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Transcription of the Herpes Simplex Virus Genome in Human Cells

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We have examined the details of the transcription of the herpes simplex virus (HSV) genome in HeLa cells using deoxyribonucleic acid-ribonucleic acid (DNA-RNA) hybridization. The following findings are reported. (i) Virus-specific RNA (vRNA) synthesized following onset of HSV-DNA replication (L-vRNA) is complementary to as much as 90% of the HSV genome. (ii) There is no significant class of virus-specific RNA synthesized later than L-vRNA. (iii) The vRNA synthesized prior to HSV-DNA replication (E-vRNA) is composed of two classes; one class comprising 75% of the total E-vRNA is found in large amounts as early as 45 min after infection, whereas the other class making up the other 25% of E-vRNA is found in only small amounts at 1.5 hr after infection. This second class of E-vRNA is found in amounts comparable to the first by 3.5 hr after infection. (iv) Inhibition of HSV-DNA synthesis results in the continued synthesis of E-vRNA, but there is no synthesis of L-vRNA. (v) Finally, there is no class of vRNA found in the nucleus that is not found associated with cytoplasmic polyribosomes either early or late after infection.

Regulated transcription of the genome of a number of deoxyribonucleic acid (DNA)-containing animal viruses has been observed (4–6, 9, 10). In all cases it has been shown that one class of virus-specific ribonucleic acid (RNA) is synthesized prior to replication of viral DNA whereas a second class is synthesized only after viral DNA synthesis has begun. Previous work from this laboratory has established that the transcription of the herpes simplex virus (HSV) genome is also controlled (11). We demonstrated that virus-specific RNA (vRNA) synthesized just prior to the inception of viral DNA (HSV-DNA) synthesis (E-vRNA) shares sequences with only 30 to 40% of that vRNA transcribed following HSV-DNA replication (L-vRNA). In this communication we have presented the results of experiments designed to further characterize the details of this transcriptional control.

MATERIALS AND METHODS

Cells. HeLa cells were grown in monolayer culture in Eagle minimal essential medium (EMEM; reference 3) containing 10% calf serum (Flow Labs, Rockville, Md.) and no antibiotics. Cells were routinely assayed for pleuropneumonialike organisms as described previously (11).

Virus. The details of the growth and assay of the F strain (type 1) of HSV adapted for growth in HeLa cells have been previously published (8, 11).

Hydroxyurea. Hydroxyurea (HU) was purchased from Calbiochem (Berkeley, Calif.). Except where noted, all experiments were carried out by adding HU immediately after infection.

Infection. Cells were infected at a multiplicity of infection of 20 to 25 plaque-forming units (PFU) per cell. Virus was absorbed for 2 hr at 37 C. The cell layer was rinsed and overlaid with EMEM containing nonessential amino acids and 5% calf serum. Time after infection was calculated from the time of addition of this overlay medium.

Radioactive labeling. Radioactive RNA was prepared, unless otherwise stated, by incubating infected cells for 60 min in overlay medium containing filter-sterilized, dialyzed calf serum and 15 μCi of ^{3}H-uridine per ml (20 Ci/mM, Schwarz Bioresearch, Orangeburg, N.J.). E-vRNA was isolated from cells incubated with ^{3}H-uridine from 2.5 to 3.5 hr after infection, whereas labeled L-vRNA was isolated from cells incubated with radioactive uridine from 5.5 to 6.5 hr after infection.

Fractionation of nuclei and cytoplasm. Isolation of cytoplasmic polyribosomes and purification of RNA for hybridization have been described in detail (11, 13). Briefly, infected cells were fractionated into nuclei and cytoplasm with 0.5% Nonidet P-40 (Shell Chemical Co.) in reticulocyte standard buffer (RSB; reference 14). Cytoplasmic polyribosomes were isolated by centrifugation of the cytoplasmic extract for 4 hr at 50,000 rev/min through a 2-ml pad of 50% (w/w) sucrose in RSB in the Spinco 50 rotor.

Purification of RNA. RNA was extracted from the

nuclear and cytoplasmic fractions by using phenol-sodium dodecyl sulfate (SDS; reference 13). Nuclear RNA sedimenting more rapidly than 12S and polyribosomal RNA sedimenting more rapidly than 8S were purified by sucrose density gradient centrifugation followed by ethanol precipitation. Precipitated RNA was collected by centrifugation and dissolved in 4 × SSC (SSC is 0.15 M sodium chloride and 0.015 M sodium citrate). Calf thymus RNA was prepared by two phenol extractions of the commercial product (Nutritional Biochemicals, Chagrin Falls, Ohio) followed by ethanol precipitation and was collected by centrifugation.

DNA-RNA hybridization. The details of the purification of HSV-DNA have been described (11). Hybridization was carried out for 18 to 22 hr at 72 C using alkali-denatured HSV-DNA immobilized on cellulose nitrate filter discs and purified RNA in 4 × SSC as described in detail elsewhere (11). Repeated control experiments using radioactive DNA bound to filter discs demonstrated that more than 90% and usually 100% of the denatured DNA remained bound to the filter discs under our hybridization conditions. Competition experiments were performed by incubating the HSV-containing filter discs with unlabeled competing RNA for 18 hr prior to the addition of radioactive RNA to the competition mixture. The amount of RNA in each unlabeled sample was adjusted to the same value with calf thymus RNA. After hybridization with radioactive RNA for 20 to 22 hr at 72 C, the discs were rinsed at 37 C with 2 × SSC, digested for 1 hr at 37 C with 250 μg of heat-treated pancreatic ribonuclease in 5 ml of 2 × SSC, and then extensively rinsed with 2 × SSC at 37 C.

RESULTS

Determination of the percentage of the HSV genome transcribed following the onset of HSV-DNA synthesis. Radioactive L-vRNA was prepared from infected cells by labeling with 10 μCi of ^{3}H-uridine per ml for 60 min at 5.5 hr after infection. Filter discs containing 1 μg of HSV-DNA were hybridized with increasing amounts of this radioactive RNA to determine the saturation level of L-vRNA (Fig. 1A). This saturation curve was replotted as a double reciprocal plot (Fig. 1B) to estimate the maximum saturation level of the L-vRNA; this value was calculated to be 3,000 counts/min of L-vRNA bound to 1 μg of HSV-DNA.

The specific radioactivity of this preparation of L-vRNA was determined as follows. A filter disc containing 25 μg of denatured HSV-DNA was preincubated for 18 hr at 72 C in 4 × SSC and extinsively rinsed with 4 × SSC. Subsequently, L-vRNA (10^6 counts/min) was hybridized to the HSV-DNA for 20 hr. The amount of ribonuclease-resistant radioactivity bound to the HSV-DNA was determined, and this RNA was eluted by incubation in 0.1 × SSC containing 1 mM ethylenediaminetetraacetic acid (EDTA) in a boiling water bath for 10 min. The amount of RNA eluted was determined by using a modified orcinol reaction (2) and its radioactivity was determined under conditions identical to those used for the determination of the amount of radioactivity bound to the discs in the saturation experiment of Fig. 1. The optical absorbance of the eluted RNA following the orcinol reaction was corrected for the absorbance of the material eluted from a filter disc containing 25 μg of HeLa cell DNA and mock hybridized in 4 × SSC alone; this value was

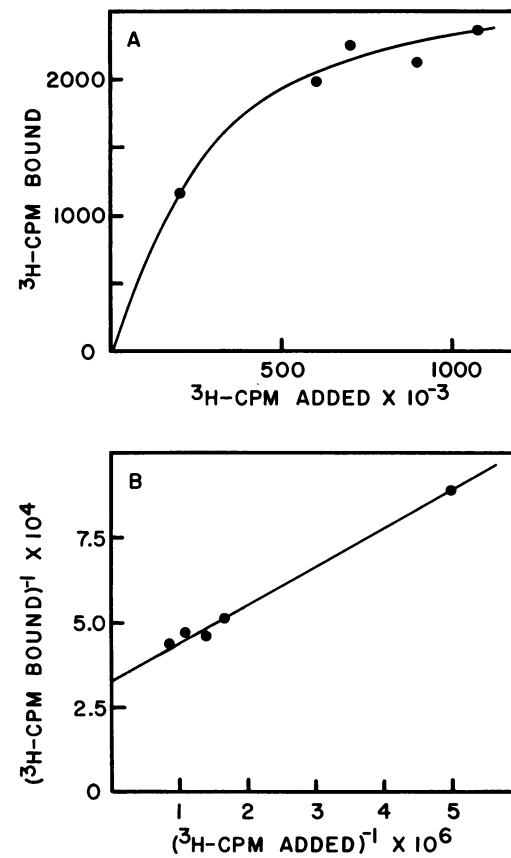

FIG. 1. *Saturation of HSV-DNA with L-vRNA. A, Nuclear L-vRNA labeled with ^{3}H-uridine was purified as described in Materials and Methods. Increasing amounts of this RNA were added to vials containing a disc with 1 μg of HSV-DNA and a blank disc. The total amount of RNA added to each vial was adjusted to 1 mg using uninfected HeLa cell nuclear RNA. Hybridization was for 22 hr at 72 C. Discs were incubated in 5 ml of 2 × SSC containing 250 μg of pancreatic ribonuclease for 1 hr at 37 C and rinsed extensively with 2 × SSC at 37 C. Background radioactivity was 0.1% or less of input radioactivity. B, Replot of the data of Fig. 1A as a double reciprocal plot. The intercept corresponds to 3,000 counts/min.*

10% of the total absorbance of the eluted RNA sample. The results of this experiment are summarized in Table 1; the specific radioactivity of the L-vRNA used in the saturation experiment was calculated to be 6,500 counts per min per μg. The maximum saturation level determined in Fig. 1B of 3,000 counts/min of L-vRNA then corresponds to 0.46 μg of L-vRNA saturating 1 μg of HSV-DNA.

Absence of a major class of vRNA synthesized subsequent to the synthesis of L-vRNA. Hybridization competition was carried out with unlabeled purified L-vRNA and radioactive purified nuclear RNA isolated from cells incubated for 90 min with ^{3}H-uridine at 9 hr after infection. As shown in Table 2, preincubation of HSV-DNA with 1.2 mg of unlabeled nuclear L-vRNA results in essentially complete competition for the hybridization of radioactive vRNA labeled at the later time.

TABLE 1. *Determination of specific radioactivity of L-vRNA*

^{3}H added (counts/min)	HSV-DNA on disc (μg)	Ribonuclease-resistant ^{3}H[a] (counts/min)		RNA eluted[b] (μg)	Specific activity of RNA (counts/min/μg)
		Bound	Eluted		
1,000,000	25	71,340	44,830	6.9	6,500
1,000,000	0	840	170	0	

[a] Hybridization was carried out in 4 × SSC (0.15 M sodium chloride and 0.015 M sodium citrate) at 72 C for 20 hr. Discs were incubated 60 min at 37 C in 10 ml of 2 × SSC containing 500 μg of pancreatic ribonuclease. Elution was carried out by incubating discs for 10 min at 100 C in 0.5 ml of 0.1 × SSC containing 1 mM EDTA, *p*H 7.4.

[b] Determined with the alcoholic orcinol method and corrected for the absorbance of the eluant of a disc containing 25 μg of HeLa DNA mock hybridized in 4 × SSC. Purified calf thymus RNA was used as a standard.

TABLE 2. *Hybridization competition between ^{3}H-nuclear vRNA labeled from 9 to 10.5 hr after infection and unlabeled L-vRNA*[a]

Unlabeled competing RNA preincubated with 1 μg HSV-DNA			^{3}H radioactive RNA added (counts/min)	Net ribonuclease-resistant ^{3}H bound[b] (counts/min) (mg)
Calf thymus	Uninfected HeLa nuclear	L-vRNA		
1.3			150,000	1,615 (70)
	1.25		150,000	1,700 (100)
		1.1	150,000	160 (40)

[a] Competition experiments were carried out as described in Materials and Methods and Fig. 2.

[b] Number in parentheses indicates radioactivity measured in counts per minute bound to blank disc.

Evidence for the existence of two classes of E-vRNA. A study was made of the sequence homology between E-vRNA isolated at 3.5 hr after infection and vRNA synthesized prior to 2.5 hr after infection. Purified nuclear RNA was isolated from HeLa cells at 45 min and at 90 min after infection. Nucleotide sequences of the vRNA in these preparations were compared with those of unlabeled E-vRNA by hybridization competition with radioactive E-vRNA (Fig. 2). The 45-min RNA competes less efficiently than does 3.5-hr RNA isolated from a comparable number of cells. It is also evident that there are vRNA sequences synthesized between 2.5 and 3.5 hr after infection which are not present in the 45-min RNA since the competition is not complete. The competition by the 90-min RNA shows equivalent amounts of the vRNA present after 45 min since the amount of competition with small amounts of both types of RNA is nearly identical. Also, in the 90-min

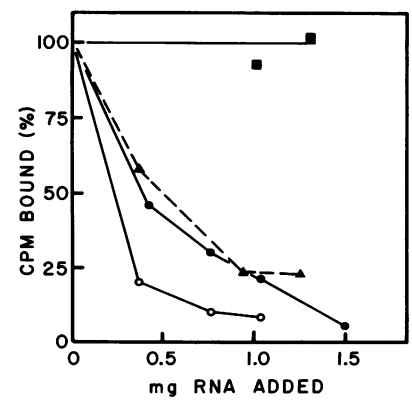

FIG. 2. *Competition between unlabeled nuclear vRNA isolated very early after infection and radioactive E-vRNA. Vials containing a disc with 1 μg of HSV-DNA and a blank disc were preincubated for 18 hr at 72 C with increasing amounts of: (i) unlabeled nuclear RNA from cells 45 min after infection (--▲--▲--); (ii) unlabeled nuclear RNA from cells 1.5 hr after infection (-●-●-); (iii) unlabeled nuclear RNA from cells 3.5 hr after infection (-○-○-); or (iv) uninfected HeLa cell RNA (-■-■-). The total amount of unlabeled RNA in each viral was adjusted to 1.5 mg with calf thymus RNA. After preincubation, 110,000 counts/min of ^{3}H-uridine labeled nuclear E-vRNA was added to each vial and hybridization carried out for 22 hr. Discs were incubated with pancreatic ribonuclease and rinsed with 2 × SSC as described in Fig. 1. Percentage net ribonuclease resistant radioactivity bound was calculated from the value found for a disc preincubated with 1.2 mg of calf thymus RNA (800 counts/min). Background varied from 40 to 60 counts/min.*

sample, there are now present those other sequences which are present in the 3.5-hr vRNA. The efficiency of competition is low, however, indicating that this vRNA is present in smaller amounts than in the 3.5-hr sample.

A second experiment was carried out to confirm the finding that the vRNA synthesized at 45 min after infection does not contain all the sequences in 90-min or later E-vRNA. Unlabeled purified nuclear RNA was isolated from cells at 45 min after infection and its sequences were compared to nuclear RNA labeled from 1 to 2 hr after infection by hybridization competition (Table 3); it can be seen that amounts of unlabeled 45 min RNA equivalent to that required for full competition with later unlabeled RNA do not completely compete with the hybridization of radioactive 90-min vRNA, but rather reach a maximum of 70% competition. This level of competition with the 45-min RNA is the same as that found when it is used to compete for hybridization of E-vRNA (Fig. 2).

Effect of HU on DNA synthesis and virus production in HSV-infected HeLa cells. A culture of HeLa cells was incubated for 15 min with 5 μCi of ^{3}H-thymidine 2 hr after the addition of 2.5 mM HU. The culture was lysed with 1% sarcosyl and sonically treated. Incorporation of radioactivity into DNA was determined by trichloroacetic acid precipitation and compared to that of an untreated control culture. The presence of 2.5 mM HU resulted in a 95% inhibition of incorporation of ^{3}H-thymidine into DNA (175 counts per min per 10^6 cells vs. 3,865 counts per min per 10^6 cells in the untreated control). In a second similar experiment, the effect of 2.5 mM HU on ^{3}H-thymidine incorporation into DNA of HSV-infected HeLa cells at 6 hr after infection was determined. In the culture incubated with HU immediately after infection, 196 counts per min per 10^6 cells of ^{3}H-thymidine was incorporated whereas, in the untreated infected culture, 2,196 counts per min per 10^6 cells were recovered in DNA.

TABLE 3. *Hybridization competition between ^{3}H-nuclear vRNA labeled from 1 to 2 hr after infection and unlabeled 45 min vRNA*[a]

Unlabeled competing RNA preincubated with 1 μg of HSV-DNA[b] (mg)			^{3}H radioactive RNA added (counts/min)	Net ribonuclease-resistant ^{3}H bound[c] (counts/min)	Maximum hybridization (%)
Uninfected HeLa nuclear RNA	90-min vRNA	45-min vRNA			
0.8			80,000	800 (30)	100
	1.0		80,000	99 (47)	12
		0.36	80,000	300 (50)	37
		1.1	80,000	230 (30)	29

[a] Competition experiments were carried out as described in Materials and Methods and Fig. 2.

[b] Total concentration of RNA in each vial was adjusted to 1.2 mg with calf thymus RNA.

[c] Number in parentheses indicates radioactivity measured in counts per minute bound to blank disc.

Cultures of HeLa cells were infected with 0.1 PFU per cell of HSV and incubated for various times with 2.5 mM HU as shown in Table 4. Continued presence of this drug throughout a 20-hr incubation period results in better than a 100-fold decrease in infectious virus production. The effects of the drug are reversible, however, as seen by the fact that the presence of the drug only during the first 6 hr after infection, or a portion thereof, followed by its removal does not affect virus yield (Table 4).

Size distribution of RNA made late after infection in the presence of HU. A culture of infected HeLa cells was incubated with 2.5 mM HU. At 5.5 hr after infection, the cells were incubated for 60 min with ^{3}H-uridine in the presence of HU, and nuclear RNA was isolated. The size distribution of this nuclear RNA was determined by sucrose gradient centrifugation (Fig. 3). In contrast to the sedimentation pattern for radioactive RNA from cells labeled late after infection in the absence of HU (Fig. 3A), there is an unresolved peak of radioactive 32*S* and 28*S* precursor to cytoplasmic ribosomal RNA (rRNA) (Fig. 3B). This is similar to the sedimentation pattern of nucear RNA labeled early after infection (13).

The size distribution of nuclear vRNA in HU-treated cells at this time after infection was determined by hybridizing subsaturating amounts of radioactive nuclear RNA with vDNA. The results of this experiment are shown in Table 5; it is evident that vRNA is found throughout all size ranges of RNA tested in relative amounts comparable to those reported previously for E-vRNA (11).

Sequence homology between L-vRNA synthesized in the presence of HU and E-vRNA. Hybrid-

TABLE 4. *Effect of HU on HSV production in infected HeLa cells*[a]

Time of incubation of cells with 2.5 mM HU (hr after infection)	Total HSV produced/culture (PFU)[b]
Control	8×10^5
0 to 4	10^6
0 to 6	1.2×10^6
4 to 6	6×10^5
0 to 20	$< 10^4$

[a] Cultures were infected at a multiplicity of 0.1 PFU/cell.

[b] Titrations were carried out as described (11).

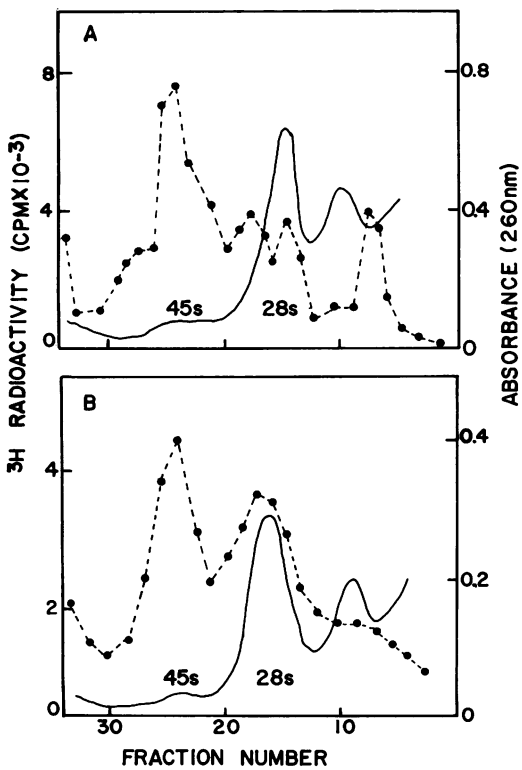

FIG. 3. *The effect of HU on the size distribution of nuclear RNA labeled late after infection. A, A culture of 3 × 10⁷ HeLa cells was incubated with 5 μCi/ml ³H-uridine for 60 min at 5.5 hr after infection. Nuclear RNA was extracted and fractionated by centrifugation on a 16-ml 15–30% sucrose, 0.5% SDS gradient for 18 hr at 20,000 rev/min in the Spinco SW27 rotor. The gradient was collected through a Gilford recording spectrophotometer and 0.6 ml fractions collected. The RNA in each fraction was precipitated with 5% trichloroacetic acid and collected by filtration onto glass fiber filters. Radioactivity was determined in a Beckman scintillation counter using the toluene scintillator described previously (12) (——, absorbance at 260 nm; -●--●--, radioactivity). B, A culture of 10⁷ infected HeLa cells was incubated at 2.5 mM HU and then incubated with ³H-uridine at 5.5 hr after infection for 60 min. Fractionation was carried out as in A.*

ization competition experiments were carried out using unlabeled purified nuclear RNA isolated from HeLa cells incubated with 2.5 mM HU for 7 hr after infection and radioactive purified L-vRNA. As seen in Fig. 4, it is apparent that vRNA synthesized in the presence of HU competes only to a level of approximately 35% when challenged with L-vRNA—essentially the same amount of competition found when unlabeled E-vRNA is used as the competitor (11).

The competition between unlabeled E-vRNA and radioactive vRNA isolated from HU-treated cells labeled for 60 min at 6 hr after infection was compared (Fig. 5). The competition between these species is essentially complete. This result implies

TABLE 5. *Size distribution of late HSV specific nuclear RNA in HeLa cells incubated with 2.5 mM HU after infection*[a]

Size range of RNA (S)	³H RNA incubated with HSV-DNA (counts/min)	Ribonuclease-resistant ³H bound[b] (counts/min)	Comparable percentage of ribonuclease-resistant ³H bound (counts/min) using:[c]	
			E-vRNA	L-vRNA
10 to 28	100,000	800 (0.8)	1	2
28 to 45	160,000	620 (0.4)	0.5	0.5
>45	80,000	350 (0.4)	0.5	1.0

[a] Hybridization was carried out by using 2 μg of HSV-DNA per filter disc. Nuclear RNA was extracted from 4 × 10⁷ infected HeLa cells labeled with 15 μCi/ml of ³H-uridine for 60 min at 5.5 hr after infection. RNA was fractionated on a 34-ml, 15 to 30% sucrose 0.5% SDS gradient by centrifugation for 18 hr at 22,000 rev/min in the Spinco SW27 rotor. RNA was precipitated with 2 volumes of ice cold 95% ethanol, collected by centrifugation, and dissolved in 4 × SSC.

[b] Average of two experiments. Number in parenthesis is percentage input radioactivity bound.

[c] Data from Wagner (11).

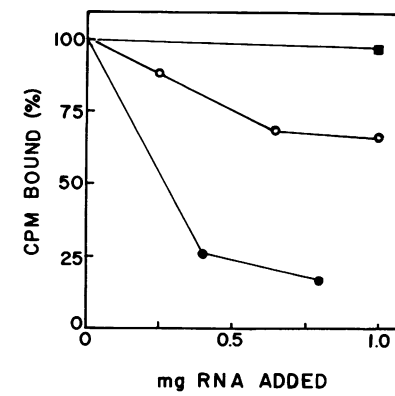

FIG. 4. *Competition between unlabeled nuclear L-vRNA from cells incubated with HU and normal radioactive L-vRNA. Vials containing a disc with 1 μg of HSV-DNA and a blank disc were preincubated with: (i) unlabeled nuclear RNA isolated at 6.5 hr after infection from infected cells incubated with 2.5 mM HU (-○-○-); (ii) normal unlabeled L-vRNA (-●-●-); or (iii) uninfected HeLa cell RNA (-■-). Total amount of unlabeled RNA in each vial was adjusted to 1 mg with calf thymus RNA. After preincubation, 160,000 counts/min of ³H-uridine-labeled L-vRNA was added. The maximum radioactivity bound was 840 counts/min. Details of the experiment are described in Fig. 2.*

that there are no sequences of vRNA synthesized in the presence of HU which are not represented in the vRNA synthesized prior to vDNA replication.

Sequence homology between nuclear and polyribosome-associated vRNA. Sequence homology between nuclear and polyribosome-associated vRNA was examined using hybridization competition experiments with both E-vRNA and L-vRNA; both gave similar results. Unlabeled nuclear polyribosomal RNA isolated from cells at 3.5 hr after infection was tested for competition with radioactive E-vRNA isolated from purified nuclei (Fig. 6). Although the relative amount of RNA in the polyribosome fraction required to give essentially complete competition is greater than that in the unlabeled nuclear fraction, it is evident that there is no class of E-vRNA in the nucleus which is not found associated with polyribosomes at this time after infection.

The same result was obtained in a second parallel experiment using unlabeled nuclear and polyribosomal L-vRNA isolated at 6.5 hr after infection to compete with the hybridization of radioactive nuclear L-vRNA (Fig. 7). In this experiment, also, there is complete competition between polyribosomal and nuclear L-vRNA in hybridization with HSV-DNA. It is also seen late

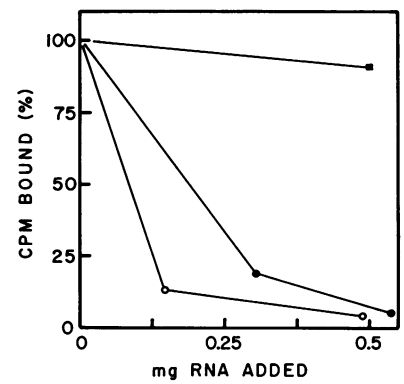

FIG. 6. *Competition between unlabeled polyribosomal E-vRNA and radioactive nuclear E-vRNA. Vials containing a disc with 0.5 μg of HSV-DNA and a blank disc were preincubated with; (i) unlabeled polyribosome-associated RNA from cells isolated at 3.5 hr after infection (-●-); (ii) unlabeled nuclear RNA from cells isolated at 3.5 hr after infection (-○-); or (iii) uninfected HeLa cell RNA (-■-). Total amount of unlabeled RNA in each vial was adjusted to 0.6 mg with calf thymus RNA. After preincubation, 96,000 counts/min of ^{3}H-uridine-labeled nuclear E-vRNA was added to each vial. The maximum radioactivity bound was 250 counts/min. Details of the experiment are as described in Fig. 2.*

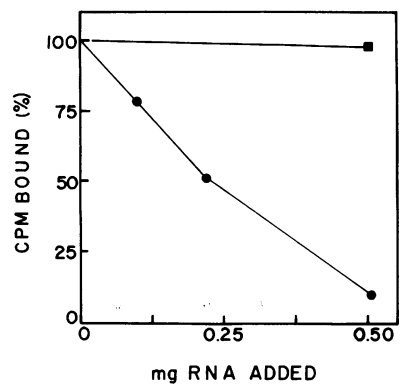

FIG. 5. *Competition between unlabeled nuclear E-vRNA and radioactive L-vRNA synthesized in the presence of HU. Vials containing a disc with 0.5 μg of HSV-DNA and a blank disc were preincubated with: (i), unlabeled nuclear RNA from cells early after infection (-●-); or (ii), RNA from uninfected HeLa cells (-■-). The total amount of RNA was adjusted to 0.5 mg with calf thymus RNA. After preincubation, 45,000 counts/min of ^{3}H-uridine-labeled RNA from infected cells treated with 2.5 mM HU and labeled from 6 to 7 hr after infection was added. The maximum radioactivity bound was 250 counts/min. Details of the experiment are as described in Fig. 2.*

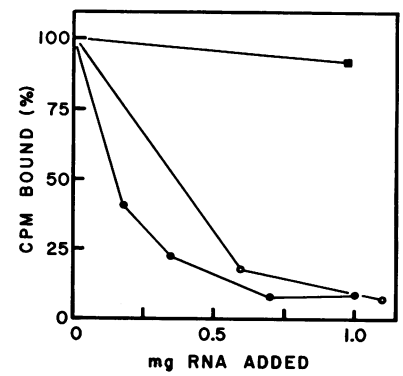

FIG. 7. *Competition between unlabeled polyribosomal L-vRNA and radioactive nuclear L-vRNA. Vials containing a disc with 1 μg of HSV-DNA and a blank disc were preincubated with; (i) unlabeled polyribosome-associated RNA from cells isolated at 6.5 hr after infection (-○-); (ii) unlabeled nuclear RNA from cells isolated at 6.5 hr after infection (-●-); or (iii) uninfected HeLa cell RNA (-■-). Total amount of unlabeled RNA in each vial was adjusted to 1.0 mg with calf thymus RNA. After preincubation, 45,000 counts/min of ^{3}H-uridine-labeled nuclear L-vRNA was added to each vial. The maximum radioactivity bound was 400 counts/min. Details of the experiment are as described in Fig. 2.*

after infection that the relative amount of polyribosomal RNA required for complete competition is greater than that from the nuclear fraction.

DISCUSSION

Extent of transcription of the HSV genome after initiation of HSV-DNA synthesis. It has been previously established that, under the present experimental conditions, HSV-DNA synthesis begins at 4 hr after infection (11); at 5.5 to 6.5 hr after infection, viral DNA synthesis is proceeding at the maximum rate. The amount of the HSV genome transcribed at this time after infection was measured by saturation of HSV-DNA with L-vRNA of known specific activity, in this instance 6,500 counts per min per μg (Table 1). The maximum measured amount of radioactivity bound to 1 μg of HSV-DNA was 2,250 counts/min (Fig. 1A) or 0.35 μg of vRNA. This may be taken as a minimum value. A maximum value was estimated using the double reciprocal plot of the saturation data (Fig. 1B); this value was 3,000 counts/min or 0.46 μg of vRNA saturating 1 μg of HSV-DNA. These values suggest that at least 70% and as much as 90% of a single strand equivalent of the HSV-genome is transcribed after initiation of vDNA synthesis. This range may be taken as the minimum one because such saturation values are insensitive to the presence of classes of vRNA synthesized in significantly smaller amounts than the majority of sequences transcribed during the labeling period.

In HeLa cells at the multiplicity of infection used in these experiments (25 PFU/cell), total RNA synthesis falls to less than 5% of uninfected control values by 11 hr after infection (*unpublished results*). Therefore, the complete competition by unlabeled L-vRNA isolated at 6.5 hr for the hybridization of radioactive vRNA synthesized between 9 and 10.5 hr after infection (Table 2) establishes that there are no additional portions of the HSV genome transcribed very late after infection. We cannot, however, rule out the possibility that there is a selective restriction of the synthesis of some class of E-vRNA by 5 to 6 hr after infection.

Classes of E-vRNA. The saturation level of L-vRNA discussed above and the maximum amount of competition between E-vRNA and L-vRNA reported previously (11) indicate that between 25 and 35% of the HSV genome is transcribed prior to HSV-DNA replication. This E-vRNA can be divided into two classes; 45-min vRNA and later E-vRNA. The 45-min vRNA competes with 75% of the E-vRNA synthesized between 2.5 and 3.5 hr after infection (Fig. 2). This result indicates that the major class of the HSV genes, which are transcribed prior to DNA replication, is transcribed very early after infection. By 90 min after infection, a second class of E-vRNA comprising about 25% of the total is being synthesized. The relative amount of this second class of E-vRNA by 90 min after infection is much lower than it is by 3.5 hr after infection as seen by the slope of the competition curve for the 25% of the radioactive E-vRNA with 90-min RNA as compared to 3.5-hr E-vRNA. There are two obvious explanations for this: (i) the maximum steady state concentraton of this class of vRNA has not been attained by 90 min after infection because its synthesis has just begun, or (ii) the rate of synthesis of this second class of E-vRNA is lower at 90 min than at 3.5 hr after infection. It is not possible, with the experiments described in this paper, to distinguish between these two not necessarily exclusive alternatives.

Requirement for HSV-DNA replication for synthesis of L-vRNA. HU in concentrations between 2 and 5 mM has been shown to inhibit DNA synthesis in HeLa cells without grossly affecting RNA synthesis (7). It is shown in Tables 3 and 4 that, in our hands, 2.5 mM HU effectively inhibited DNA synthesis in uninfected and infected HeLa cells and production of infectious HSV. The sucrose density gradient fractionation of radioactive nuclear RNA labeled in HU-treated cells from 5.5 to 6.5 hr after infection (Fig. 3B) shows that the RNA synthesized in the presence of this drug late after infection is similar in size distribution to uninfected cells. In contrast to this, the sedimentation pattern of radioactive RNA in untreated cells incubated with ^{3}H-uridine at this time after infection (Fig. 3A) shows the characteristic interruption of processing of 45*S* precursor to rRNA characteristic of HSV type I infection and described in detail previously (13).

The vRNA synthesized from 5.5 to 6.5 hr after infection in HU-treated cells has a size distribution similar to that of normal HSV-specific RNA. The data shown in Table 5 demonstrate that there is appreciable high-molecular-weight vRNA synthesized in HU-treated cells as has been previously shown to be the case with vRNA both early and late after infection (11). The sequence homology between vRNA isolated from HU-treated infected cells and normal E-vRNA and L-vRNA was examined using hybridization competition as shown in Fig. 4 and 5. The level of competition between late RNA from HU-treated cells and radioactive L-vRNA is the same as that reported for the competition between unlabeled E-vRNA and radioactive L-vRNA (Fig. 4; reference 11). The complete competition by unlabeled E-vRNA for the hybridization between radioactive late vRNA and

HSV-DNA (Fig. 5) demonstrates that late vRNA from HU-treated cells contains all the sequences of E-vRNA. These results suggest that HSV-DNA replication is a requirement for the synthesis of L-vRNA. Such a requirement for viral DNA synthesis for the synthesis of late viral messenger RNA is a common feature in DNA bacteriophage infection.

Lack of restriction of any sequences of vRNA to the nucleus. The possibility of restriction of a class of vRNA to the nucleus of the infected host was investigated both early and late after infection. It has been shown that use of the detergent NP-40 results in the effective separation of nuclei and cytoplasm (1). It has also been demonstrated that nuclear vRNA has a significantly different size distribution than that of vRNA associated with polyribosomes (13). Although it is not clear that this detergent completely removes all ribosomes and polyribosomes from the nuclear fraction, it is clear that there is no high-molecular-weight nuclear vRNA found in the cytoplasmic fraction. Therefore, the presence of sequences of vRNA restricted to the nucleus would be readily detectable by the less than complete competition by polyribosomal vRNA in the hybridization of vRNA from the nuclear fraction to HSV-DNA. The complete competition shown in Fig. 6 and 7 demonstrates that all sequences of nuclear vRNA synthesized at either time after infection can be found on virus-specific polyribosomes. This result, along with the fact that vRNA associated with polyribosomes effectively competes with the hybridization of fractionated high-molecular-weight nuclear vRNA from cells late after infection (14) shows that there is no portion of the HSV genome transcribed which does not code for viral protein.

It is clear, then, that restricted transport of some classes of vRNA from the nucleus to the cytoplasm has no role in the control of HSV gene function following infection. This is in striking contrast to the situation in adenovirus infection where there is strong evidence that some virus-specific RNA synthesized early after infection does not become available for translation in the cytoplasm until a later time (10).

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