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Sequence Relationships Between Adenovirus 2 Early RNA and Viral RNA Size Classes Synthesized at 18 Hours After Infection

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Synthesis of cytoplasmic viral RNA was studied during infection of cultured human (KB) cells with adenovirus 2. At 6 h, before viral DNA synthesis began, 5% of the poly(A)-containing RNA hybridized to viral DNA; by 12 h and at later times more than 80% was virus specified. At 18 h after infection, four major size classes of cytoplasmic viral RNA were identified among the poly(A)-containing molecules. These size classes migrated as 27*S*, 24*S*, 19*S*, and 12 to 15*S* in polyacrylamide gels. The three larger size classes could also be identified in denaturing formamide gels. Hybridization of the 27*S*, 24*S*, and 19*S* viral RNAs was not inhibited by RNA harvested from cells at early times in infection. Therefore, these three major RNAs must code for late viral proteins. Hybridization of the 12 to 15*S* RNA was partially inhibited by RNA from cultures harvested at early times, suggesting that in this size class some of the RNA labeled at 18 h codes for early viral proteins.

The replication of adenovirus 2 in cultured human cells requires the synthesis of at least two classes of virus-specified mRNA and protein. At early times in infection, cytoplasmic viral mRNA's are derived from a limited portion of the viral genome (7, 10, 32). By 6 to 8 h after infection, early viral proteins have been synthesized (28, 33) and viral DNA replication begins (12, 31). At late times (16 to 18 h), the viral genome dominates macromolecular synthesis; a high percentage of the newly synthesized DNA (23), polysomal RNA (3, 14), and polypeptides are virus specified (1, 13, 33). At these late times, transcripts of a larger portion of the genome are present as cytoplasmic mRNA's (7, 10, 32), and as many as 22 virus-induced polypeptides are synthesized in vivo (1, 33). Some of these peptides correspond to virion polypeptides and presumably are coded for by virus-specified mRNA's.

Several reports have described the size distribution of cytoplasmic viral RNAs synthesized at early (7, 14, 19) and late times (14, 18, 26) in infection. We have previously studied the relationship between the size classes of cytoplasmic viral RNA synthesized early and those viral RNA sequences present in the cell at late times (7). The current study includes a further analysis of the size distribution of cytoplasmic viral RNAs synthesized at late times (18 h). We have also determined the sequence relationship between the major cytoplasmic size classes synthesized late in infection and those viral RNAs present at early times. The results indicate that the role of three major size classes of cytoplasmic viral RNA (27*S*, 24*S*, and 19*S*) is restricted to late times in infection. It is likely that these RNAs code for viral proteins essential for virion assembly. (The experiment documented in Fig. 2A was presented at the 2nd ICN-UCLA Symposium on Molecular Biology and is described in a chapter in Virus Research [26].)

MATERIALS AND METHODS

Cell culture and virus infection. Exponentially growing KB suspension cultures were concentrated to 1.2×10^7 cells per ml and infected with adenovirus 2 (40 PFU/cell) purified by centrifugation in cesium chloride (11, 23). After a 1-h adsorption period, the culture was diluted to 3×10^5 cells per ml. Unless otherwise indicated, RNA synthesized at late times was labeled with [^{3}H]uridine (25 μCi/ml; 40 C/mM; New England Nuclear Co.) from 18 to 21 h after infection. Before labeling, cells were concentrated to 9×10^5 cells per ml. RNA synthesized at early times was labeled 2 to 6 h after infection. All early RNA preparations were obtained from infections performed in the presence of 25 μg of cycloheximide per ml as previously described (6).

RNA labeled with ^{32}P was prepared using the following procedure: for virus adsorption, standard, Joklik minimal essential medium was used without any addition of serum. At the end of the adsorption period, the cells were collected by centrifugation and resuspended in phosphate-free Joklik minimal essential medium supplemented with 3% horse serum. At

18 h, the cells were concentrated three to fourfold and 0.1 to 0.15 mCi of ^{32}P per ml (New England Nuclear, carrier free) was added. The ^{32}P solution was neutralized before addition to the culture. In initial experiments, phosphate-free medium was used for virus adsorption as well as the infection. With this procedure, the yield of virus specific ^{32}P-labeled RNA was variable and often much reduced.

Cell fractionation. Cytoplasmic extracts were prepared by resuspending the cells in hypotonic buffer (RSB; 0.01 M Tris-hydochloride, pH 7.4, 0.01 M NaCl, 0.0015 M $MgCl_2$) (20) containing 0.3% diethylpyrocarbonate. The swollen cells were disrupted by addition of Nonidet P-40 to a final concentration of 0.5% (5). Cell disruption was monitored by microscope examination. Nuclei and cellular debris were removed by centrifugation at 20,000 × *g* for 20 min.

RNA purification, fractionation, and hybridization. Purification of cytoplasmic RNA (6), selection of poly(A)-containing molecules by oligo dT-cellulose chromatography (6) and hybridization to adenovirus 2 DNA were all performed as previously described (6, 24).

Electrophoresis of RNA. RNA was fractionated on 7- and 11-cm 3.2% polyacrylamide-ethylene diacrylate gels containing 10% glycerol (4, 15, 34). Electrophoresis was performed in E buffer (0.04 M Tris acetate, pH 7.2, 0.02 M sodium acetate, 0.001 M EDTA) containing 0.2% sodium dodecyl sulfate. Gel slices (2 mm) were solubilized by incubating for 10 h at 60 C in 100 μliters of 6 × SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate), pH 7.8, containing 0.1% sodium dodecyl sulfate. RNA in solubilized gel slices was hybridized directly by addition of a membrane containing immobilized viral DNA.

Agarose (Seakem) gels were prepared in E buffer. A clear solution was obtained by autoclaving the agarose suspension for 10 min. The solution was then placed in a 50 C water bath for 20 to 30 min before preparation of the gels. In general, the profile of late viral RNA obtained by use of agarose gels was essentially the same as with acrylamide gels. Although the separation between size classes was slightly reduced, electrophoresis in agarose gels is convenient because considerably less time is required for the electrophoresis (see below).

Gels made in formamide were prepared as described (9, 30). Prior to electrophoresis through formamide gels, RNA was treated with 90% formamide for 5 min at room temperature; electrophoresis was performed at 22 C.

RESULTS

Time course of cytoplasmic viral RNA synthesis during productive infection. To obtain the time course of viral RNA synthesis, cultures were labeled with [^{3}H]uridine for 2-h intervals at different times in infection, and the total cytoplasmic RNA annealed to excess viral DNA (Fig. 1). Hybridization to viral DNA increased as the infection progressed, reaching a level of approximately 15% at 15 to 18 h after infection. This time course is similar to that previously described (17, 31).

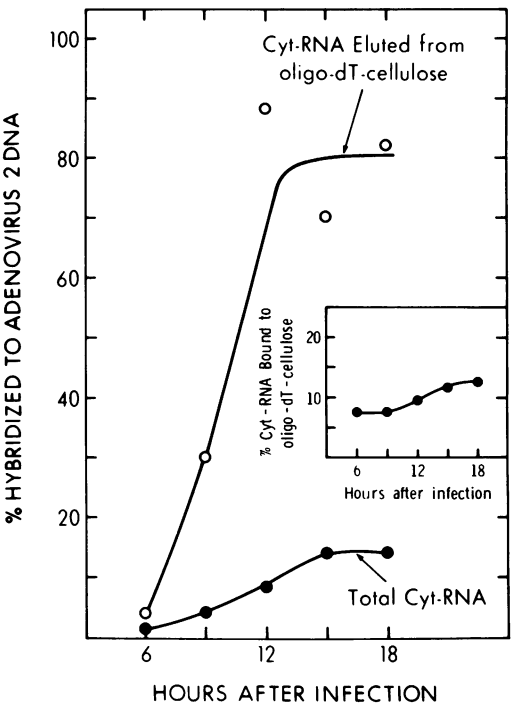

FIG. 1. *Time course of cytoplasmic viral RNA synthesis during productive infection. At the indicated times, portions of KB cells infected with adenovirus 2 were concentrated fourfold and labeled with [^{3}H]uridine for two h. The size of the culture samples was as follows: 800 ml at 6 and 9 h after infection, 600 ml at 12 h, and 400 ml at 15 and 18 h. Cytoplasmic RNA was purified from each sample and a portion was used to prepare poly(A)-containing molecules. For each time point, the percentage of RNA binding to oligo dT-cellulose was determined and is shown in the inset. Total cytoplasmic RNA and oligo dT selected RNA were then hybridized to adenovirus 2 DNA. Input counts per minute for total cytoplasmic RNA ranged from 10,000 to 60,000 counts/min, with higher inputs used for the early time samples. For oligo dT selected RNA, the input counts per minute varied from 1,400 to 3,100. For total cytoplasmic RNA, three DNA concentrations were used, 0.1, 0.5, and 1.0 μg; for oligo dT selected RNA 0.5, 1.0, and 4.0 μg were used. Maximal hybridized counts per minute for total cytoplasmic RNA ranged from 857 to 2,500; for poly(A)-containing RNA the values were from 80 to 2,658. Hybridizations were performed in triplicate. The hybridization at infinite DNA concentration was obtained by plotting the reciprocal of the DNA concentration versus the counts per minute hybridized. Hybridization conditions were chosen such that the maximal value hybridized was at least 90% of the calculated value.*

Since most adenovirus mRNA contains poly(A) (3, 14, 22) and total cytoplasmic RNA includes large amounts of ribosomal and tRNA in addition to mRNA, the poly(A)-containing molecules were hybridized to viral DNA to

obtain a time course of viral synthesis. The poly(A)-containing portion of each labeled sample was selected by oligo dT-cellulose chromatography. The fraction of the labeled material binding to oligo dT-cellulose increased during the infection, from 7.5% in the 6- to 8-h labeling period to 13% during the 18- to 20-h interval (Fig. 1 inset). This increased binding presumably reflects the inhibition of cellular ribosomal RNA synthesis which occurs during the course of infection (27). Hybridization of the poly(A)-containing molecules indicated a somewhat different time course for synthesis of cytoplasmic viral RNA. RNA labeled from 6 to 8 h, when viral DNA replication is just beginning, hybridized only 5%. By 12 to 14 h the newly synthesized RNA had already attained its maximal viral content, being 80 to 90% virus specified.

Size distribution of cytoplasmic viral RNA synthesized at 18 h. The size distribution of viral RNAs synthesized at late times in infection was obtained by fractionating RNA on 3.2% polyacrylamide or 2% agarose gels, eluting the RNA in each slice, and hybridizing the RNA to viral DNA. When total cytoplasmic RNA was analyzed by this procedure (Fig. 2A), viral RNA size classes migrating as 27*S*, 24*S*, and 19*S* could be identified. In addition, slower migrating viral RNA and some viral RNA migrating faster than 18*S* ribosomal RNA were also present.

To allow further analysis of the size classes of viral RNA, the poly(A)-containing portion of the cytoplasmic RNA was purified using oligo dT-cellulose chromatography (2, 6). Previous studies have demonstrated that 85 to 90% of the cytoplasmic viral RNA synthesized at late times is polyadenylated (3, 14). Size analysis of the poly(A)-containing molecules confirmed the presence of the 27*S*, 24*S*, and 19*S* size classes (Fig. 2B). In addition, a peak migrating at 11 to 15*S* could also be identified, both in the agarose gel of Fig. 2B and in polyacrylamide gels (26). Shorter electrophoresis times showed that there were not any viral mRNA's migrating more rapidly than the 11 to 15*S* size class identified in the data of Fig. 2B (data not shown). As expected, because of the high viral content of poly(A)-containing RNA synthesized at 18 h (Fig. 1), the profile of total radioactive RNA generally corresponded to the profile obtained by hybridization. The viral RNA migrating slower than 28*S* RNA was very much reduced in the oligo dT selected material. Because of their large size, such RNAs may represent leakage of high-molecular-weight RNAs from nuclei during cell fractionation. Alternatively, the slower migrating RNA may be molecules which ag-

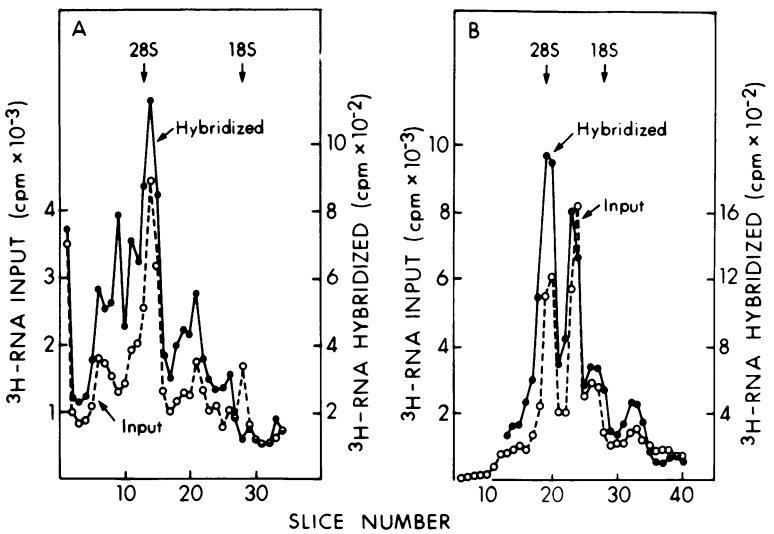

FIG. 2. *Size distribution of cytoplasmic viral RNA synthesized late in infection. As described, cultures were infected with adenovirus 2 and labeled with [^{3}H]uridine. Panel A shows the size distribution of viral RNA in total cytoplasmic RNA. For this preparation a culture was labeled for 30 min; the cells were then collected by centrifugation and incubated for an additional 2 h in fresh medium containing 50 μg of cold uridine per ml, and the culture then was harvested (19). The distribution of viral RNA in oligo dT selected RNA is presented in panel B. For this preparation a culture was labeled from 18 to 21 h after infection as described. The fractionation shown in panel A utilized an acrylamide gel whereas for panel B an agarose gel was used. In both instances, the RNA in each gel slice was hybridized to 1 μg of adenovirus 2 DNA. For the analysis in panel A, electrophoresis was for 7 h at 5 mA on a 7-cm 3.2% acrylamide gel. For panel B, the conditions were 2 h at 5 mA on an 11- cm 2% agarose gel. Symbols: O, input counts per minute; ●, hybridized counts per minute.*

gregate during electrophoresis (see Fig. 3); such aggregation might be reduced considerably in the RNA samples selected by oligo dT-cellulose.

Analysis of cytoplasmic RNA by electrophoresis in polyacrylamide gels containing 95% formamide confirmed that the major RNA size classes are not aggregates. The three major size classes of viral RNA could be identified in these denaturing conditions (Fig. 3). There were some differences in the migration of the viral RNAs relative to the marker ribosomal RNAs. This change in relative mobility in denaturing conditions is not unexpected since ribosomal RNA and mRNA's have different amounts of secondary structure.

The sequence relationship between cytoplasmic viral RNAs synthesized at late times and RNA present at early times in infection. To compare directly the size distribution of early and late cytoplasmic mRNA's, ^{32}P-labeled RNA synthesized at late times and RNA labeled with ^{3}H early in infection were coelectrophoresed on the same gel. The early RNA was prepared by labeling from 2 to 5 h after an infection performed in the presence of cycloheximide. The addition of cycloheximide to the medium inhibits early viral protein synthesis, thus preventing viral DNA replication and the synthesis of late viral mRNA's (12). In addition, the cytoplasmic poly(A)-containing RNA synthesized in the presence of cycloheximide hybridizes 10-fold more to viral DNA than control preparations from infections performed in the absence of cycloheximide, thus facilitating analysis of early viral transcripts (6, 19).

The size distribution of oligo dT selected RNA from cultures labeled at early and late times is shown in Fig. 4A. The RNA in each gel slice was hybridized to viral DNA and the profile of ^{32}P-labeled late and ^{3}H-labeled early viral mRNA obtained (Fig. 4B). In confirmation of earlier studies, the early viral RNA size classes migrate as approximately 12 to 15*S*, 19 to 20*S*, and a heterogeneous RNA migrating in the range of 26*S* (7, 14, 19). The major early size class (19 to 20*S*) has a migration more rapid than the late 24*S* size class and slightly slower than the late 19*S* RNA. Of the faster migrating RNAs, both early and late size classes have molecules in the range 11 to 16*S*; the late RNA appears to be resolved into two classes of 11 to 13*S* and 14 to 16*S*.

Since size analysis alone cannot provide information concerning the sequence content of fractionated RNAs, hybridization-inhibition studies were performed to determine the relationship between viral RNAs synthesized at late and early times. For these studies, we determined the ability of early whole cell RNA to inhibit the hybridization of ^{3}H-labeled cytoplasmic RNA synthesized at late times. As a control, whole cell RNA from cultures harvested

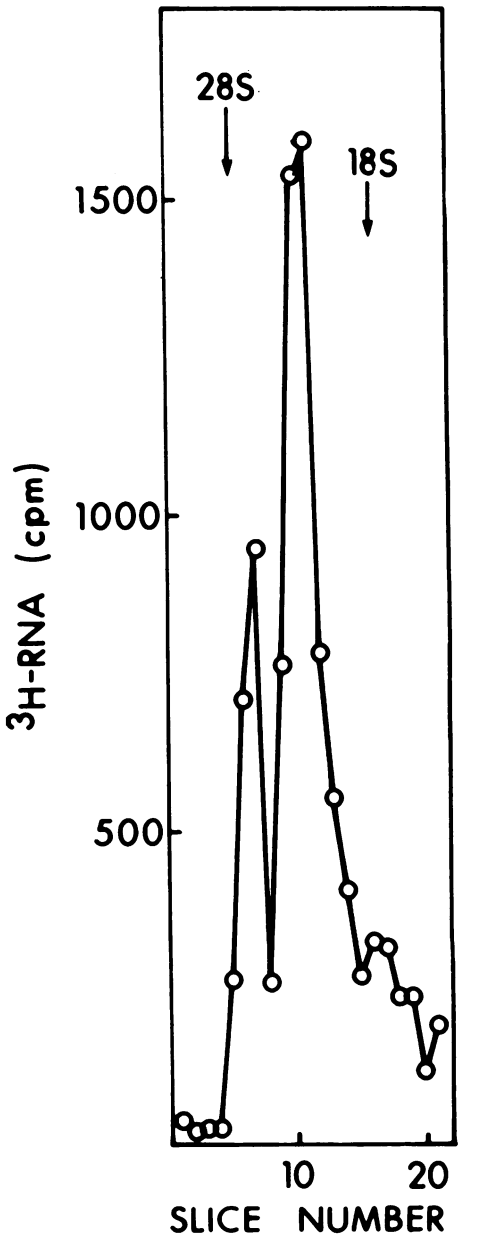

FIG. 3. *Formamide gel analysis of oligo dT selected cytoplasmic RNA synthesized at late times in infection. Cytoplasmic RNA synthesized late in infection was labeled, purified, and fractionated by oligo dT-cellulose chromatography as described. The poly(A)-containing molecules were treated with formamide and analyzed on polyacrylamide gels containing 95% formamide. Electrophoresis was at room temperature for 13 h at 5 mA/gel.*

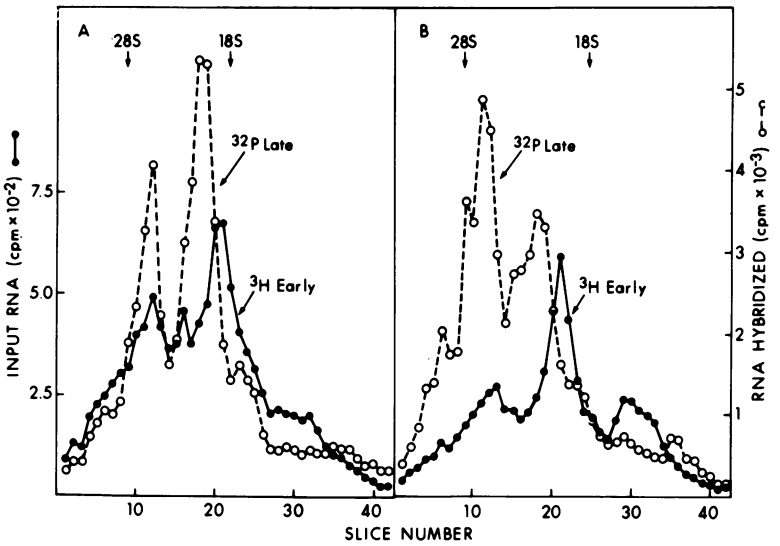

FIG. 4. *Coelectrophoresis of ^{3}H-labeled early and ^{32}P-labeled late cytoplasmic viral RNAs. ^{3}H-labeled early and ^{32}P-labeled late cytoplasmic RNAs were prepared as described, and the poly(A)-containing molecules were selected by oligo dT-cellulose chromatography. ^{32}P-labeled late RNA (550,000 counts/min) and ^{3}H-labeled early RNA (600,000 counts/min) were applied to an 11-cm 3.2% acrylamide gel. After electrophoresis for 7 h at room temperature (5 mA/gel), 5-μliter portions from each gel slice were sampled for input radioactivity (panel A) and the remainder of the RNA from each gel slice was hybridized to 0.5 μg of adenovirus 2 DNA (panel B). Symbols: O, ^{32}P-labeled late RNA; ●, ^{3}H-labeled early RNA.*

18 h was used as inhibitor; hybridization was reduced more than 80% with 0.6 mg of RNA per ml (Fig. 5A). Whole cell RNA from cultures harvested at early times, at 6 h, inhibited hybridization of late RNA only 20% at concentrations of 2.5 mg of inhibitor RNA per ml. This result agrees with the previous study of Thomas and Green (31) and indicates that 20% of the cytoplasmic viral RNA sequences synthesized at late times correspond to viral RNAs transcribed in abundance at early times.

Similar hybridization-inhibition experiments were performed with the size classes of ^{3}H-labeled late RNA obtained from an acrylamide gel (as in Fig. 2). Such experiments might identify some viral RNAs which are present as functional mRNA only at late times. Because of the limitations in obtaining large amounts of radioactive size-fractionated viral RNA, these hybridization-inhibition experiments were performed in nonsaturating conditions. The results (Fig. 5B) demonstrate that early whole cell RNA was unable to prevent the hybridization of the three major size classes of viral RNA, those molecules migrating as 27*S*, 24*S*, and 19*S*. In contrast, the hybridization of the more rapidly migrating late viral RNAs was significantly inhibited by early RNA. Hybridization of RNAs migrating 14 to 16*S* and 11 to 13*S* was inhibited 20 and 50%, respectively.

DISCUSSION

The time course of cytoplasmic viral RNA synthesis appears considerably different when the analysis is performed on the fraction of cytoplasmic RNA which contains poly(A) as compared to total cytoplasmic RNA. Maximal hybridization of labeled total cytoplasmic RNA occurred at 15 to 18 h after infection (Fig. 1; 17, 31). At the same time that the rate of viral RNA synthesis is increasing, cellular ribosomal RNA (27) and presumably host cell mRNA synthesis are decreasing. The combination of these events complicates analysis of the time course of cytoplasmic viral RNA synthesis. Since the poly(A) fraction of cytoplasmic RNA contains most of the cellular (8) and viral (3, 14) mRNA and not ribosomal RNA, use of this fraction to obtain a time course provided a more useful measurement of cytoplasmic viral RNA synthesis. Such experiments demonstrated that maximal hybridization of the poly(A)-containing fraction was attained by 12 to 14 h. The shift of mRNA synthesis to viral-specified molecules by 12 to 14 h is consistent with protein synthesis studies which indicate that viral capsid proteins are synthesized as early as 12 to 14 h after infection (13, 28, 33). In addition, the time course illustrates the magnitude and rapidity of the change in mRNA metabolism which occurs after the beginning of viral DNA replication at 8 to 9 h

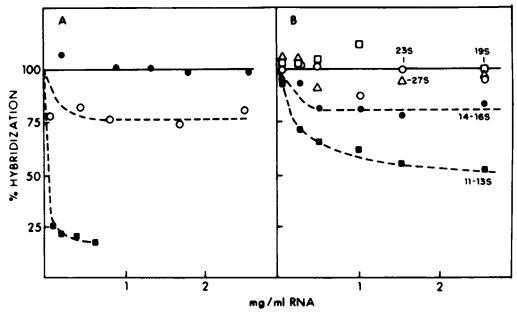

FIG. 5. *Sequence relationships between ^{3}H-labeled late RNAs and early RNA. In the experiment shown in panel A, the relationship between ^{3}H-labeled cytoplasmic late RNA and nonradioactive early RNA was determined. Total ^{3}H-labeled cytoplasmic RNA (10,000 counts/min per μg) was hybridized to adenovirus 2 DNA in saturating conditions; 0.04 μg of DNA were saturated at appoximately 35 μg of RNA. Approximately 60 μg were used in the inhibition studies. Uninfected KB cell RNA (●), whole cell late infected RNA (■), and whole cell early RNA (○) were used as inhibitors. Incubation volumes were 100 μliters; 100% hybridization corresponded to 420 counts/min. For panel B, oligo dT selected ^{3}H-labeled late RNA (1.5 × 10^6 counts/min) was fractionated by electrophoresis on a 3.2% gel. The gel was sliced and solubilized, portions were counted to determine the profile of RNA, and the fractions corresponding to 27S, 19S, 14 to 16S, and 11 to 13S RNA were pooled for use in hybridization inhibition studies. Hybridizations to 0.5 μg of DNA were performed with increasing concentrations of whole cell early RNA followed by RNase treatment and RNase inactivation (6). The radioactive size fractionated RNAs were then annealed to the DNA. Hybridization (100%), when inhibitor RNA was omitted in step one, corresponded to 510 counts/min for 27S RNA, 555 counts/min for 25S RNA, 670 counts/min for 19S RNA, 360 counts/min for 14 to 16S RNA, and 410 counts/min for 11-13S RNA.*

after infection (17, 31).

The present analysis of the size classes of cytoplasmic viral RNA synthesized late in infection supplements other studies (14, 18, 26). The 27S, 24S, 19S, and 12 to 15S size classes we have identified probably correspond to the 26S, 22S, 19S, 12S, and 16S described by Lindberg et al. (14). That the three larger size classes are not aggregates was demonstrated by electrophoresis in gels made in formamide; even in these denaturing conditions the same size classes existed (Fig. 3). Lindberg et al. (14) showed that poly(A)-containing cytoplasmic viral RNA labeled late in infection sedimented as 19 to 26S in Me_2SO gradients. Two dimensional fingerprints of the 27S, 24S, and 19S viral RNAs have provided further evidence that these RNAs contain different sequences (J. Tal, P. Jacobi, H. J. Raskas, and E. Wimmer, manuscript in preparation).

Although the size distribution of cytoplasmic viral RNA synthesized at early and late times is considerably different (Fig. 4), other hybridization experiments are required to provide conclusive evidence that RNAs synthesized at early and late times differ in sequence content as well as in size. Previous hybridization studies relating early and late viral RNAs have yielded two different results: Lucas and Ginsberg (16) and, more recently, Craig and Raskas (7) were able to distinguish two classes of early cytoplasmic viral RNA; class I RNA was present in greatly reduced concentrations at 18 h after infection, whereas class II RNA remained at high concentrations even at late times. In contrast, Fujinaga and Green (10) and Tibbetts et al. (32) did not detect early RNA which was present in reduced concentrations at 18 h. However, these differences do not affect the interpretation of the hybridization-inhibition studies described here, for the present experiments are concerned with viral mRNA that is newly synthesized at 18 h, not the composition of the total cytoplasmic viral RNA present by 18 h after infection.

Previous hybridization-inhibition studies in our laboratory suggested that the 19 to 20S and 12 to 15S early RNAs consist of more than one molecular species, for only half of the sequences in these two size classes were present in high concentrations at late times (7). The present study demonstrates that the sequences in the major 27S, 24S, and 19S RNAs synthesized at late times are not related to early RNAs. Since it is not possible to obtain sufficient amounts of labeled, size-fractionated RNA to perform these hybridization-inhibition experiments in saturating conditions, we certainly cannot exclude the possibility that at late times small amounts of early RNA sequences are still synthesized, but these sequences are not detected in nonsaturating hybridization-inhibition studies. However, most of the ^{3}H-labeled RNA in the three larger size classes must represent viral RNAs coding for late viral proteins. Direct evidence that 27S, 24S, and 19S RNAs code for different late viral proteins has been obtained by using size-fractionated RNA in an in vitro protein synthesizing system (C. W. Anderson, J. B. Lewis, J. F. Atkins, and R. F. Gesteland, Proc. Nat. Acad. Sci. U.S.A., in press).

Hybridization of the late 12 to 15S RNAs was inhibited significantly by early RNA, suggesting that some of these mRNA's are early viral RNAs that are also synthesized at late times. The detection of some early RNA sequences in size-fractionated RNA synthesized at late times is consistent with the finding that the hybridization of ^{3}H-labeled late cytoplasmic RNA is inhibited 20% by early cytoplasmic RNA (Fig.

5A; 31). Although cytoplasmic viral RNA size classes have identified, the precise number of viral mRNA species is not known. For this reason a more detailed comparison cannot be made as yet between hybridization-inhibition studies with total cytoplasmic RNA and size-fractionated cytoplasmic RNA.

Even though the results of studies of viral RNA size classes and in vitro protein synthesis may be consistent with a particular size RNA being a single mRNA species, definite proof requires analysis of the sequences in the RNA. For example, two mRNA species may have the same migration rate and therefore not be separated by electrophoresis. Likewise, a size class of RNA may contain two species, one in much higher concentration than the other so that only one polypeptide is identified in protein synthesis studies. Use of the unique viral DNA fragments generated by digestion with restriction enzymes is potentially able to resolve such questions (21, 29). Initial studies with size-fractionated RNA and adenovirus 2 DNA fragments generated by digestion with Eco R·R1 have succeeded in localizing some viral RNAs to defined portions of the genome (J. Tal, E. A. Craig, S. Zimmer, and H. J. Raskas. Proc. Nat. Acad. Sci. U.S.A., in press).

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