA. By 6 h after infection, poly(A+), poly(A−), and total polyribosomal RNA anneal to between 35 and 40% of the total viral DNA. The poly(A−) RNA from the nucleus isolated early after infection drives 20% or more of viral DNA into hybrids. The corresponding fraction isolated late after infection drives at least 50% of HSV DNA into DNA-RNA hybrids. The rate plots of such reactions are virtually indistinguishable from those reported earlier for total viral RNA isolated at these times after infection (21) and are not shown.

Size distribution of HSV-specific poly(A−) RNA. Radioactive HSV-specific poly(A−) RNA from the nucleus and cytoplasm was isolated by DNA excess hybridization and formaldehyde denaturation of the hybrids as described above for poly(A+) RNA. The viral RNA was fractionated on 5 to 20% sucrose gradients containing 1% formaldehyde, and the size distribution of this material is shown in Fig. 6. The viral poly(A−) RNA synthesized early after infection contains a larger proportion of more slowly sedimenting species than does the poly(A−) viral material synthesized late after infection. This result is analogous to that found with the viral poly(A+) species.

Both early and late after infection, the poly(A−) viral RNA contains a larger proportion of rapidly sedimenting species than is found in poly(A+) viral RNA. The fast-sedi-

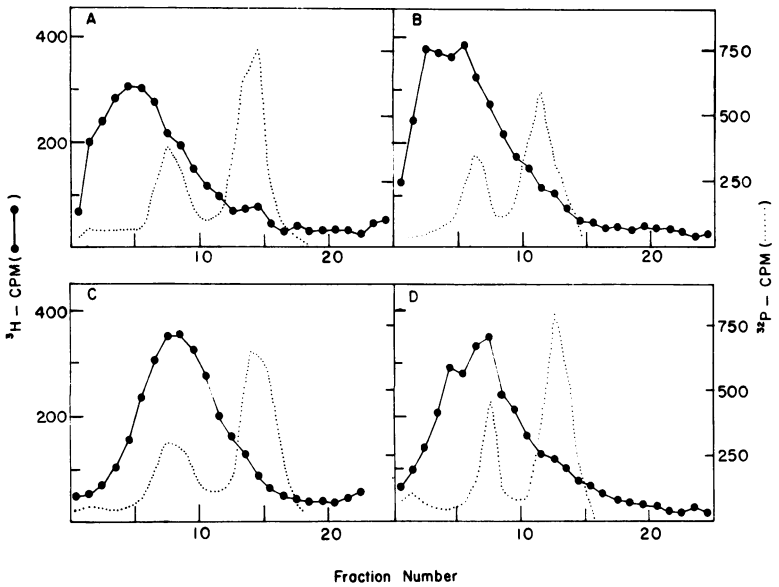

FIG. 3. *HSV poly A(+) RNA size distributions. Cultures of 4 × 10⁷ HeLa cells were incubated with 15 μCi of [³H]uridine per ml for 60 min at 2 h after infection or at 5 h after infection. Nuclear and polyribosomal poly(A+) RNA was extracted, and viral RNA was purified by preparative hybridization in the presence of excess viral DNA as described in Materials and Methods. The hybrids were heat denatured in formaldehyde and layered over 18-ml 5 to 20% sucrose-1% formaldehyde gradients. Centrifugation was at 4°C for 20 h at 24,000 rpm in a Spinco SW27 rotor. The gradient was collected through a Gilford recording spectrophotometer, and 0.7-ml fractions were collected. The radioactivity in each fraction was determined as described. Symbols:. . . . , ³²P-labeled HeLa rRNA added prior to formaldehyde treatment; ●——●, [³H]uridine-labeled viral RNA from: (A) early polyribosomes, (B) early nuclei, (C) late polyribosomes, and (D) late nuclei.*

menting HSV RNA, found both in the nucleus and associated with polyribosomes, contains a significant amount of material that sediments at least as rapidly as 45*S* rRNA (data not shown). As is the case with the viral poly(A+) species, there is no gross difference between the size distribution of the nuclear and polyribosome-associated viral poly(A−) RNA either early or late after infection.

Distribution of viral sequences within polyribosome-associated RNA. We carried out the following experiment to determine whether the viral poly(A−) sequences were confined to a specific fraction of total polyribosome-associated RNA. Cells were labeled with [³H]uridine at 5 h after infection, and the total polyribosome pellet was isolated as described above. The pelleted material was fractionated by centrifugation on a 15 to 40% sucrose gradient as shown in Fig. 7. The fractions sedimenting with single ribosomes and polyribosomes were isolated by pooling the fractions indicated by the bars in Fig. 7. The amount of HSV-specific poly(A+) and poly(A−) RNA was determined by poly(U) chromatography and analytical DNA excess hybridization. The results of the experiment (Table 3) indicate that 80% of the viral polyribosome pellet-associated RNA is found sedimenting with polyribosome structures and that the proportion of viral poly(A−) RNA is invarient between the monosome and polyribosome fractions.

We next examined the properties of the HSV-specific poly(A+) and poly(A−) RNA released by EDTA treatment of the viral material cosedimenting with polyribosomes. Such treatment releases mRNA from polyribosomes but does not disaggregate other cytoplasmic ribonucleoprotein complexes (13). The pooled polyribosome-associated material fractionated in the same manner as that shown in Fig. 7 was pelleted by centrifugation for 60 min at 50,000 rpm at 4°C in a Spinco 65 rotor. The pelleted material was resuspended in 20 mM EDTA (pH 7.5) and fractionated by centrifugation for 75 min at 40,000 rpm on a 15 to 40% sucrose gradient containing 0.1 M NaCl, 0.01 M Tris, and 0.020 M EDTA, pH 7.5, in an SW41 rotor. The amount of HSV-specific RNA in the top one-third of the gradient (EDTA released) was determined, and the proportion of HSV-specific poly(A+) and poly(A−) RNA in the EDTA-released fraction was identical to that found in total polyribosomes (Table 3). We conclude,

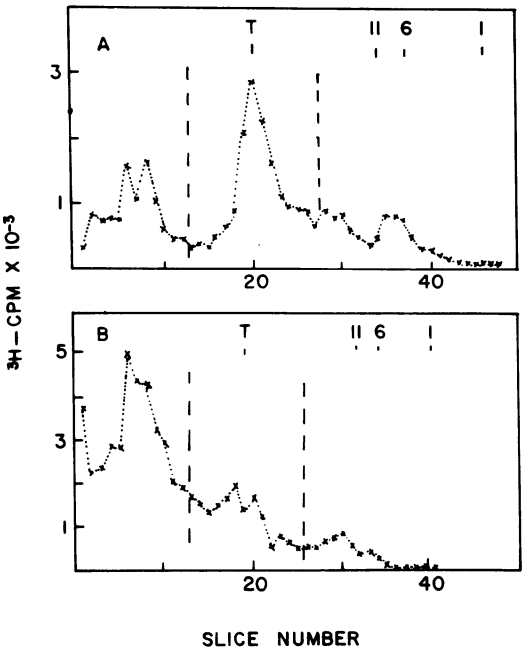

FIG. 4. *Size distribution of poly(A) sequences present on polyribosome-associated RNA after infection with HSV. (A) HSV-infected HeLa cells (4 × 10^7) were labeled with 50 μCi of [^{3}H]adenosine from 2 to 3 h after infection. Cytoplasmic RNA was extracted, and the poly(A+) RNA was removed by hybridization chromatography onto poly(U) filters. The poly(A+) RNA was digested with RNase A and RNase T_1. RNase-resistant material was phenol extracted and subjected to electrophoresis in a 12% polyacrylamide gel, and radioactivity was measured as described in Materials and Methods. AMP (1), $(A_p)_5A$ (6), $(A_p)_{10}A$ (11), and tRNA (T) were added as internal size markers, and their positions are shown by the respective symbols. (B) Size distribution of poly(A) sequences present late after infection. Cells were labeled with [^{3}H]adenosine from 5 to 6 h after infection. All other conditions are as described for (A). The dashed lines on all three panels show the rough size ranges discussed in the text.*

therefore, that a significant proportion of poly(A−) viral RNA is actually bound to polyribosome structures in the same manner as is the poly(A+) RNA. The size distributions of EDTA-released HSV poly(A+) and poly(A−) RNA were determined by sedimentation in sucrose-formaldehyde gradients as described above. Polyadenylated and nonpolyadenylated viral RNA released from polyribosomes by EDTA display the same size distributions as viral RNA isolated from total polyribosome-associated material (Fig. 3 and 6).

Metabolic relationship between HSV poly(A+) and poly(A−) RNA. We examined the effect of cordycepin on the synthesis and appearance of HSV RNA in poly(A+) and poly(A−) fractions in infected cells. Cells were labeled for 45 min with [^{3}H]uridine either in the presence or absence of 50 μg of cordycepin per ml at 5 h after infection. After labeling, polyribosome RNA was extracted and separated into poly(A+) and poly(A−) fractions, and the amount of HSV-specific RNA in each fraction was determined (Table 4). The results of this experiment demonstrate that there is a general reduction of label incorporation into poly(A−) RNA in nucleus and polyribosomes (50 to 60%) in the presence of the drug and that the amount of radioactivity incorporated into HSV-specific poly(A−) RNA is reduced by a similar amount. In contrast, there is a reduction of incorporation of radioactivity into viral poly(A+) RNA by about 85% in both the nucleus and on polyribosomes. Since there is a specific inhibition of viral poly(A+) RNA in the presence of the drug, whereas the appearance of the viral poly(A−) RNA is inhibited only at the same level as overall RNA synthesis, we conclude that no large amount of viral poly(A−) RNA is derived from the poly(A+) fraction during a short (1-h) labeling time.

DISCUSSION

DNA excess hybridization in solution in the presence of formamide is a highly efficient and specific technique that is well suited for quantitative analysis and mild enough for preparative applications. We have utilized this technique to quantitate the amount of HSV RNA in subcel-

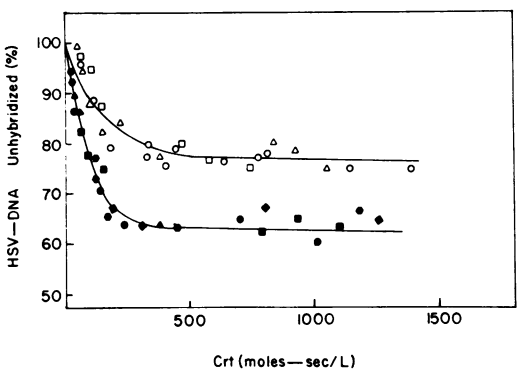

FIG. 5. *Sequence specificity of poly(A−) and total HSV RNA associated with polyribosomes. RNA excess hybridization was as described in the legend to Fig. 2. The concentrations of early RNA (open symbols) used were 4.1 mg and 2.3 mg of poly(A−) RNA per ml (△, ○) and 17 mg of total RNA per ml (□). The concentrations of late RNA (closed symbols) used were 3.8, 3.6, 2.7, and 1.2 mg of poly(A−) RNA (▲, ●, ■, ◆) and 27 mg total of RNA per ml (■).*

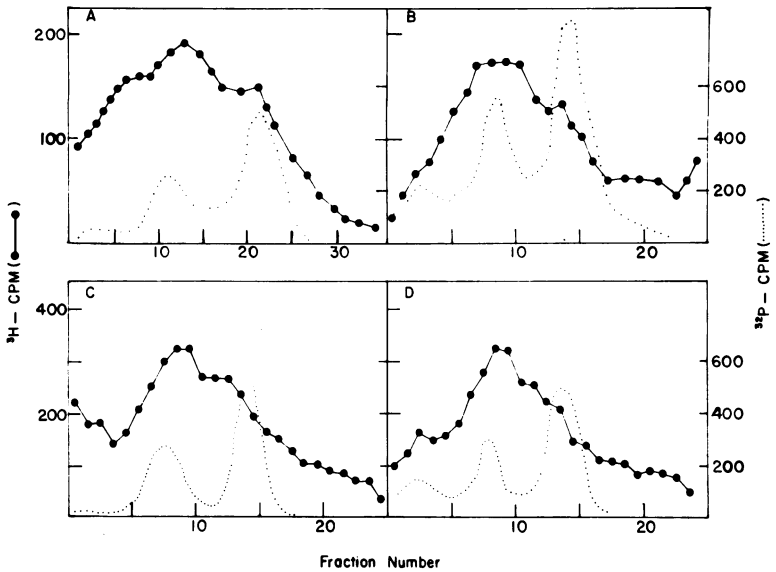

FIG. 6. *HSV poly(A −) RNA size distribution. [^{3}H]uridine-labeled HSV poly(A −) RNA was prepared as described in the legend to Fig. 3. Centrifugation was through 5 to 20% sucrose-formaldehyde gradients at 4°C for 20 h at 24,000 in a Spinco SW27 rotor. Gradients were collected and fractionated, and fractions were counted as described in the legend to Fig. 3. Symbols: . . . . , ^{32}P-labeled HeLa rRNA added prior to formaldehyde treatment; ●, [^{3}H]uridine-labeled viral RNA from: (A) early polyribosomes, (B) early nuclei, (C) late polyribosomes, and (D) late nuclei.*

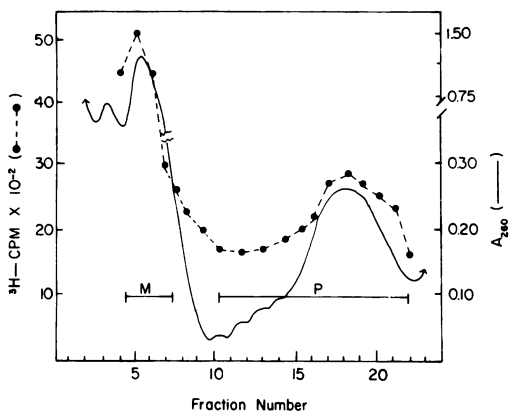

FIG. 7. *Polyribosome sedimentation profile from HeLa cells late after infection. [^{3}H]uridine-labeled, polyribosome-associated RNA was prepared and fractionated by centrifugation on 12-ml gradients of 15 to 40% sucrose in 25 mM NaCl, 25 mM Tris, 5 mM $MgCl_2$, and 200 μg of heparin per ml. Centrifugation was at 4°C for 75 min at 40,000 rpm in a Spinco SW40 rotor. The gradient was separated into 0.5-ml fractions collected through a Gilford recording spectrophotometer. ^{3}H radioactivity was determined by counting 100-μl portions of each fraction in Aquasol. The horizontal bars indicate: M, fractions pooled as RNA associated with not more than one ribosome; P, fractions pooled as RNA associated with more than one ribosome.*

lular fractions of infected HeLa cells and to isolate intact viral RNA for direct size analysis.

The extent of DNA-RNA annealing under conditions of DNA excess depends both on the initial ratio of DNA to RNA and on the ratio of the rate constants of the two competing annealing reactions (8, 19). Under our analytical conditions we can drive 65 to 75% of purified HSV RNA into hybrid in a single cycle and better than 90% into hybrid in three such cycles (Fig. 1). The experiments summarized in Table 1 demonstrate that increasing the input amount of viral RNA by 10- to 15-fold has little effect on the maximum efficiency of hybridizaton of the viral RNA. We conclude that the range of total input HSV RNA used in our experiments results in sufficiently high DNA excess to allow valid quantitation of this RNA in a given sample.

Several lines of evidence support the accuracy of our measurements of the relative amounts of viral RNA in different cell fractions. We find that the relative amount of HeLa cell-specific sequences in samples of infected RNA is the same when determined by cell DNA excess hybridization, as is inferred from results of HSV DNA excess hybridization of the same sample (see Table 2 and Results). Also, we have measured the relative amounts of viral RNA in

cellular fractions using filter disk hybridization either at high temperatures, as has been described previously (20, 27), or at low temperatures in 50% formamide. The results of such experiments are fully consistent with those reported here, although the maximum efficiency of hybridization of pure viral RNA to DNA is on the order of 10% (J. R. Stringer and E. K. Wagner, unpublished data).

The distribution of RNA synthesized during a 6-h labeling period in HeLa cells early and late after infection is shown in Table 2. Total incorporation of [^{3}H]uridine declines markedly late after infection due to the inhibition of synthesis and processing of the 45S rRNA precursor (25). The total amount of viral RNA synthesized in the infected cells increases slightly from 2 to 5 h after infection; however, the amount of viral RNA associated with cytoplasmic polyribosomes is virtually constant during this period. Although at least 60% of the total poly(A+) RNA on polyribosomes is viral by 2 h after infection, there is still a detectable amount of cellular sequences being synthesized and appearing as poly(A+) RNA on polyribosomes even late after infection. The functional significance of this continued cellular mRNA synthesis is unknown, but it is unlikely that it is due to a fraction of cells remaining uninfected, since it can be observed at higher multiplicities of infection and under conditions where cell density in the culture flask is relatively low to maximize cell-virus interaction.

TABLE 3. *Distribution of polyribosome-associated HSV-specific RNA*[a]

Distribution	Amt of HSV RNA			
	(A+)		(A−)	
	cpm	%	cpm	%
Association with total polyribosomal pellet[b]	126,000	55	102,000	45
Cosedimenting with single ribosomes	26,000	56	20,000	44
Cosedimenting with polyribosomes	100,000	55	82,400	45
Released by EDTA treatment of polyribosomes[c]	100,000	59	68,000	41

[a] Fractionation was carried out as shown in Fig. 7. Cells were labeled with 15 μCi of [^{3}H]uridine per ml at 5 h after infection, and the polyribosomal pellet was isolated as described in Materials and Methods.

[b] Data are expressed as total viral radioactivity per 4 × 10^7 cells; viral RNA was calculated by DNA excess hybridization as described.

[c] Data from a separate experiment.

As shown in Fig. 3, HSV-specific poly(A+) RNA found early after infection has a measurably smaller weight average *s* value than viral poly(A+) RNA synthesized late after infection. Some of this difference is likely due to the smaller average number of (A) residues on poly(A+) RNA early after infection (Fig. 4). It is unlikely that this is the whole explanation, since there is a readily detectable light component of viral RNA seen in early poly(A−) RNA also (Fig. 6). Silverstein et al. (18) have found that the poly(A) tracts in HSV-infected cells labeled from 2 to 8 h after infection are similar to that shown in Fig. 4B. This suggests that, whatever the reason for shorter poly(A) tails being on RNA made early after infection, these short poly(A) sequences are transitory.

Although we can readily detect polyadenylated HSV RNA, we have also observed the presence of large amounts of HSV RNA lacking these sequences on polyribosomes both early and late after infection (Table 2). The extent of the annealing of excess RNA to HSV DNA shown in Fig. 2 and 5 demonstrates that there is little, if any, difference in the sequences of poly(A+) and poly(A−) HSV RNA from polyri-

TABLE 4. *Effect of cordycepin on proportion of Poly(A+) HSV RNA in infected HeLa cells*[a]

Expt	Nucleus (cpm × 10^{-5})[a]						Polyribosomes (cpm × 10^{-4})					
	Total RNA			HSV RNA			Total RNA			HSV RNA		
	(A+)	(A−)	Total	(A+)	(A−)	Total	(A+)	(A−)	Total	(A+)	(A−)	Total
Cells labeled 5 h after infection[b]	6.4	39	45	2.7	5.8	8.5	10	10	20	8.7	4.7	13.4
Cells labeled 5 h after infection in the presence of 50 μg of cordycepin per ml[c]	2.5	15.8	18.3	0.38	3.2	3.6	1.6	5	6.6	0.7	2.6	3.3
Inhibition (%)	61%	60%	60%	86%	45%	58%	84%	50%	67%	92%	45%	75%

[a] Data are given as total radioactivity/4 × 10^7 cells; HSV-specific RNA was calculated from DNA excess hybridization as described.

[b] Cells were infected at a multiplicity of infection of 30 PFU HSV-1/cell. Cells were labeled with 15 μCi of [^{3}H]uridine per ml for 45 min.

[c] Cordycepin was added 5 min prior to labeling and was present during the labeling period.

bosomes at either time after infection. It is clear, however, that there is a significant fraction of the polyribosome-associated viral poly(A−) RNA that is larger in size than the corresponding viral poly(A+) material (Fig. 3 and 6).

The larger poly(A−) viral transcripts found on polyribosomes approach the size of the large viral poly(A−) RNA found in the nucleus, which we have observed to sediment as fast as the 45*S* precursor to rRNA. Even though there are viral sequences on the polyribosomes significantly larger in size than 28*S* rRNA, the proportion of total polyribosomal HSV RNA of this size is low compared to the major fraction cosedimenting in the size range of 18*S* rRNA or smaller. Since poly(A−) RNA is the predominate class of viral RNA in the nucleus (ca. 80%) and the majority of viral RNA is found in the nuclear fraction (Table 2), it is clear that, as has been previously estimated (25), the weight average *s* value of total viral RNA in the nucleus exceeds that of total viral RNA found on polyribosomes.

Early after infection, the poly(A+) and poly(A−) HSV RNAs from polyribosomes have sequences homologous to 20% of the viral DNA. This value corresponds to the major class of total viral RNA found at this time after infection (4, 11, 20, 21, 23). Late after infection, viral RNA homologous to 35 to 40% of HSV DNA is found on polyribosomes. More of the viral DNA is represented as poly(A−) and poly(A+) transcripts in the nucleus late after infection (50%) than on polyribosomes (35 to 40%; Fig. 2 and 5). This result indicates a restriction of some viral sequences to the nucleus late after infection. Roizman et al. (15) first described such a restriction of sequences to the nucleus in studying total polyribosome-associated HSV RNA. The restriction of some poly(A)-containing sequences to the nucleus indicates that, with HSV, polyadenylation of RNA is not in itself sufficient to insure transport to the cytoplasm, in agreement with the finding with simian virus 40 by Aloni (1).

The results reported here clearly demonstrate that a significant portion of the HSV-specific poly(A−) RNA associated with the polyribosomes is actually bound to polyribosomes and not in a ribonucleoprotein complex of unknown function. The data of Fig. 7 and Table 3 show that the major portion of viral poly(A−) RNA sediments with the polyribosomes themselves. It is also shown in Table 3 that EDTA treatment of material sedimenting as polyribosomes releases viral poly(A+) and poly(A−) RNA in the same proportion as found in the intact polyribosomes. Further, this viral poly(A−) RNA found on polyribosomes contains methylated 5′ caps and internally located methylated species as do viral poly(A+) mRNA molecules (Moss et al., manuscript in preparation). Such methylation is consistent with this poly(A−) RNA functioning as mRNA.

HSV-specific poly(A−) RNA is not a metabolic derivative of viral poly(A+) RNA. In infected cells labeled in the presence of cordycepin, the appearance of viral poly(A−) RNA on polyribosomes is inhibited at the same level as is total RNA synthesis, whereas there is a virtually complete inhibition of appearance of radioactivity into the poly(A+) fraction in both the nucleus and cytoplasm (Table 4). Such a result demonstrates that poly(A−) RNA does not require polyadenylation for transport to the cytoplasm.

HSV-specific poly(A−) RNA reported in this paper shares many features with the uninfected HeLa cell cytoplasmic poly(A−) RNA described by Milcarek et al. (9); it is associated with polyribosomes in a manner expected for mRNA, it is larger than polyribosomal poly(A+) RNA, and its appearance is resistant to the action of cordycepin. Although this material appears similar to that found in uninfected cells, it is clear from the data of Fig. 2A and 5 that the HSV-specific poly(A−) RNA on polyribosomes shares the same sequences as the viral poly(A+) RNA, in contrast to the situation in uninfected cells as reported by Penman and coworkers (9).

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