JOURNAL OF VIROLOGY, May 1975, p. 1202-1213
Vol. 15, No. 5

Printed in U.S.A.

Analysis of Early Adenovirus 2 RNA Using Eco R·R1 Viral DNA Fragments

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Received for publication 30 December 1974

Adenovirus 2 RNA synthesized early in productive infection was analyzed by RNA-DNA hybridization. Hybridization experiments were performed with adenovirus 2 DNA and with the six adenovirus 2 DNA fragments generated by digestion with the restriction endonuclease Eco R·R1. Duplex formation between RNA and ^{32}P-labeled viral DNA was assayed by S_1 nuclease digestion. RNA from the cytoplasm annealed 12% of the total viral DNA and the following percentage of each of the R·R1 fragments: 6% of R1-A, 24% of R1-B, 0% of R1-F, 40% of R1-D, 13% of R1-E, and 22% of R1-C. The early cytoplasmic RNA is composed of two sequence classes: class I, present in greatly reduced quantities at late times in infection (18 h), and class II, which remains at high concentrations at 18 h. In hybridization-inhibition experiments, hybridization of class II RNA is inhibited by late cytoplasmic RNA, whereas hybridization of class I RNA is not blocked by late cytoplasmic RNA (J. J. Lucas and H. S. Ginsberg, 1971; E. A. Craig and H. J. Raskas, 1974). To determine the location of class I and II sequences on the genome, membrane bound DNA fragments were used in hybridization-inhibition experiments. These studies demonstrated that the early cytoplasmic transcripts of R1-D belong to class II, whereas R1-C transcripts are class I sequences. The cytoplasmic RNAs transcribed from fragments A and B contain both class I and class II sequences. Analysis of cytoplasmic RNA fractionated by size demonstrated that the class I sequences include a 19S RNA transcribed from R1-B and class II sequences include a 20S RNA derived from R1-D. Nuclear RNA purified from cultures early in infection was annealed with ^{32}P-labeled R1 fragments. With all six fragments the nuclear RNA annealed as much or more of the DNA than did cytoplasmic RNA. Eco R1-F annealed at least 25% with early nuclear RNA, whereas no sequences homologous to R1-F were detected in early cytoplasmic RNA. When cultures were labeled from 2 to 6 h after infection, at least 5% of the ^{3}H-labeled early nuclear viral RNA annealed to Eco R1-F. Some of these nuclear transcripts from R1-F appear to be covalently linked to sequences transcribed from a contiguous region of the genome (Eco R1-B); 8.4% of the RNA selected by hybridization of R1-F reannealed to R1-B, whereas no more than 1.5% reannealed to R1 fragments A, D, E, or C.

The early phase of adenovirus 2 replication in cultured human cells lasts for approximately 6 h, until the onset of viral DNA replication. DNA-RNA hybridization studies have demonstrated that early cytoplasmic viral RNA represents only a limited portion of the genome (4, 5, 23), and that the early cytoplasmic viral RNAs are found as two major size classes, 19 to 20S and 11 to 15S (4, 9, 14). These two size classes have been further resolved into at least six viral RNA species by hybridization of size fractionated RNA to the unique adenovirus 2 DNA fragments generated by endonuclease Eco R·R1 (21). The early cytoplasmic RNA includes two subclasses: class I RNA, which is present in at least a 16-fold lower concentration at 18 h than at 6 h, and class II RNA, which remains at high concentrations at late times (4, 10). In the present study we have extended the analysis of early cytoplasmic RNA by performing additional RNA-DNA hybridization studies with the six Eco R·R1 adenovirus 2 DNA fragments (15). The results of these studies allow the assignment of class I and class II RNAs to specific regions of the genome.

Earlier studies have established that the early nuclear viral RNA purified from cultures labeled for short intervals is considerably larger

than the largest of the early cytoplasmic mRNAs (12, 24). These high-molecular-weight nuclear RNAs contain all the viral sequences present in early cytoplasmic viral RNAs as well as additional viral sequences (22, 24). These additional sequences present in the nucleus have been characterized further in hybridization experiments using the R1 DNA fragments.

(A preliminary report of this work was presented at the Cold Spring Harbor Symposium on Quantitative Biology, June 1974.)

MATERIALS AND METHODS

Cell culture, virus infection, and virus purification. Suspension cultures of KB cells were maintained in Joklik minimal essential medium supplemented with horse serum. Infections were performed as described previously (17, 18).

Stock adenovirus 2 was purified from cultures infected at a multiplicity of 15 PFU/cell and harvested 30 h later (6, 7). The infected cells were collected by centrifugation and suspended in 0.01 M Tris-hydrochloride (pH 8.1) at a concentration of 3×10^7 cells/ml. The suspension was frozen and thawed three times using an acetone-dry ice bath, and then sonically treated for sufficient time to destroy all cells; cell disruption was verified microscopically. After sonic treatment the extract was diluted twofold with 0.01 M Tris (pH 8.1) and homogenized with an equal volume of fluorocarbon (Trichlorotrifluoroethane, Allied Chemical Co.). The mixture was centrifuged at 2,000 rpm for 5 min at 5 C in a no. 269 rotor in the IEC PRJ centrifuge. The virus in the supernatant fluid was then concentrated by centrifugation onto a 5-ml cesium chloride cushion of density 1.43 g/cm^3. Centrifugation was for 1 h at 20,000 rpm in a Spinco SW25.1 rotor. The band containing virus was further purified by two equilibrium centrifugations in cesium chloride of density 1.34 g/cm^3. Centrifugation was performed for 18 h in a Spinco type 65 rotor at 30,000 rpm, 5 C. Virus to be used as stock for further infections was diluted 10-fold into 0.01 M Tris (pH 8.1), 0.15 M NaCl, 0.1% serum albumin, and 50% glycerol and stored at −20 C.

Cultures to be harvested 6 h after infection were infected at a multiplicity of 100 PFU/cell and diluted to 9×10^5 cells/ml after a 1-h adsorption period. In most experiments, 25 μg of cycloheximide (Sigma Chemical Co.) per ml was added at the time of dilution (3). Early RNA was labeled by exposing cultures to 12 μCi of [^{3}H]uridine (40 Ci/mmol; New England Nuclear) per ml from 2 to 6 h after infection. Cultures to be harvested at late times, 18 to 22 h after infection, were diluted to 3×10^5 cell/ml after the 1-h adsorption period.

Cell fractionation and RNA purification. Cultures were harvested, and cytoplasmic and nuclear extracts were prepared as described by Craig and Raskas (3). RNA in cytoplasmic extracts was purified by the addition of 0.5% sodium dodecyl sulfate (SDS), followed by three extractions at room temperature with an equal volume of water-saturated phenol and 24:1 chloroform/isoamyl alcohol (for details see Craig and Raskas [3]). The RNA was precipitated by the addition of two volumes of 95% ethanol in the presence of 0.15 M NaCl. RNA containing poly(A) was separated from RNA lacking poly(A) by selective retention on oligo(dT)-cellulose (1, 3).

Nuclei were lysed in high salt buffer (0.5 M NaCl, 0.05 M $MgCl_2$, 0.05 M Tris-hydrochloride, pH 7.4), treated with DNase and then diluted 10-fold with TES buffer (0.05 M Tris-hydrochloride, pH 7.4, 0.02 M EDTA; 0.5% SDS) (3). Following dilution, the preparations were extracted three times at 60 C with water-saturated phenol and chloroform/isoamyl alcohol. The RNA was precipitated with ethanol, resuspended in high salt buffer, and incubated a second time with DNase. The RNA was then re-extracted with water-saturated phenol and precipitated with ethanol.

Nucleic acid concentrations were determined by optical density measurements at 260 nm, assuming 43 μg of RNA per optical density unit. The yield of RNA per 3×10^6 cells was approximately 1.3 mg of cytoplasmic RNA from cultures harvested at 6 h and 2.0 mg of cytoplasmic RNA from cultures harvested at 18 h. Nucleic acids purified from nuclei of cultures harvested at 6 h amounted to 0.75 mg and 1.3 mg per 3×10^6 cells from cells harvested at 18 h.

Purification of viral DNA. Viral DNA was extracted from purified virus essentially as described by Green and Pina (7). The purified virus in cesium chloride was dialyzed against several changes of 0.01 M Tris-hydrochloride (pH 8.1) for a minimum of 2 h and then incubated at 37 C with 0.6 mg of papain per ml (Sigma Chemical Co.) in 0.0075 EDTA, 0.015 M cysteine-hydrochloride and 0.15 M sodium phosphate (pH 6.9). After 1 h SDS was added to a concentration of 0.75%, and the incubation was continued for an additional 30 min. The sample was extracted twice with gentle agitation at 4 C using an equal volume of phenol saturated with 0.05 M Tris-hydrochloride (pH 7.4), 0.1 M NaCl, and 0.001 M EDTA. The final aqueous layer was dialyzed against 0.1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) at 4 C for 24 h; during this time the 300 ml of buffer was changed six times. The concentration of DNA was determined by optical density measurements at 260 nm assuming 50 μg of DNA per optical density unit.

Preparation of R1 DNA fragments. Digestion of adenovirus 2 DNA by endonuclease R·R1 was carried out as described by Pettersson et al. (15). The standard reaction mixture contained 10 mM $MgCl_2$, 90 mM Tris-hydrochloride (pH 7.6) and 2 units of enzyme per 1 μg of DNA with a maximum of 150 μg of DNA/ml. One unit of enzyme was defined as the amount needed to cleave 1 mg of adenovirus 2 DNA to completion with no detectable intermediates remaining. Incubations with enzyme were for 60 min at 37 C; digestion was terminated by addition of EDTA to a concentration of 20 mM.

A similar procedure was used to prepare ^{32}P-labeled DNA fragments. Radioactive virions were purified from cultures exposed to ^{32}P (60 μCi/ml; New England Nuclear) as described by Craig and Raskas (4), and DNA was extracted as described above. When

necessary to concentrate DNA before electrophoresis, the digested ^{32}P-labeled DNA was extracted with an equal volume of chloroform/isoamyl alcohol (at a ratio of 24:1) and precipitated with 0.1 M NaCl and 2 volumes of ethanol.

The Eco R·R1 DNA fragments were separated by using the agarose gel system developed by Sharp et al. (19). The gels (0.8 by 12 cm) contained 1.4% agarose (Seakem), 0.04 M Tris-hydrochloride (pH 7.6), 0.005 M sodium acetate, 0.001 M EDTA, and 0.5 μg of ethidium bromide per ml (Sigma Chemical Co.). Electrophoresis was performed for 4.5 h at 5 mA/tube in buffer containing 0.04 M Tris-hydrochloride (pH 7.6), 0.005 M sodium acetate, 0.001 M EDTA, and 0.5 μg of ethidium bromide per ml. After electrophoresis the DNA fragments were visualized as fluorescent bands using a high intensity UV light, and portions of the gel containing DNA fragments were excised. ^{32}P-labeled DNA fragments were fractionated in the same manner except that the gel was cut into 2 mm slices, and the position of the fragments was determined by Cerenkov radiation in a liquid scintillation counter. The DNA in gel fractions was eluted by electrophoresis at 100 V for 2 h (15, 21).

DNA:RNA hybridizations with membrane-bound DNA. Hybridizations were performed with adenovirus 2 DNA immobilized on 6.5-mm cellulose nitrate membranes (Schleicher and Schuell, type B6) as described by Craig and Raskas (3). Two-step hybridization-inhibition experiments were performed in 30% formamide (Matheson, Coleman and Bell), 3× SSC, 0.1% SDS, and 0.01 M TES [*N*-tris(hydroxymethyl)methyl-2-amino-ethane sulfonic acid] (pH 7.4) at 45 C. The formamide was further purified as described by Tibbetts et al. (23) except that Amberlite MB3 was used and the ether extractions were omitted.

To determine the relationship between RNA sequences present in different preparations, hybridization-inhibition experiments were performed. In the first step of such experiments increasing concentrations of unlabeled RNA were incubated for 24 h with filters containing R1 DNA fragments derived from 0.5 μg of total viral DNA (e.g., 0.5-μg equivalents). The RNA used as inhibitor had been previously treated with alkali (1 M NaOH for 90 s at room temperature) as described by Lucas and Ginsberg (10). This treatment reduced the average size to 4*S* (as determined by gel electrophoresis) but did not reduce the amount of acid-precipitable RNA. After the first incubation the filters were washed at 60 C for 1.5 h with 8 ml of 2× SSC. During this time the buffer was changed three times. ^{3}H-labeled RNA was then added for a second incubation at 45 C for 24 h. The filter was washed three times at 60 C with 2× SSC, treated with 20 μg of pancreatic RNase (Worthington Biochemical Co.) per ml for 1 h at room temperature, and then washed again with 2× SSC. The filters were dried and counted in a liquid scintillation counter.

In some experiments hybridization-inhibition studies were performed with the RNA in individual fractions from acrylamide gels. For these experiments ^{3}H-labeled RNA was fractionated on 3.2% ethylene diacrylate cross-linked acrylamide gels as described previously (4). The gel slices were solubilized by addition of 6× SSC containing 0.1% SDS and subsequent incubation at 60 C for 16 h. Portions were then hybridized at 66 C for 20 h to membrane-bound DNA which had been preincubated with unlabeled alkali-treated RNA. The first hybridization was performed at 45 C for 20 h in 30% formamide as described above. Two types of inhibitor RNAs were used: cytoplasmic RNA from uninfected KB cultures and from cultures harvested 18 h after infection. The filters prehybridized to the same type of inhibitor were incubated simultaneously in one reaction mixture of 300 μl. After 20 h the filters were removed, washed at 60 C with 2× SSC, and then added to the solubilized gel slice for the second incubation.

For some experiments, RNA was hybridized and then eluted and rehybridized essentially as described by Weinberg et al. (25). Hybridizations were carried out as described above in 30% formamide at 45 C but using 25-mm membranes and incubating for 36 h. Both sides of the filters were then washed by filtration with 50 ml of 2× SSC containing 0.2% SDS. The filter was then incubated for 2 h at 45 C with 10 ml of hybridization buffer. The filter was again washed on both sides, first with 20 ml of 2× SSC plus 0.2% SDS, and then with 10 ml of hybridization buffer. RNA was eluted by incubation in 1 ml of 90% formamide, 0.01 M $NaPO_4$ (pH 7.0), and 0.2% SDS for 1 h at 45 C. At least 80% of the hybridized RNA was recovered. The eluted RNA was diluted 1:3 with 0.01 M Tris-hydrochloride (pH 7.4), 5 μg of carrier KB RNA per ml was added, and the RNA was precipitated by ethanol; subsequent rehybridization was performed in 30% formamide as described above.

Liquid phase hybridizations were performed with ^{32}P-labeled DNA. Before hybridization, the DNA was fragmented by boiling in 0.2 M NaOH for 1 h. The DNA was neutralized by the addition of hydrochloride. DNA denatured by this procedure reannealed as expected. Hybridizations were performed in 1.2 M NaCl and 0.01 M Tris-hydrochloride (pH 7.0) at 66 C. The fraction of the DNA present as hybrid was determined by digestion with the single-strand specific nuclease (S_1) from *Aspergillus oryzae*. S_1 nuclease was purified from diastase powder (Sigma Chemical Co.) as described by Sutton (20). The reaction mixtures contained 500 U of enzyme, 0.03 M sodium acetate buffer (pH 4.5). 3×10^{-5} M $ZnCl_2$, 0.12 M NaCl, 20 μg of calf thymus DNA per ml, and 1,000 to 10,000 counts per min of ^{32}P-labeled DNA (specific activity 5×10^{5} counts per min/μg). Nuclease digestion was performed for 2.5 h at 50 C. In these conditions, 100% of native adenovirus DNA and only 0.2% of denatured DNA remained acid precipitable. For each experiment in which DNA was annealed with RNA from infected cells, a control hybridization was performed with RNA from uninfected cells. In general, in the time required to achieve maximal formation of RNA-DNA hybrids, less than 2% of DNA was double stranded in the presence of RNA from uninfected cells (Fig. 1, 2, and 5).

The hybridization reactions were carried out in 0.7- to 1 ml volumes. Samples (25 μl) were removed in triplicate for a determination of input counts; 100-μl

samples were removed at various times and diluted into 0.9 ml of S_1 nuclease reaction mixture. After digestion with nuclease, the samples were precipitated with 5% trichloroacetic acid, collected by filtration, dried, and counted in a liquid scintillation counter.

RESULTS

Liquid hybridization of total viral DNA with nuclear and cytoplasmic RNAs from cultures harvested 6 h after infection. To compare viral transcripts present in the nucleus and cytoplasm at early times in infection, early RNAs were prepared from cultures infected in the presence of cycloheximide. Cycloheximide, an inhibitor of protein synthesis, prevents the onset of viral DNA synthesis (8). Since the initiation of viral DNA synthesis and other late events may not be synchronous in all infected cells, drug treatment eliminates possible contamination of early cytoplasmic viral RNA with low concentrations of late RNA (3).

Liquid hybridization experiments were performed with ^{32}P-labeled viral DNA, and formation of RNA-DNA hybrids was determined by resistance to digestion with the single-strand nuclease S_1 from *Aspergillus oryzae* (20). Early cytoplasmic RNA formed a hybrid with 12% of total viral DNA (Fig. 1). The same percent hybridization was obtained when the RNA concentration ranged from 0.4 to 2.0 mg/ml. Early nuclear RNA was also annealed with ^{32}P-labeled viral DNA. Although the amount of nuclear RNA used for the experiment corresponded to seven times as many cell equivalents as were used for the hybridization with early cytoplasmic RNA, a longer incubation time was required to approach maximal hybridization with the nuclear RNA; at least 26% of total viral DNA annealed with early nuclear RNA.

Liquid hybridization of cytoplasmic RNA with Eco R·R1 DNA fragments. For a more detailed analysis of the viral RNA synthesized early in infection, cytoplasmic RNAs were hybridized to ^{32}P-labeled adenovirus 2 DNA fragments generated by digestion with the restriction endonuclease Eco R·R1. The molecular weight (15) and linear order (C. Mulder, J. Arrand, H. Delius, W. Keller, U. Pettersson, R. Roberts, and Sharp, Cold Spring Harbor Symp. Quant. Biol., in press) of the six DNA fragments obtained by incubation with Eco R·R1 are reviewed in Table 1.

Each DNA fragment was annealed with cytoplasmic RNA from cultures harvested 6 h after infection and also with cytoplasmic RNA harvested from uninfected cells (Fig. 2). In reactions containing RNA from infected cells the formation of RNA-DNA hybrids tended to reach a plateau within 1 h.

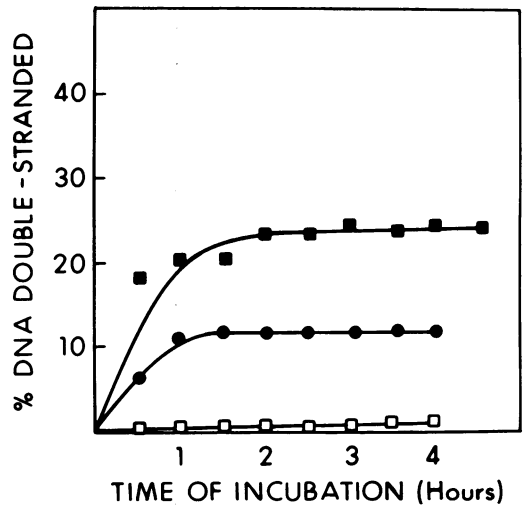

FIG. 1. *Liquid-phase hybridization of early cytoplasmic and nuclear RNA with ^{32}P-labeled adenovirus 2 DNA. RNA purified from cells harvested at 6 h after infection was hybridized to denatured ^{32}P-labeled DNA as described in Materials and Methods. 28,000 counts/min of ^{32}P-labeled DNA/ml was annealed with 0.8 mg of early cytoplasmic RNA (●) per ml or 4.6 mg of early nuclear RNA (■) per ml. The yield of RNA per 3 × 10^6 cells was approximately 1.3 mg of cytoplasmic RNA and 0.75 mg of nuclear RNA. A control reaction (□) contained 1 mg of RNA from uninfected cells per ml. At the indicated times, 100-μl samples were removed, and the percentage of the DNA forming hybrid was determined by digestion with S_1 nuclease.*

The maximal hybridization of each of the six R1 fragments with early cytoplasmic RNA is summarized in Table 1: 6% of R1-A, 24% of R1-B, 0% of R1-F, 40% of R1-D, 13% of R1-E, and 24% of R1-C. The percentage of the genome represented in early cytoplasmic RNA can be calculated by summation of the hybridizations achieved with the six R1 DNA fragments. This calculated value of 12% for early RNA agrees with the result obtained by hybridization of total viral DNA (Fig. 1).

Assignment of class I and class II early RNAs to regions of the genome defined by the Eco R·R1 fragments. The existence of class I early RNA was first demonstrated by hybridization-inhibition experiments (10). When membranes containing total viral DNA were first exposed to early cytoplasmic RNA, the subsequent hybridization of ^{3}H-labeled early cytoplasmic RNA was inhibited at least 90%. However, when the inhibitor RNA was nonradioactive late (18 h) RNA, the hybridization of ^{3}H-labeled early cytoplasmic RNA was inhib-

TABLE 1. *Hybridization of ^{32}P-labeled Eco R·R1 fragments of adenovirus 2 DNA with cytoplasmic RNA harvested early in infection*[a]

Fragment	Mol wt ($\times 10^{-6}$)	Fractional length (%)	DNA hybridized (%)	
			A	B
A	13.6	58.3	5	6
B	2.7	11.9	23	24
F	1.1	4.5	0	0
D	1.7	7.2	42	40
E	1.4	5.8	12	15
C	2.3	9.9	24	24

[a] The R1 fragments are listed in their linear order (C. Mulder, J. Arrand, H.. Delius, W. Keller, U. Pettersson, R. Roberts, and P. Sharp, Cold Spring Harbor Symp. Quant. Biol., in press). Information concerning the molecular weight and fractional length of each fragment is from Pettersson et al. (15). The percent DNA hybridized was obtained from data such as shown in Fig. 2. Data from two different experiments (A and B) are presented. The calculated percent DNA hybridized is the value hybridized at the minimum time required to reach the plateau value for hybrid formation; since DNA reannealing was generally less than 1% at these times, no correction was made for formation of DNA-DNA hybrids.

ited only 50%. Thus approximately half of the viral RNA sequences in early cytoplasmic RNA are present in greatly reduced concentrations by 18 h after infection (class I) (4, 10). The early RNA remaining in high concentrations at 18 h was designated class II (10). To localize early class I and class II RNA to segments of the viral genome, experiments were performed with the Eco R·R1 adenovirus 2 DNA fragments (Fig. 3). Nonradioactive RNAs from cells harvested early and late in infection and from uninfected cells were used in hybridization-inhibition experiments with membrane-bound DNA fragments. The ^{3}H-labeled early cytoplasmic RNA for the second step of the hybridization-inhibition experiments was utilized in less than saturating amounts; a previous analysis (4) demonstrated that the difference in concentration between class I and class II RNAs at late times could be detected even when the ^{3}H-labeled early cytoplasmic RNA was not present in saturating amounts.

In control experiments, hybridization of ^{3}H-labeled early cytoplasmic RNA to DNA fragments A, B, C, and D was not inhibited by RNA from uninfected cells and was inhibited at least 90% by homologous early cytoplasmic RNA (Fig. 3). Using late cytoplasmic RNA as inhibitor, the following results were obtained: hybridization of ^{3}H-labeled early cytoplasmic RNA to fragments A and B was partially inhibited, 63% and 59%, respectively. Hybridization of the same ^{3}H-labeled RNA to R1-C was inhibited less than 5% by late cytoplasmic RNA, whereas formation of hybrids with R1-D was inhibited at least 85%. In these experiments, hybridization to E fragment DNA was too low to permit quantitation of hybridization inhibition. Since early cytoplasmic RNA does not hybridize to F fragment DNA (Fig. 2) (21), hybridization-inhibition experiments were not performed with this DNA fragment.

In a recent study, hybridization of size-fractionated early cytoplasmic RNA to the Eco

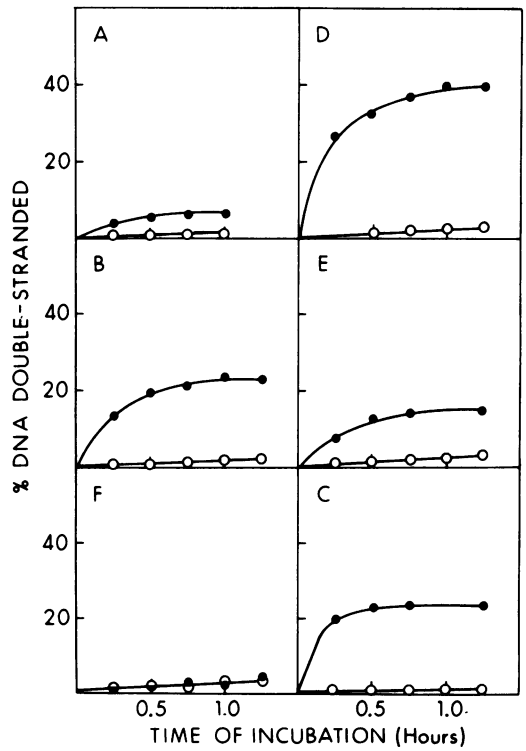

FIG. 2. *Liquid-phase hybridization of early cytoplasmic RNA with ^{32}P-labeled R1 DNA fragments. ^{32}P-labeled DNA fragments were annealed with 1 mg of early cytoplasmic RNA (●) per ml and with 1 mg of RNA from uninfected KB cells (○) per ml. 100-μl aliquots were removed at the indicated times, and the percentage of the DNA hybridized was determined by digestion with S_1 nuclease. One hundred microliters of the reaction mixtures contained the following amounts of radioactivity: 12,000 counts/min of A; 3,800 counts/min of B; 1,600 counts/min of F; 5,000 counts/min of D; 5,000 counts/min of E; and 2,000 counts/min of C. The results of the hybridizations are presented in the linear order of the fragments, A, B, F, D, E and C.*

R·R1 DNA fragments resulted in the identification of at least six early viral RNA species (21). Two of these species are a 20S RNA transcribed from R1-D and a 19S RNA from R1-B. Previous hybridization studies with total viral DNA demonstrated that the 19 to 20S early RNA contains both class I and class II sequences (4). Experiments were therefore performed to determine which of the 19 to 20S RNA species belongs to class I. ^{3}H-labeled early cytoplasmic RNA was size fractionated by polyacrylamide gel electrophoresis. The RNA in the fractions of the gel containing 19 to 20S molecules was divided into two aliquots, and the RNA in each aliquot was annealed simultaneously to two DNA filters (Fig. 4). One aliquot was annealed to B and D fragment filters which had been preincubated with saturating amounts of RNA from uninfected cells. The hybrids formed with these membranes revealed the 20S RNA transcribed from R1-D and the 19S RNA from R1-B. The RNA in the second aliquot from each gel slice was annealed to B and D fragment filters which had been preincubated with saturating amounts of cytoplasmic RNA from cultures harvested 18 h after infection. The prehybridization with late RNA did not prevent hybridization of the 19S RNA to B fragment but reduced hybridization of the 20S D fragment RNA more than 85%. This result establishes that the 19S B fragment RNA is a class I RNA and that the 20S RNA transcribed from R1-D is a class II RNA.

Liquid hybridization of early nuclear RNA with Eco R·R1 DNA fragments. In experiments analogous to those performed with cytoplasmic RNA (Fig. 2), early nuclear RNA was hybridized with ^{32}P-labeled Eco R·R1 DNA fragments (Fig. 5). For all six fragments the plateau value for hybridization with early nuclear RNA was equal to or greater than that obtained with cytoplasmic RNA. For example, 21% of A fragment was annealed by early nuclear RNA as compared to 6% with early cytoplasmic RNA (Fig. 5; Table 1). Early cytoplasmic RNA did not anneal at all to F fragment DNA (Table 1); nuclear RNA hybridized to 25% of F fragment DNA (Fig. 5).

Transcription of Eco R·R1 fragment DNA at early times in infection. The liquid hybridization studies with R1-F DNA demonstrated the cytoplasmic RNA, harvested at early times in infection, does not contain transcripts of this specific fragment, but early nuclear RNA does contain sequences homologous to F fragment DNA (Fig. 2 and 5).

The absence of a cytoplasmic viral mRNA transcribed from R1-F was previously demonstrated by hybridizing size fractionated ^{3}H-labeled early cytoplasmic RNA with F fragment DNA (21). The reduced hybridization of ^{3}H-labeled early cytoplasmic RNA to F fragment is shown in Table 2. Although F fragment comprises 4.5% of the genome, only 0.46% (20 counts per min) of the hybridized early RNA bound to F fragment DNA. In contrast, of the hybridized early nuclear RNA, 5.8% was homologous to F fragment (Table 2). Nuclear RNAs containing and lacking poly(A) were also analyzed for

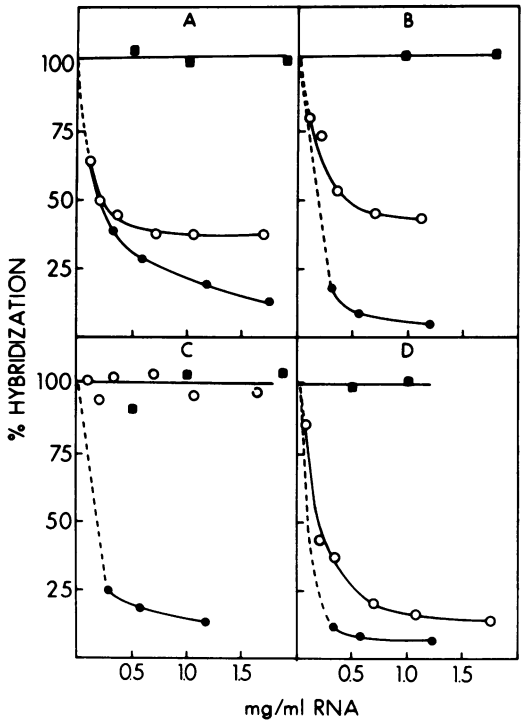

FIG. 3. *Ability of late cytoplasmic RNA to inhibit early cytoplasmic RNA hybridization to the Eco R·R1 fragments. Cytoplasmic ^{3}H-labeled RNA was purified from cells treated with cycloheximide and harvested 6 h after infection. Two-step hybridization inhibition experiments were performed with fragments A, B, C, D and E DNA immobilized on filters. The amount of fragment DNA per membrane was the amount derived from 0.5 μg of total adenovirus 2 DNA. RNA purified from uninfected KB cells (■), cells harvested at 6 h after infection (●), and cells harvested at 18 h (○) were used as inhibitors at the concentrations indicated. ^{3}H-labeled early RNA (3×10^5 counts/min; specific activity of 40,000 counts/min per μg) was added in the second hybridization step. One hundred percent hybridization corresponded to 1,000 counts/min for R1-A; 1,200 counts/min for R1-B; 1,120 counts/min for R1-C; and 1,310 counts/min for R1-D. Insufficient counts hybridized to fragment E to allow an accurate determination of inhibition.*

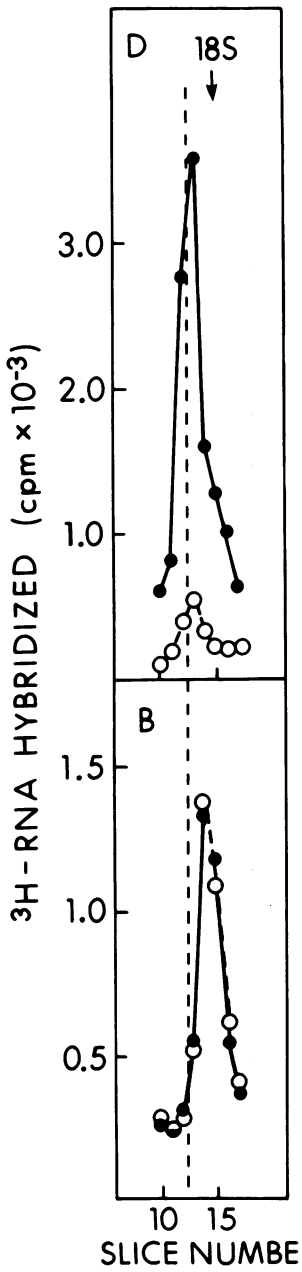

FIG. 4. *Ability of late RNA to inhibit hybridization of 19 to 20S ^{3}H-labeled early RNA to R1-B and R1-D DNA. Poly(A)-selected ^{3}H-labeled early RNA (5 × 10^5 counts/min) was fractionated on a 3.2% acrylamide gel; electrophoresis was for 4 h at 5 mA/tube. Gel slices (2 mm) were solubilized in 150 μl of 6× SSC plus 0.1% SDS. Fractions 10 to 17, which contained 18S to 21S RNA, were divided into two aliquots; the two aliquots of each fraction were hybridized simultaneously to two filters, one containing 0.5-μg equivalents of R1-B DNA and one containing 0.5-μg equivalents of R1-D RNA. One aliquot was hybridized to filters prehybridized to 3 mg of KB RNA (●) per ml; the other was annealed to filters prehybridized to 3 mg of late cytoplasmic RNA (○) per ml. A parallel gel contained ^{14}C-labeled ribosomal RNA. The arrow indicates the position of 18S RNA; fraction 6 contained 28S RNA.*

transcripts from F fragment DNA (Table 2). Of the poly(A)-containing RNA which was virus specific, 5.1% was transcribed from F fragment DNA. Analysis of nuclear RNA lacking poly(A) showed that 9.1% of the hybridizing RNA was homologous to F fragment.

The possibility that the nuclear R1-F transcripts are linked to transcripts of other portions of the genome was tested by further analysis of RNA which hybridized to F fragment DNA.

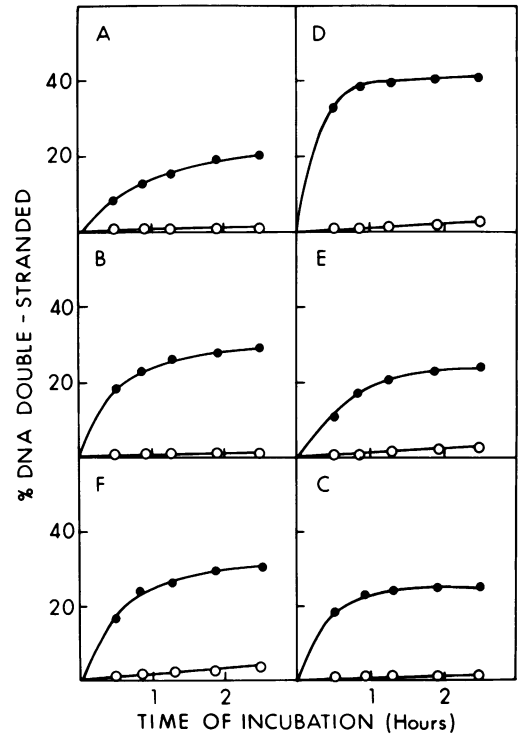

FIG. 5. *Liquid-phase hybridization of early nuclear RNA with ^{32}P-labeled R1 DNA fragments. ^{32}P-labeled DNA fragments were hybridized with 2 mg of early nuclear RNA (●) per ml and with 2 mg of RNA purified from uninfected KB cells (○) per ml. Aliquots (100 μl) removed at the indicated times, and the percentage of the DNA annealed was determined by digestion with S_1 nuclease. 100 μl of the reaction mixture contained the following amounts of radioactivity: 1,700 counts/min of R1-A; 900 counts/min of R1-B; 700 counts/min of R1-F; 900 counts/min of R1-D; 800 counts/min of R1-E; 1,000 counts/min of R1-C. The results are presented in the linear order of the fragments, A, B, F, D, E and C.*

TABLE 2. *Hybridization of cytoplasmic and nuclear ^{3}H-labeled early RNA to Eco R·R1 DNA fragments*[a]

Fragment	Cytoplasmic RNA total		Nuclear RNA					
			Total		Poly(A) containing		Non-poly(A) containing	
	Counts/min hybridized	Hybridized counts/min (%)	Counts/min hybridized	Hybridized counts/min (%)	Counts/min hybridized	Hybridized counts/min (%)	Counts/min hybridized	Hybridized counts/min (%)
A	539	12.5	1,528	13.8	597	13.2	210	15.9
B	772	17.9	2,204	20.0	1,029	22.8	202	15.3
F	20	0.46	640	5.8	229	5.1	120	9.1
D	1,300	34.5	3,391	30.8	1,170	26	320	24.8
E	143	3.3	411	3.1	328	7.3	76	5.8
C	1,484	30.2	2,781	25.2	1,148	25.5	400	30.3

[a] A culture infected in the presence of cycloheximide was labeled with [^{3}H]uridine from 2 to 6 h after infection. Cytoplasmic and nuclear RNAs were purified; a portion of the nuclear RNA was separated into poly(A)-containing and non-poly(A)-containing fractions by chromatography on oligo(dT)-cellulose. Each of these RNA samples was hybridized simultaneously to six filters, each containing 1.0-μg equivalents of one of the Eco R·R1 fragments. The hybridization reactions contained the following counts per minute: total cytoplasmic RNA, 1.4×10^6; total nuclear RNA, 2.8×10^5; poly(A)-containing nuclear RNA, 3.2×10^4; non-poly(A)-containing nuclear RNA, 3.5×10^4. The percent hybridized counts per minute bound to each fragment is the hybridization to an individual fragment divided by the total counts per minute hybridized to all six fragments. For all data shown, background was subtracted (12 counts/min).

TABLE 3. *Rehybridization of early nuclear R1-F fragment transcripts*[a]

Fragment	Counts/min hybridized		Average % hybridized
	1	2	
F	422	476	52
A	32	51	0.9
B	410	345	8.4
D	69	76	1.6
E	35	37	0.8
C	70	45	1.3

[a] A culture was infected in the presence of cycloheximide, labeled with [^{3}H]uridine from 2 to 6 h, and then harvested. Total nuclear RNA (8×10^6 counts/min) was hybridized in 30% formamide at 45 C to two membranes, each containing 4.2-μg equivalents of F fragment DNA. The hybridized RNA was eluted in 90% formamide giving a total of 8,250 counts/min. Part of the eluted RNA (870 counts/min) was rehybridized to F fragment DNA. Another portion was hybridized simultaneously to five filters, each containing 1-μg equivalents of the other five R1 fragments.

Early nuclear RNA was annealed to F fragment DNA in 30% formamide at 45 C, conditions which greatly reduce RNA degradation (2, 25). RNase treatment was omitted and the hybridized RNA was eluted by treatment with 90% formamide. The eluted RNA rehybridized 52% to F fragment DNA (Table 3). When the eluted RNA was hybridized simultaneously to the other five R1 fragments, 8.4% annealed to B fragment DNA (Table 3). The amount annealing to B fragment DNA was five times more than hybridized to any of the other four fragments (A, D, E, C).

DISCUSSION

The results of liquid hybridization with R1 fragments as compared to the viral mRNAs assigned to R1 fragments. In agreement with the result obtained when total viral DNA was annealed with early cytoplasmic RNA (Fig. 1) (4), studies with the R1 fragments demonstrated that early cytoplasmic RNA contains transcripts corresponding to 24% of the coding capacity of the genome (Table 1). This result is in reasonable agreement with studies performed in two other laboratories (L. Philipson, U. Pettersson, U. Lindberg, C. Tibbetts, B. Vennstrom, and T. Persson, Cold Spring Harbor Symp. Quant. Biol., in press; and P. Sharp, P. Gallimore, and S. Flint, Cold Spring Harbor Symp. Quant. Biol., in press). The studies with DNA fragments show that even though only a small portion of the genome codes for early cytoplasmic viral RNA, the segments of the genome coding for early RNA are widely distributed. Of the six R1 fragments, only F fragment

did not code for early cytoplasmic RNA (Fig. 2). Thus the genes coding for early functions are not clustered in one region of the genome.

The liquid hybridization studies supplement a previous report in which ^{3}H-labeled early viral mRNAs were annealed to the R1 fragments (21). Using size fractionated ^{3}H-labeled early RNA, hybridization showed that 13*S* and 11*S* molecules are transcribed from R1-A, 19*S* and 11*S* from R1-B, 20*S* and 13*S* from R1-D, 13*S* from R1-E, and heterogeneous transcripts (10*S* to 24*S*) from R1-C (21). Analysis of the size of these RNAs and the segments of the genome from which they are transcribed suggests that the six RNAs from fragments A, B, and D represent at least six different viral species, and that the transcripts from R1-E are part of the 13*S* molecules from R1-D (21; E. A. Craig, M. McGrogan, C. Mulder, and H. J. Raskas, manuscript in preparation). Assuming that the migration of these RNA species in acrylamide gels is proportional to the logarithm of their molecular weights, a comparison can be made of the viral mRNAs transcribed from each fragment and the percentage of each fragment DNA which anneals to early cytoplasmic RNA (Table 4). Although there is reasonable agreement between the two sets of data, in several instances (R1-B and R1-D) the sum of the molecular weights of the viral mRNAs is greater than would be expected on the basis of the liquid hybridizations. Either the liquid hybridizations may not have reached completion or, more likely, the molecular weight estimates for at least several of the RNAs may be too high. In fact, several of the viral RNA size classes migrate more rapidly relative to ribosomal RNA when analyzed in formamide gels (unpublished data).

Early cytoplasmic RNA contains two subclasses. As originally reported by Lucas and Ginsberg (10) and confirmed by Craig and Raskas (4), the cytoplasmic viral RNA sequences synthesized at early times consist of two subclasses. Class I RNA, the RNA present in reduced concentrations at 18 h, had not been detected in early hybridization-inhibition experiments (5). Subsequent studies showed that precautions must be taken to avoid nonspecific inhibition in the first step of hybridization-inhibition experiments (10); either the nonradioactive RNA must be degraded to small fragments before use, or alternatively the membranes must be treated with RNase after exposure to the inhibitor RNA and before addition of the ^{3}H-labeled RNA.

Liquid hybridization experiments with ^{32}P-labeled viral DNA (4) demonstrated that a mixture of early and late cytoplasmic RNA annealed more viral DNA than late RNA alone, as would be expected if part of the early RNA is in reduced concentrations at 18 h. However other experiments (23; Sharp, Gallimore, and Flint, Cold Spring Harbor Symp. Quant. Biol., in press), using a similar ratio of viral RNA: DNA, did not reveal the existence of class I RNA. It is likely that this discrepancy can be explained in the following manner: since the experiments of Tibbetts et al. (23) and Sharp et al. (Cold Spring Harbor Symp. Quant. Biol., in press) utilized separated strands of DNA, it was possible to continue the hybridization reactions for 36 h, as compared to 3 to 4 h for the annealing reactions performed by Craig and Raskas (4). Such hybridizations with separated DNA strands might detect viral RNAs present in very low concentrations, i.e., the early class I RNA and possibly other RNAs. This explana-

TABLE 4. *Comparison of early cytoplasmic viral RNAs assigned to R·R1 DNA fragments and results of liquid hybridizations with cytoplasmic RNA and R·R1 fragments*[a]

Fragment	DNA hybridized (%)	Mol wt of cytoplasmic RNA transcribed from hybridized DNA	Viral mRNAs	Mol wt of viral mRNAs
A	6	0.81×10^6	13*S*	0.38×10^6
			11*S*	0.30×10^6
B	24	0.65×10^6	19*S*	0.71×10^6
			11*S*	0.30×10^6
F	0			
D	40	0.68×10^6	20*S*	0.79×10^6
			(⅔) 13*S*	0.24×10^6
E	14	0.20×10^6	(⅓) 13*S*	0.12×10^6
C	22	0.50×10^6	Heterogenous	

[a] The data for the first two columns of this table were obtained from the information in Table 1. The assignment of mRNAs to the R·R1 fragments is based on the studies of Tal et al. (21; see Fig. 6); the presence of two 11*S* species, one from R1-A and one from R1-B, has been recently established (Craig, McGrogan, Mulder and Raskas, manuscript in preparation). It is likely that there is only one 13*S* molecule which overlaps the D-E cleavage site, with approximately ⅓ of the molecule originating from R1-E (21). Molecular weights were assigned to the viral RNAs assuming the migration in acrylamide gels with aqueous buffers is proportional to the logarithm of molecular weights and that 28*S* and 18*S* ribosomal RNAs are appropriate markers; since the poly(A) tract is not transcribed from the genome (16), 50,000 was subtracted from the molecular weight of each RNA.

tion is consistent with the quantitation of the liquid hybridizations using late cytoplasmic RNA: Tibbetts et al. (23) and Sharp et al. (Cold Spring Harbor Symp. Quant. Biol., in press) both found that at least 45% of viral DNA could be annealed to late RNA, whereas Craig and Raskas reported 24% (4). Indeed, more recent experiments by Tibbetts, Pettersson, and Philipson (personal communication) have shown that some cytoplasmic RNAs present at 18 h are at lower concentrations, a finding compatible with the existence of class I RNA.

Localization of class I and class II early mRNAs to regions of the genome. The hybridization-inhibition studies with ^{3}H-labeled early cytoplasmic RNA have allowed the assignment of class I early RNA to regions of the genome (Fig. 3). The early transcripts from Eco R·R1-D DNA belong to class II, whereas those from Eco R R1-C appear to be class I. The cytoplasmic RNAs transcribed from R1-A and R1-B contain both class I and class II sequences. Combining these results with the hybridization-inhibition experiments utilizing 19 to 20*S* RNA (Fig. 4), many of the early mRNA species (see Table 4) can be assigned unambiguously to these two sequence classes (Fig. 6). The 20*S* and 13*S* RNAs transcribed from R1-D are both class II RNAs. The assignment of R1-D 20*S* RNA to class II has been confirmed by direct hybridization of 20*S* RNA to D fragment DNA (Fig. 4). The 13*S* RNA transcribed from R1-E migrates at exactly the same rate as the 13*S* R1-D RNA and is present only in very small amounts. For this reason it is likely that the 13*S* R1-E and R1-D RNAs are the same molecule which overlaps the R1 cleavage site (21). As also shown directly by hybridization of size-fractionated RNA (Fig. 4), the 19*S* RNA transcribed from R1-B is a class I RNA. Since the early cytoplasmic transcripts from R1-B contain both class I and class II sequences, the second mRNA transcribed from R1-B, an 11*S* RNA, should be a class II molecule. In our previous study (21) the R1-A fragment appeared to code for 13*S* and 11*S* RNAs with the 11*S* RNA comigrating with the R1-B 11*S* RNA. Recent results demonstrate that the R1-A and R1-B 11*S* RNAs are different molecules (Craig, McGrogan, Mulder, and Raskas, manuscript in preparation). Experiments are in progress to determine which of the R1-A RNAs are class I and class II.

A comparison of the genome distribution of class I and class II early RNAs (Fig. 6) shows that these sequences, like the total cytoplasmic early RNA, are not clustered in a particular region of the genome. Using separated DNA strands, the early cytoplasmic RNA transcripts have been assigned to the two strands (Philipson et al. Cold spring Harbor Symp. Quant. Biol., in press; and Sharp et al. Cold Spring Harbor Symp. Quant. Biol., in press). The transcripts from R1 fragments A, D, and E are from the l strand, whereas the RNAs derived

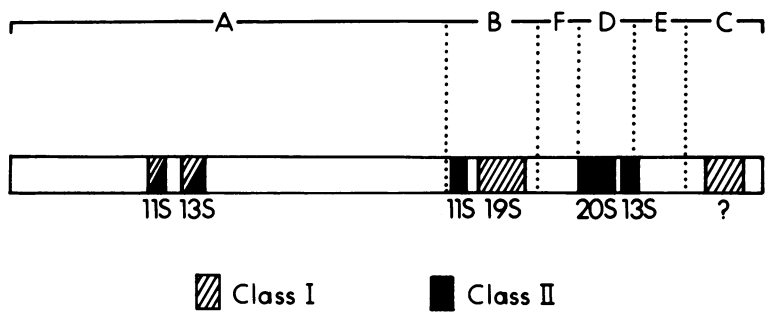

FIG. 6. *Localization of early class I and class II viral RNAs to R1 segments of the adenovirus genome. The data of Fig. 2, 3 and 4 were combined with a previous study which assigned individual viral mRNAs to the R1 fragments (21). The genome location of individual mRNAs is based on the results of Tal et al. (21) with the following exceptions: recent experiments (Craig, McGrogan, Mulder and Raskas, manuscript in preparation) have shown that the 11S RNA transcribed from R1-B is different from the R1-A 11S RNA. The length of the RNAs is proportional to their molecular weights (Table 4) as calculated from electrophoretic mobility. The size of a unique early RNA transcribed from R1-C has not been identified (21), and therefore a question mark is positioned under that fragment; the size of cytoplasmic transcripts from R1-C shown on the map is based on the liquid hybridization experiments with R1-C (Fig. 2) assuming that the 24% of R1-C which is transcribed into cytoplasmic RNA codes for a single mRNA. Within R1 fragments the precise location of the sequences coding for individual mRNAs has not been determined; for example, within the R1-A and R1-B fragments the relative position of the mRNAs is not yet known. Closed areas represent class I RNAs. Viral mRNAs which could not be placed in class I or II were drawn half closed and half cross-hatched.*

from DNA fragments B and C are from the h strand. Since more than 95% of the cytoplasmic viral mRNA synthesized at late times is derived from the l strand (Philipson et al. Cold Spring Harbor Symp. Quant. Biol., in press), one possible model is that the early mRNAs transcribed from the l strand remain at high concentrations at late times, i.e., class II. This model is consistent with what we have found for transcripts from R1 fragments C and D but not for the transcripts from A and B. Thus it is unlikely that the different metabolism of class I and class II RNAs is determined solely by the strand transcribed. In formulating models for the differential control of early RNAs it should be noted that the decreased concentration of class I RNA at late times may be caused either by reduced formation or greater instability of class I RNA or a combination of these two factors.

Analysis of early nuclear RNA using R1 DNA fragments. The liquid hybridizations with early nuclear RNA (Fig. 5) detected transcripts that were not represented in early cytoplasmic RNA (Fig. 1). These additional sequences are present in relatively high concentrations, for they could be detected in hybridizations using denatured duplex DNA. Viral RNA sequences which are restricted to the nucleus were first identified by hybridization-inhibition experiments (11, 12, 22, 24); the hybridization of ^{3}H-labeled early or ^{3}H-labeled late nuclear RNAs was inhibited only partially by early or late cytoplasmic RNA.

The RNA sequences restricted to the nucleus at early times are transcribed from at least four of the adenovirus 2 R1 DNA fragments (Fig. 1 and 5). The values obtained for hybridization of the R1 fragments by nuclear RNA are minimal estimates. In hybridization studies with separated strands of R1 fragments, Sharp et al. (Cold Spring Harbor Symp. Quant. Biol., in press) have achieved a higher percent hybridization with early nuclear RNA, reaching a level of 80 to 100% of an asymmetric transcript of the DNA in each fragment. The nuclear RNA from cultures harvested late in infection (18 h) contains symmetrical transcripts of the entire viral genome (Philipson et al. Cold Spring Harbor Symp. Quant. Biol., in press; Sharp et al. Cold Spring Harbor Symp. Quant. Biol., in press). Therefore the possibility of symmetrical transcription at early times merits serious consideration.

Both liquid-phase hybridizations and hybridizations with membrane-bound R1-F DNA demonstrated that early nuclear but not cytoplasmic RNA contains transcripts of R1-F (Fig. 2 and 5; Table 2) (21). The nuclear F transcripts comprise approximately 5% of the nuclear viral RNA labeled from 2 to 6 h after infection. As shown by the following calculation, these transcripts are found predominantly in the non-poly(A)-containing portion of nuclear RNA: approximately 11% of early nuclear RNA labeled in the presence of cycloheximide binds to oligo (dT)-cellulose (3); 17% of the poly(A)-containing RNA anneals to viral DNA, whereas 3% of the non-poly(A)-containing RNA is virus specified. Therefore at least 40% of the nuclear viral RNA labeled from 2 to 6 h after infection is polyadenylated. When nuclear poly(A)-containing RNA was annealed to R1 fragments (Table 2), 5% of the hybridized RNA bound to R1-F. Using non-polyadenylated RNA, 9% of virus-specified RNA was bound to R1-F. Normalizing these results to the input counts per minute for the hybridizations (see Table 2) and again using 11% as the percent of nuclear RNA binding to oligo (dT)-cellulose, we calculate that 80% of the nuclear F transcripts are in non-polyadenylated molecules.

Since the early cytoplasmic RNA does not contain transcripts of R1-F, the early nuclear R1-F RNAs are not utilized in early viral mRNA. For this reason the possible relationship of nuclear F transcripts to other RNA sequences has been investigated. F fragment RNA was selected by hybridization and elution in buffers containing formamide, conditions which aid in preserving the integrity of RNA molecules (2, 25). At least some nuclear F fragment transcripts obtained by this procedure may be covalently linked to RNA transcribed from R1-B (Table 3). An alternative explanation of this data is that nuclear R1-F transcripts are bound to R1-B transcripts by hydrogen bonds. The role of nuclear transcripts from R1-F is not clear. These transcripts might be part of precursors to the cytoplasmic mRNAs. For example, the F transcripts might be found in molecules which are precursors to the mRNAs derived from regions to the right or left of R1-F, i.e., R1-B or R1-D. Either of these possibilities is compatible with the predominance of R1-F transcripts in the non-polyadenylated fraction of nuclear RNA, since the F sequences might be cleaved early in processing of the mRNA. Further studies with other viral DNA fragments and separated strands of DNA fragments can distinguish between various models for the structure of nuclear R1-F transcripts. However, it should be noted that even though the structure of such nuclear RNAs may be compatible

with the role of precursor to a cytoplasmic RNA, metabolic studies are necessary to establish such a relationship.

ACKNOWLEDGMENTS

We thank C. Mulder, L. Philipson, P. Sharp and their collaborators for communication of their data prior to publication. We also thank Joe Kelley and Mark Telle for technical assistance.

This study was supported by grants from the National Cancer Institute (CA16007) and the American Cancer Society (VC-94A). Elizabeth Craig is supported by a postdoctoral fellowship from the National Institute of Allergy and Infectious Diseases. Stephen Zimmer is the recipient of a postdoctoral fellowship from the National Cancer Institute. Cell culture media were prepared in a facility funded by a National Science Foundation grant (GB-38657). This study was also supported by grants from the following companies: Brown & Williamson Tobacco Corp.; Larus and Brother Co., Inc.; Liggett & Myers, Inc.; Lorillard, a Division of Loews Theatres Inc.; Philip Morris, Inc.; R. J. Reynoldss Tobacco Co.; United States Tobacco Co.; and Tobacco Associates, Inc.

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