Two Classes of Cytoplasmic Viral RNA Synthesized Early in Productive Infection with Adenovirus 2

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Received for publication 14 May 1974

The RNA sequences and RNA size classes transcribed early in productive infection with adenovirus 2 were analyzed by RNA-DNA hybridization. Two independent procedures demonstrated that early cytoplasmic viral RNA is composed of two sequence classes, class I which is absent or present in greatly reduced quantities at 18 h, and class II which persists throughout the infection. When the sequences in early viral RNA were analyzed by hybridization-inhibition studies, the hybridization of early [^3H]RNA was inhibited only 50% by RNA from cultures harvested late (18 h) in infection. Liquid hybridizations with radioactive viral DNA confirmed that early RNA includes two classes. Duplex formation of RNA with ^{32}P -labeled viral DNA was assayed by hydroxylapatite chromatography and resistance to S_1 nuclease digestion. Both methods showed that the cytoplasmic RNA present early in infection annealed 12 to 15% of the viral DNA; late cytoplasmic RNA hybridized 21 to 25% of the DNA. Mixtures of early plus late cytoplasmic RNAs hybridized 30 to 34% of the viral DNA, demonstrating the reduced concentration of early class I RNA in the late RNA preparations. Experiments were performed to correlate class I and class II early RNA with size-fractionated cytoplasmic RNA synthesized early in infection. Fractionation of RNA by gel electrophoresis or sucrose gradient centrifugation confirmed three major size classes, 12 to 15S, 19 to 20S, and 26S. Total cytoplasmic RNA and RNA selected on the basis of poly(A) content contained the same size classes of viral RNA. In standard electrophoresis conditions, the 19 to 20S viral RNA could be resolved into two size classes, and the distribution of 12 to 15S RNA also indicated the presence of more than one size component. Hybridization-inhibition studies under nonsaturating conditions were performed with 26S, 19 to 20S, and 12 to 15S viral RNAs fractionated by gel electrophoresis. Late RNA inhibited the hybridization of 26S RNA only 20%, 19 to 20S RNA was inhibited 45%, and 12 to 15S RNA was inhibited 50%. When 18 to 19S and 12 to 15S viral RNAs purified by sucrose gradient centrifugation were similarly analyzed, late RNA inhibited hybridization of 18 to 19S RNA 50%, and the annealing of 12 to 15S RNA was inhibited 70%.

The productive infection of cultured human cells by adenovirus 2 includes at least two distinct phases. During the early period, which precedes the onset of DNA replication, only a limited portion of the genome is transcribed into functional viral mRNA (6, 19). In the late period, transcripts of a larger portion of the genome are represented in cytoplasmic mRNA (6, 19). The relationship between the RNA sequences synthesized during the early period, the first 5 to 6 h of infection, and those present much later, at 18 h, is not fully clarified. Lucas and Ginsberg (10) were able to identify a class of RNA sequences which were synthesized between 2 and 6 h after infection, but were either

absent or present in greatly reduced quantities at late times. Other studies concluded that all RNA sequences present at 6 h are also present in high concentrations after 18 h of infection (6, 18, 19).

We have further investigated the relationship between RNA sequences present at early and late times. Detailed studies of transcription early in infection are greatly facilitated by the use of drugs that inhibit protein or DNA synthesis and, therefore, prevent the onset of viral DNA replication (3, 10, 13, 20). Since the beginning of viral DNA synthesis may not be synchronous in all infected cells, drug treatment eliminates possible contamination of early

viral RNA sequences with low concentrations of the sequences synthesized late in infection (3). In these experiments, we analyzed the cytoplasmic viral RNA synthesized in the presence of cycloheximide (CH). The use of CH, an inhibitor of protein synthesis, is particularly advantageous in studying radioactive early viral RNA; RNA labeled in the presence of CH hybridizes to viral DNA approximately 10-fold more than control RNA (3, 11). The cytoplasmic viral RNA synthesized in the presence of CH contains the same early viral sequences as RNA prepared from control-infected cultures (3, 11).

Using both liquid and membrane hybridization techniques to determine sequence relationships, we confirmed the existence of a class of early RNA sequences which was present in at least a 16-fold lower concentration at 18 h than at 6 h. In addition, we extended the previous size characterizations of the early cytoplasmic RNAs (8, 11). Hybridization-inhibition experiments were performed to begin to define the relationship between the size-fractionated early RNAs and the two sequence classes of early RNA.

MATERIALS AND METHODS

Cell culture, virus infection, and cell fractionation. Exponentially growing KB suspension cultures were infected with adenovirus 2 purified by equilibrium centrifugation in cesium chloride (3, 7, 15). Cultures harvested 6 h after infection were infected at a multiplicity of 100 PFU per cell and were diluted to 9×10^5 cells per ml after a 1-h adsorption period. In many experiments, 25 μ g of CH per ml (Sigma Chemical Co., St. Louis, Mo.) were added at the time of dilution. Cultures were labeled with 12.5 μ Ci of [3 H]uridine per ml (40 Ci/mmol, New England Nuclear Corp., Boston, Mass.) from 2 to 6 h after infection. Cultures harvested 18 h after infection were diluted to 3×10^5 cells per ml after the 1-h adsorption period. Cytoplasmic extracts were prepared as described by Craig and Raskas (3).

RNA purification and fractionation. RNA in cytoplasmic extracts was purified by the addition of 0.5% sodium dodecyl sulfate (SDS), followed by three extractions with equal volumes of water-saturated phenol and chloroform-isoamyl alcohol (at a ratio of 24:1) at room temperature (3). The RNA was precipitated by the addition of 2 volumes of 95% ethanol in the presence of 0.15 M NaCl.

Molecules containing poly(A) were separated from molecules lacking poly(A) by selective retention on oligo(dT)-cellulose (Collaborative Research, Inc.) (1). RNA was applied to an oligo(dT)-cellulose column (0.5 by 1 cm) in 0.5 M KCl and 0.01 M Tris-hydrochloride (pH 7.5). The column was then washed with 15 ml of the same buffer, and the poly(A)-containing RNA was eluted in 2 ml of 0.01 M Tris-hydrochloride (pH 7.5).

Early [3 H]RNA was fractionated on 11-cm 3.2 and 4.8% ethylene diacrylate cross-linked acrylamide gels containing 10% glycerol (2, 9, 21). Gels were frozen and sliced into 2-mm fractions. Gel samples to be counted directly were solubilized in ammonium hydroxide and counted in a scintillation counter (15). Unless noted otherwise, gel slices used in the hybridizations were solubilized by incubating at 60°C in $6 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS. Slices from 3.2% gels were solubilized in 100 μ liters of liquid; 200 μ liters were added to 4.8% gel slices. Solubilization required a minimum of 8 h, but generally, gel slices were incubated overnight prior to further analysis.

RNA-DNA hybridizations. All filter hybridizations were performed with adenovirus 2 DNA immobilized on 6.5-mm cellulose nitrate membranes (type B6, Schleicher and Schuell Co., Keene, N.H.) (14). Hybridizations of [3 H]RNA from solubilized gel slices were incubated for 20 h at 66°C. Filters were then washed with $2 \times$ SSC, treated with pancreatic RNase (20 μ g per ml in $2 \times$ SSC) for 1 h at room temperature, and washed again with $2 \times$ SSC.

Two-step hybridization-inhibition experiments were performed in a solution containing 30% formamide, $3 \times$ SSC, 0.1% SDS, 0.01 M *N*-Tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid, pH 7.4, at 45°C. In the first step, increasing concentrations of unlabeled RNA in 100 μ liters were incubated for 20 h with filters containing 0.02 or 0.5 μ g of adenovirus 2 DNA. After incubation, the filter was washed three times with $2 \times$ SSC and treated with 20 μ g of pancreatic RNase per ml for 45 min at room temperature (11). The filter was then treated with 0.15 M iodoacetic acid to inhibit RNase activity (17, 20) and was washed again with $2 \times$ SSC. 3 H-labeled RNA was then added for a second incubation of 20 h, after which the filter was washed three times at 60°C with $2 \times$ SSC and treated with pancreatic RNase as before. The hybridizations performed with 0.02 μ g of DNA were performed by using saturating amounts of [3 H]RNA. The amount of RNA required to saturate 0.02 μ g of DNA was determined before the hybridization-inhibition experiments were performed. Typical saturation profiles are shown in Craig and Raskas (3).

Liquid phase hybridizations were carried out with 32 P-labeled adenovirus DNA (0.5 $\times 10^6$ counts per min per μ g). 32 P-labeled virus was prepared by performing infections in phosphate-free Joklik minimal essential medium supplemented with 2.5% horse serum. [32 P]phosphoric acid (60 μ Ci/ml; New England Nuclear) was added 6 h after infection, and the cultures were harvested after 30 h. The virus was purified, and viral DNA was extracted as described previously (7). Prior to hybridizations, DNA was fragmented by sonic treatment and was denatured by boiling for 20 min.

The fraction of DNA present as hybrid was determined by two methods, hydroxylapatite chromatography or digestion with the single-strand-specific nuclease (S_1) from *Aspergillus oryzae*. Preparations to be analyzed by hydroxylapatite chromatography were hybridized in 0.4 M sodium phosphate, pH 6.8.

Hybridizations were terminated by diluting 100- μ l samples into 5 ml of 0.12 M sodium phosphate buffer containing 0.4% SDS. Each sample was absorbed to 0.6 g of hydroxylapatite (Biorad, DNA grade). The binding and all subsequent washings were carried out at 60 C in centrifuge tubes as described by Fanshier et al. (4). Single-stranded DNA was removed by a 5-ml wash with 0.12 M phosphate buffer containing 0.4% SDS. Complete elution (100%) of double-stranded material was achieved by two successive washes with 5 ml of 0.4 M sodium phosphate, pH 6.8. Calf thymus DNA (5 μ g/ml) was added as carrier, and the two fractions were precipitated with 5% trichloroacetic acid, filtered, dried, and counted.

Preparations analyzed by S_1 nuclease digestion were hybridized in 1.2 M NaCl and 0.01 M Tris-hydrochloride, pH 7.0. S_1 nuclease was purified from diastase powder (Sigma Chemical Co.) as described by Sutton (16). The reaction mixtures contained 500 U of enzyme, 0.03 M sodium acetate buffer (pH 4.5), 3×10^{-6} M $ZnCl_2$, 0.12 M NaCl, 20 μ g of calf thymus DNA, and 1,000 to 10,000 counts of [^{32}P]DNA per min. Nuclease digestion was performed for 2.5 h at 50 C. In these conditions, 100% of native adenovirus DNA and only 0 to 2% of denatured DNA remained acid precipitable.

RESULTS

Relationship between RNA sequences synthesized early after infection and those sequences present at late times. Two types of experimental procedures were used to determine the relationship between the RNA sequences transcribed early and those present at late times.

First, hybridization-inhibition experiments were performed with membrane-bound viral DNA. When saturating amounts of early cytoplasmic [3H]RNA were used, whole-cell RNA synthesized at early times inhibited hybridization more than 90%. Whole-cell RNA from late times reduced the hybridization of early RNA only 40 to 50% (Fig. 1A). Hybridizations performed under nonsaturating conditions yielded the same results: when 25 times more DNA and 5 times less [3H]RNA were used (Fig. 1B), late RNA inhibited hybridization only 50 to 60%. Similar results were obtained when hybridization-inhibition experiments were carried out using poly(A)-containing RNA as both inhibitor and [3H]RNA (data not shown).

Second, liquid-phase hybridizations confirmed the existence of two classes of early RNA. ^{32}P -labeled viral DNA was annealed with early cytoplasmic RNA, late cytoplasmic RNA, and a mixture of the early and late RNA. The resulting hybrids were detected by two methods, chromatography on hydroxylapatite (Fig. 2A) and digestion by the single-strand-

FIG. 1. Inhibition of early cytoplasmic RNA hybridization by late whole-cell RNA. Cytoplasmic [3H]RNA was isolated from cells treated with CH and harvested 6 h after infection. Increasing amounts of [3H]RNA (specific activity of 40,000 counts per min per μ g) were hybridized to 0.02 μ g of adenovirus DNA; 7.5 μ g of RNA were required for saturation. A two-step hybridization-inhibition experiment was performed with 0.02 (A) and 0.5 μ g (B) of adenovirus 2 DNA immobilized on a filter. RNA from uninfected cultured human cells ($\square$), cells treated with CH and harvested 6 h after infection ($\bullet$), and cells harvested 18 h after infection ($\circ$) were used as competitor RNAs. The hybridization performed in saturating conditions (A) used 4×10^5 counts of input [3H]RNA per min, and 100% hybridization corresponded to 410 counts/min. In nonsaturating conditions (B), 6×10^3 counts of [3H]RNA per min were used, and 2,300 counts/min hybridized to 0.5 μ g of DNA.

specific S_1 nuclease from *A. oryzae* (Fig. 2B). With both techniques, early RNA formed a hybrid with 12 to 15% of the [^{32}P]DNA. Late cytoplasmic RNA converted 21 to 25% of the DNA to double-stranded material. The mixture of early plus late RNA increased the hybrid formation 30 to 34%, the amount expected if approximately half of the early RNA sequences were absent or in greatly reduced quantities 18 h after infection.

Size distribution of viral RNA synthesized early in infection. To enable further analysis of early viral RNA, the size distribution of the early viral mRNA was studied. Since most adenovirus mRNA contains poly(A) (8, 13), studies of viral RNA size classes were performed on the poly(A)-containing fraction of cytoplasmic RNA synthesized early in infection. Preparations labeled in the absence or presence (Fig. 3A and B) of CH were fractionated by polyacrylamide gel electrophoresis, and the RNA in each gel slice annealed to viral DNA. The size distribution of viral RNA in both types of preparations was similar, containing three size classes of viral RNA which migrated as 26S,

FIG. 2. Liquid-phase hybridization of ^{32}P -labeled adenovirus 2 DNA with early, late, and a mixture of early and late cytoplasmic RNAs. Formation of RNA-DNA hybrids was quantitated by hydroxylapatite chromatography (A) and single-strand-specific S_1 nuclease digestion (B). The samples assayed by hydroxylapatite chromatography (A) were obtained by incubating 56,000 counts of ^{32}P -DNA per min per ml with 0.66 mg of early RNA per ml ($\square$), 1.0 mg of late RNA per ml ($\circ$), or a mixture of 0.66 mg of early RNA per ml and 1.0 mg of late RNA per ml ($\blacktriangle$). (B) depicts the results of hybridization of 50,000 counts of ^{32}P -DNA per min per ml with 1 mg of early RNA per ml ($\square$), 1 mg of late RNA per ml ($\circ$), or a mixture of 1 mg of early RNA per ml and 1 mg of late RNA per ml ($\blacktriangle$). Hybridizations were performed at 66°C for the indicated times. The fraction of the DNA-forming hybrid was determined by incubation with S_1 nuclease. RNAs prepared from two different sets of infections were used for the two experiments shown. In both cases, a sample of DNA alone was incubated to determine the background of DNA-DNA reannealing. A portion from this tube was analyzed for each time point, and the amount of RNA-DNA hybrid was corrected for the DNA-DNA duplexes. The maximum correction was 3% of the input DNA counts/min.

19S, and 12 to 15S. The smaller size class often separated into at least two peaks. The same size distribution of viral RNA was obtained when total cytoplasmic RNA was analyzed. These results are basically in agreement with the previous studies of Parsons and Green (11) and Lindberg et al. (8).

When analyzed on 3.2% acrylamide gels, the RNA migrating as 19S sometimes appeared to be split into two components. Therefore, early RNA was analyzed on 4.8% acrylamide gels in an attempt to separate more clearly the two size classes. The partial resolution of the 19S material into two peaks is shown in Fig. 4.

The third size class of viral RNA formed a rather broad peak in the 26S region. The amount of material in this region relative to the 19S peak varied from preparation to preparation. The variation was about the same in preparations purified from control- or CH-treated cultures.

Relationship between size-fractionated early and late RNA. Hybridization-inhibition experiments were performed with the three size classes of ^3H RNA obtained from an acrylamide gel similar to that shown in Fig. 3B. Such experiments might identify early viral RNAs which are no longer present at 18 h. The RNA equivalent to those found in fractions 12 to 15 for 26S viral RNA, in fractions 19 to 22 for 19S RNA, and in fractions 27 to 32 for 12 to 15S molecules was pooled. Because of the limitations in obtaining large amounts of radioactive size-fractionated viral RNA, the hybridization-inhibition experiments were performed under nonsaturating conditions. Although early RNA fully inhibited the hybridization of each size class, late RNA inhibited the hybridization of 26S RNA only 20%, of 19S RNA about 45%, and of 12 to 15S RNA about 55% (Fig. 5A).

FIG. 3. Size fractionation of cytoplasmic viral RNAs synthesized in the presence and absence of CH. Following a 1-h virus adsorption, two cultures were diluted to 165 ml (9×10^6 cells per ml); 25 μg of CH per ml was added to one culture. ^3H uridine (12.5 $\mu\text{Ci/ml}$) was added to both cultures at 2 h. The cells were harvested at 6 h, the cytoplasmic RNA was purified, and the poly(A)-containing RNA was selected by oligo(dT)-cellulose chromatography. ^3H RNA (1.5×10^6 counts/min) harvested from the control culture and 3×10^6 counts of ^3H RNA per min from the culture treated with CH were analyzed on a 3.2% acrylamide gel. Electrophoresis was performed for 6.5 h at 5 mA per gel. After electrophoresis, the gels were sliced and solubilized in 100 μl of $6 \times \text{SSC}$ containing 0.1% SDS. The profile of the total poly(A)-containing RNA ($\circ$) was obtained by counting directly 2 μl from each fraction of the control gel (A) and 10 μl from the gel containing RNA synthesized in the presence of CH (B). The remainder was hybridized to 0.5 μg of adenovirus 2 DNA ($\bullet$). ^{14}C -rRNA was applied to a third gel; the arrows indicate the positions of 28S and 18S rRNA.

The hybridization-inhibition experiments were repeated with 19S and 12 to 15S viral RNA fractionated on sucrose gradients. The three size classes of viral RNA were again detected, although the major viral RNA peak sedimented as 18 to 19S relative to rRNA rather than the 19- to 20S-value obtained from electrophoresis (Fig. 6A). To insure the purity of the 18 to 19S and 12 to 15S size classes, the [^3H]RNA was rerun on a second sucrose gradient (Fig. 6B and C). Using these repurified RNAs, late RNA inhibited the hybridization of the 18 to 19S class about 30%, and inhibited the 12 to 15S class about 60% (Fig. 5B).

DISCUSSION

Two independent hybridization procedures used in this study confirmed that the viral RNA sequences transcribed early in adenovirus 2 infection consist of at least two classes. Using the terminology of Lucas and Ginsberg (10), class I sequences represent cytoplasmic RNAs that are transcribed prior to the onset of DNA synthesis, but are absent or greatly reduced in concentration in cultures harvested late in in-

FIG. 4. Analysis of early cytoplasmic 19 to 20S RNA by electrophoresis on 4.8% acrylamide gels. Cytoplasmic poly(A)-containing [^3H]RNA was purified from infected cells harvested 6 h after infection. [^3H]RNA (5×10^3 counts/min) were analyzed on a 4.8% acrylamide gel (20 h, 5 mA per tube). The RNA in each gel slice was annealed to 0.5 μg of adenovirus 2 DNA ^{14}C -labeled rRNA was applied to a second gel; although the position of the 28S RNA is shown, these molecules did not migrate sufficiently far into the gel to be a useful reference.

FIG. 5. Sequence relationships between size-fractionated early [^3H]RNA and late RNA. Poly(A)-containing early cytoplasmic RNA (1.5×10^6 counts/min) was fractionated by electrophoresis for 6.5 h at 5 mA per gel on a 3.2% acrylamide gel. The gel was sliced, and the RNA was eluted by incubation in 1 ml of high salt-EDTA buffer (0.5 NaCl, 0.01 mM EDTA, and 0.05 M sodium acetate, pH 5.5) for 16 h at 60 C. Portions (90 μl) were removed and hybridized to 0.5 μg of adenovirus DNA. The viral RNA peaks migrating as 24 to 27S, 19 to 20S, and 10 to 15S were pooled, and the RNA was precipitated in ethanol. Hybridization-inhibition experiments were then performed with the fractionated RNAs (A). Preliminary incubations to 0.5 μg of DNA were performed in the presence of increasing concentrations of RNA isolated from cells harvested 18 h after infection, 6 h after infection, or from uninfected cultured human cells. Radioactive 26S RNA (circles), 19 to 20S RNA (triangles), and 12 to 15S RNA (squares) were then annealed. Closed symbols ($\bullet$, $\blacktriangle$, $\blacksquare$) represent inhibition by cultured human RNA; partially closed symbols ($\circ$, $\triangle$, $\square$) show inhibition by early RNA; and open symbols ($\circ$, Δ , $\square$) represent inhibition by late RNA. When inhibitor RNA was omitted from the preliminary incubation, 395 counts of 26S RNA per min hybridized to filters, 1,050 counts of 19 to 21S RNA per min hybridized to filters, and 595 counts of 13 to 15S RNA per min hybridized to filters. These numbers are the 100% values for the hybridizations shown in (A). In (B), [^3H]RNA was size fractionated by two sucrose gradient centrifugations (see Fig. 6). Hybridizations were carried out as in (A); 520 counts of 19 to 21S RNA per min and 450 counts of 13 to 15S RNA per min hybridized to 0.5- μg filters after a preliminary incubation without RNA.

fection (18 h). Early class II RNA, which appears to correspond to approximately 50% of the early cytoplasmic RNA sequences, is present in high concentrations at both late and early times.

From the hybridization studies, we can calculate the maximal amount of early class I RNA still present at 18 h. With ^{32}P -labeled viral DNA and late RNA, no detectable increase in duplex formation occurred over a fourfold increase in hybridization time (1 to 4 h; Fig. 2A), whereas

Fig. 6. Size fractionation of early cytoplasmic [^3H]RNA by sucrose gradient centrifugation. Early [^3H]RNA (2.1×10^7 counts/min) were centrifuged in a 15 to 30% sucrose gradient (0.01 M Tris-hydrochloride (pH 7.3) and 0.05 M NaCl with 0.5% SDS) (A). Samples were centrifuged for 17 h at 24,500 rpm in a Spinco SW25.1 rotor at 20°C. The gradient was fractionated; 5- μl samples were counted directly (●); 35- μl samples were hybridized to 0.5 μg of adenovirus DNA in $6 \times \text{SSC}$ with 0.1% SDS (○). Fractions identified by the bars in (A) were precipitated in ethanol and were rerun under the same conditions to further purify 18 to 19S and 12 to 15S viral RNA. After fractionation of these gradients (B and C), 5 μl of each fraction was counted directly (●); 30 μl of each fraction was hybridized (○). Fractions 19 to 25 (B) and 27 to 33 (C) were pooled, precipitated in ethanol, and used as the 18 to 19S and 12 to 15S size-fractionated RNA in hybridization-inhibition experiments shown in Fig. 5B.

the addition of early RNA did increase the percentage of viral DNA hybridized under the same conditions. In hybridization-inhibition reactions (Fig. 1A), early class I RNA sequences were not detected when the concentration of late RNA was increased 16-fold (0.1 to 1.6 mg of RNA). Thus, the first method indicates at least a fourfold reduction in class I sequences at 18 h, whereas the second method suggests a 16-fold reduction. Since neither of these methods detected any class I RNA at 18 h, these estimates are certainly upper limits.

The existence of two classes of early RNA is consistent with some, but not all, previous studies. Initial experiments using hybridization-inhibition techniques indicate that all early viral RNA sequences remain at high concentrations late in infection (6, 18). Recently, Tibbetts et al. (19) used radioactive separated strands of viral DNA to analyze transcription during productive infection. Their studies also indicated that all early cytoplasmic RNAs are present in comparable concentrations at 18 h. In contrast to the above results, Lucas and Ginsberg (10) used modified hybridization-

inhibition methods and were able to identify the two classes of early RNA. In our study, both types of hybridizations, membrane and liquid, were used, and the results concur with those of Lucas and Ginsberg (10). For this reason, we do not feel the discrepancies can be attributed to hybridization methodology or other technical variations. Rather, the differences may arise from unknown variations in the virus-cell systems used. In fact, the quantitation of our liquid hybridizations differs from that reported by Tibbetts et al. (19). In our system, early cytoplasmic RNA corresponded to 24 to 30% of the asymmetrical coding capacity of the genome (Fig. 2), whereas the comparable data reported by Tibbetts et al. (19) is 43 to 53%.

The size distribution of cytoplasmic viral RNA presented here is in agreement with and supplements those previously reported. The two major size classes, 19 to 20S and 12 to 15S, appear to be heterogeneous, each containing at least two components. Assuming a molecular weight of 0.75×10^6 for each of two 19 to 20S species and 0.35×10^6 for at least two 12 to 15S species, these RNAs would correspond to a molecular weight of 2.2×10^6 for single-stranded DNA or 19% of the coding capacity of the genome.

The nature of the 26S viral RNA is unknown. Parsons and Green (11) could identify this component only in infections performed in the presence of CH. Lindberg et al. (9) suggested that 26S RNA is preferentially synthesized very early in infection, prior to 2 h. We have found no difference in the appearance of 26S RNA at different early times or in the absence or presence of CH. Recent studies in our laboratory suggest that conditions of electrophoresis may alter the migration of the 26S viral RNA (Tal, Craig, Zimmer, and Raskas, manuscript in preparation).

Hybridization-inhibition studies performed with the size-fractionated 19 to 20S and 12 to 15S viral RNAs are consistent with sequence heterogeneity, for hybridization of only a portion of the sequences present in each size class could be inhibited by late RNA. Thus, both 19 to 20S and 12 to 15S RNAs appear to be composites of early class I and II RNA. Since the hybridization-inhibition studies were performed under nonsaturating conditions, it is not possible to estimate the percentage of class I sequences in the size-fractionated RNAs. However, it is not surprising that class I RNA could be detected in these conditions, for class I RNA could be identified in hybridizations of total cytoplasmic RNA hybridized under nonsaturat-

ing conditions (Fig. 1). When hybridization-inhibition experiments were performed with 26S RNA, 20% was inhibited by late RNA, but the significance of this result is unclear because of our uncertainty concerning the *in vivo* existence of this RNA as a 26S molecule.

This study has identified two classes of early RNA with respect to intracellular concentrations at late times, but we have not analyzed directly for the transcription of these two classes at late times. Some early RNA continues to be transcribed at late times (10, 18), and thus, even class I RNA may still be synthesized at 18 h, although at greatly reduced levels. Abundance classes of viral RNAs have been demonstrated in the replication of herpesviruses, where the differences between the RNA concentration of abundance classes appears to vary as much as 140-fold (5). Further identification of the viral RNA species in each class will await the use of specific fragments of the viral genome (12) in hybridization studies.

ACKNOWLEDGMENTS

We thank David Schlessinger for a critical reading of the manuscript. Mark Telle and Joe Kelley provided able technical assistance.

This study was supported by Public Health Service grants from the National Cancer Institute (CA 12560) and American Cancer Society (VC-94). Elizabeth Craig was a recipient of a postdoctoral fellowship from the National Institute of Allergy and Infectious Diseases.

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