Species Identification and Genome Mapping of Cytoplasmic Adenovirus Type 2 RNAs Synthesized Late in Infection

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Adenovirus type 2 cytoplasmic RNAs synthesized late in productive infection were resolved by electrophoresis on formamide gels. Regions of the adenovirus 2 genome specifying RNAs of distinct size were determined by hybridization to specific DNA fragments generated by cleavage with endo R·EcoRI and endo R·SmaI. From these studies 13 distinct viral RNA species were identified. A 26S to 28S size class and a 21S to 23S size class were each found to consist of four distinct RNA species. Three RNA species were identified in a 16S to 18S size class, and a fourth size class, 11S to 13S, was resolved into two components. The SmaI-D region (0.38 to 0.51 on the unit genome) and the EcoRI-F, D region (0.70 to 0.83) of the genome were found to code for multiple transcripts. Three RNAs (28S, 22S, and 18S) are specified by SmaI-D, and four components, 28S, 22S, 18S, and 16S, are encoded by EcoRI-F,D. The RNA represented by each set of multiple transcripts exceeds the coding capacity of the respective region, and the species within each set of RNAs appear to contain common sequences. The relationship between the cytoplasmic RNA species synthesized at late times and early cytoplasmic RNAs was determined by hybridization-inhibition experiments. The multiple transcripts encoded by the EcoRI-D fragment were found to contain sequences that are present in early cytoplasmic RNA. These studies enabled preparation of a map which accounts for transcription of approximately 67% of the r strand of the adenovirus 2 genome.

Expression of the adenovirus type 2 genome during productive infection is under temporal control (18, 47). At early times, before viral DNA replication begins, seven to eight viral mRNA species are transcribed (8, 12, 43). These mRNA's include transcripts from limited regions of both strands of the genome (33, 38). The cytoplasmic RNA isolated late in infection contains transcripts of approximately 90% of the single-strand coding capacity (33, 38). These RNAs include sequences transcribed exclusively at late times as well as RNAs that are also present early in infection. Of the cytoplasmic viral RNA synthesized at a typical late time, 17 h, 99% is specified by one strand (30), the DNA strand transcribed in the rightward direction (r strand) (38).

At late times, expression of viral genes predominates in the infected cell. More than 80% of the polyadenylated cytoplasmic RNA synthesized at these times is virus specified (6, 22, 42). The adenovirus mRNA labeled at 18 h consists of four major size classes, which migrate as 13S to 27S RNAs on polyacrylamide gels (22, 36, 42). These major size classes are likely to contain multiple species, for more than 20 virus-induced or -specified polypeptides have been

shown to be synthesized late in infection (2, 20, 28). At least 14 of these polypeptides can be identified as structural components of the virion (15, 20).

To establish the size of individual viral mRNA species and identify the genome sites coding for viral mRNA's, RNAs can be fractionated by polyacrylamide gel electrophoresis and then hybridized to specific DNA fragments. In an initial study using this procedure, RNAs were fractionated in aqueous gel systems and hybridized to DNA fragments (43) generated by digestion of adenovirus 2 DNA with endo R·EcoRI (27). The only unique species identified was a 24S RNA detected by hybridization with EcoRI-E DNA (0.83 to 0.90 on the unit genome). Here we have used 14 DNA fragments to characterize cytoplasmic viral RNAs fractionated in 98% formamide gels. The present studies have confirmed the previous results and identified at least 12 additional cytoplasmic viral RNAs. Two regions of the genome specify several size classes of RNA, whose total length exceeds the transcriptional capacity of the region. These RNAs of different sizes apparently contain common sequences. The results of these studies suggest a transcription map for late

RNA species.

MATERIALS AND METHODS

Cell culture and virus infection. Maintenance of KB cell suspension cultures, virus infection, and purification of adenovirus type 2 were performed as described previously (13). Prior to labeling, cultures were concentrated to 9×10^5 cells/ml. [³H]uridine (50 μCi/ml, 40 Ci/mmol; New England Nuclear Corp.) was added either 12 to 14 h or 17 to 19 h after infection.

Cell fractionation, RNA purification, and fractionation. Cultures were harvested at 4°C, and cytoplasmic fractions were prepared by suspending cells in isotonic buffer (0.15 M NaCl, 0.01 M Tris, pH 7.5, 0.0015 M MgCl₂, 0.1% diethylpyrocarbonate) and 0.1% Nonidet P-40 (Shell Chemical Co.). Nuclei were removed by centrifugation at $800 \times g$ for 10 min, and the supernatant was clarified by centrifugation at $18,000 \times g$ for 20 min. RNA was purified, after the addition of 0.002 M EDTA and 0.2% sodium dodecyl sulfate, by multiple extractions at room temperature with an equal volume of phenol (saturated with 0.1 M NaCl, 0.01 M Tris, pH 7.5, and 0.002 M EDTA) and chloroform-isoamyl alcohol (at a ratio of 24:1) as previously described (9).

RNA containing polyadenylic acid [poly(A)] was selected by chromatography on oligodeoxythymidylic acid-cellulose (Collaborative Research, Inc.) (4) and precipitated with 2 volumes of 95% ethanol in the presence of 0.1 M NaCl at −20°C.

Polyadenylated [³H]RNA was denatured (11) and fractionated on 3.0 or 3.5% bisacrylamide gels (0.6 by 10 cm) containing 98% formamide (Eastman Co.) (14, 41). The electrophoresis was performed in 0.04 M sodium phosphate (pH 6.8) for 4 to 6 h at 2 to 3 mA/gel. To provide reference positions of 28S and 18S rRNA, ¹⁴C-labeled cytoplasmic RNA isolated from uninfected KB cells was added to a sample of the [³H]RNA and fractionated in a parallel gel. In some experiments, *E. coli* [¹⁴C]RNA was also added to provide 23S and 16S bacterial rRNA's as additional markers. For these four rRNA's the relationship between distance migrated and S value was linear. To establish the ability of these gels to fractionate RNAs larger than 28S, KB cell 45S and 32S ribosomal precursor RNAs were also fractionated in this gel system and could be separated as distinct peaks migrating slower than 28S RNA. Gels were fractionated into 1-mm slices, and the RNA was eluted from each slice by incubation for 36 h at 66°C in 6× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate. Prior to performing hybridization experiments with RNAs fractionated by size, a sample of the RNA eluted from each fraction was counted directly to establish that the RNA profile was characteristic of the pattern reproducibly obtained late in adenovirus 2 infection (42).

Purification of viral DNA and preparation of DNA fragments. Adenovirus 2 DNA was extracted from CsCl-purified virus by the procedure of Tibbetts et al. (45). Viral DNA was digested with either endo R·*Eco*RI or endo R·*Sma*I, and the DNA fragments were isolated by electrophoresis on 1% agarose gels as described previously (11, 39). Trace amounts of ³²P-labeled adenovirus DNA (100 cpm/μg) were added before digestion to quantitate recovery. DNA fragments were routinely subjected to a second electrophoresis on agarose gels to insure their purity. DNA was eluted from gel slices by crushing the gel in 2 volumes of 0.01 M Tris, pH 7.5, 0.002 M EDTA followed by three cycles of freezing and thawing. The gel suspension was then forced through a syringe plugged with glass wool to retain gel particles. The eluted DNA was clarified by centrifugation at $15,000 \times g$ for 15 min. Recovery of DNA fragments by this procedure was greater than 70%.

All *Sma*I DNA fragments were prepared by cleavage of *Eco*RI-A. To purify the *Eco*RI-A fragment for subcleavage, the eluted DNA was passed through a 1-ml hydroxylapatite column (Bio-Rad) at room temperature; the DNA was applied in 0.01 M potassium phosphate, pH 7.0, washed with 0.01 M potassium phosphate, pH 7.0, and eluted with 0.5 M potassium phosphate, pH 7.0, according to the procedure of Ludwig and Summers (24). After passage through the column, the DNA was dialyzed at 4°C against four changes of 0.01 M Tris, pH 7.5, 0.002 M EDTA for a total of 24 h. The dialyzed DNA was adjusted to 0.1 M NaCl and precipitated with 2 volumes of ethanol at −20°C.

RNA-DNA hybridization. Hybridization experiments utilized adenovirus 2 DNA immobilized on 6.5-mm cellulose nitrate membranes (type B6, Schleicher and Schuell Co.) (9, 35). For hybridization with whole adenovirus DNA, membranes contained from 0.5 to 2 μg of DNA. For hybridizations with DNA fragments, amounts of DNA were defined as microgram equivalents; a 1-μg equivalent of a DNA fragment is the amount of DNA derived from 1 μg of whole adenovirus DNA. All DNA membrane filters were tested for competence by hybridization to ³²P-labeled DNA (34). [³H]RNA eluted from gel slices was hybridized for 24 h at 66°C in 6× SSC, 0.1% sodium dodecyl sulfate. After incubation, filters were washed with 2× SSC, treated with 20 μg of pancreatic RNase per ml (Worthington Biochemicals Co.), and processed as described previously (10). When membranes containing different DNA fragments were hybridized simultaneously, each DNA filter was processed separately. Filters were dried and counted in a liquid scintillation counter.

Hybridization-inhibition experiments were performed in two steps as described by Craig et al. (13). Unlabeled alkali-fragmented RNA was first hybridized to filters containing either 0.5 μg of whole viral DNA or 0.5-μg equivalents of a particular DNA fragment. Three different types of unlabeled cytoplasmic RNAs were used: RNA from uninfected KB cells, RNA from cultures treated with 25 μg of cycloheximide per ml and harvested at 6 h after infection, or RNA from cultures harvested at 18 h after infection. In the second step, ³H-cytoplasmic RNA labeled late in infection was added and incubated with the prehybridized DNA filters for 24 h at 66°C. Filters were washed and treated with RNase as described above. For some experiments the [³H]RNA was first fractionated on gels, then eluted, and hybridized to membranes that had been preannealed with inhibitor RNA.

RESULTS

Since previous studies had not identified any viral mRNA's lacking poly(A) (22), late cytoplasmic viral RNA could be selected by oligodeoxythymidylic acid-cellulose chromatography. When the poly(A)-containing [³H]RNA was analyzed by electrophoresis on 98% formamide gels, four major size classes of viral RNA were resolved: 26S to 28S, 21S to 23S, 16S to 18S RNAs, and a small virus-specific peak of 11S to 13S RNA (data not shown). Late in adenovirus 2 infection, more than 80% of the polyadenylated cytoplasmic RNA is virus specified (22, 42); therefore, the total profile of poly(A)+ cytoplasmic RNA is essentially identical to the profile of viral RNA. Although the size distribution obtained with formamide gels was in general agreement with previous studies using aqueous gel systems (6, 22, 29, 36, 42), one consistent difference was revealed. Although several earlier studies reported that some cytoplasmic viral RNA migrated significantly slower than 28S rRNA (22, 29, 42), such size classes have not been detected when formamide gels are used.

For the studies reported here, we have used two restriction endonucleases, endo R·EcoRI and endo R·SmaI. Cleavage maps of adenovirus 2 DNA for these two enzymes are shown in Fig. 1. The viral genome is cleaved at five sites by endo R·EcoRI (27) and at twelve sites by endo R·SmaI (C. Mulder, personal communication). Nine of the endo R·SmaI sites are located in the left-hand 58% of the viral genome. Cleavage of purified EcoRI-A fragment with endo R·SmaI produces nine SmaI fragments and one new fragment, designated A-A, which is generated from the region of EcoRI-A that overlaps SmaI-A (0.561 to 0.585 on the unit genome).

Use of SmaI fragments to map cytoplasmic RNAs transcribed from the left-hand 58% of the genome. When cytoplasmic RNA was analyzed by hybridization to EcoRI-A, a fragment containing the left-hand 58% of the genome, the size distribution obtained was essentially the same as that obtained with whole adenovi-

rus DNA. To localize RNAs of different sizes to specific regions within EcoRI-A, hybridization experiments were performed with the eight largest SmaI fragments generated by subcleaving EcoRI-A (Fig. 2). For each experiment in which multiple panels are shown, the experiments were performed by eluting RNA from individual gel slices and hybridizing the eluted RNA simultaneously to a series of membranes containing different DNA fragments. The results with the SmaI fragments are presented in the genomic order of the fragments (see Fig. 1). The size of the RNAs detected by hybridization with the SmaI fragments and the coding capacity of these fragments are compared in Table 1.

Although SmaI-J specifies early mRNA's (11), the level of synthesis of cytoplasmic RNA at late times was too low to be detected in these experiments. The hybridization profile with the SmaI-E fragment includes a major peak that migrates as 13S RNA. Of all the late-specific sequences, only those sequences contained in SmaI-F and part of SmaI-B are transcribed from the l strand rather than the r strand (32). SmaI-F DNA gives a low level of hybridization that is heterogeneous in size. Hybridization with the SmaI-B fragment reproducibly resolved 26S, 21S, and 13S RNAs superimposed on a background of heterogeneous RNAs.

The SmaI-I fragment contains the sequences in 0.354 to 0.384 on the unit genome map (see Fig. 1). Hybridization with this fragment yielded a broad 27-26S peak and also a 21S peak. Hybridization to SmaI-D DNA was 10-fold higher than to the other SmaI fragments. The SmaI-D profile consisted of 27S, 22S, and 18S RNAs. A single 28S peak was found to hybridize to both SmaI-H and SmaI-A-A fragments. This RNA size class consistently migrated slower than the 27S SmaI-D peak.

From the summary in Table 1, it is clear that the total molecular weight of the RNA size classes transcribed from some SmaI fragments is greater than their coding capacity. This result would be obtained if (i) some mRNA's are encoded by sequences present in two adjacent fragments or (ii) some RNAs contain common sequences.
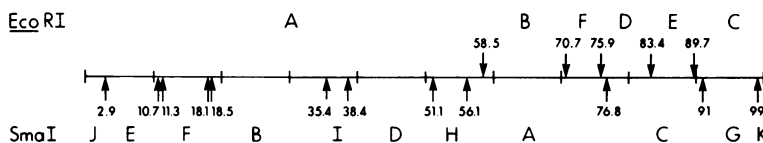

FIG. 1. Restriction endonuclease cleavage maps of the adenovirus 2 genome. The cleavage sites for endo R·EcoRI were determined by Mulder et al. (27). The endo R·SmaI sites are based on studies of Mulder, Green, and Delius (C. Mulder, personal communication). The cleavage sites are designated by arrows and a number corresponding to the map position of the site. The space between each pair of small vertical bars represents 10% of the length of the viral genome.
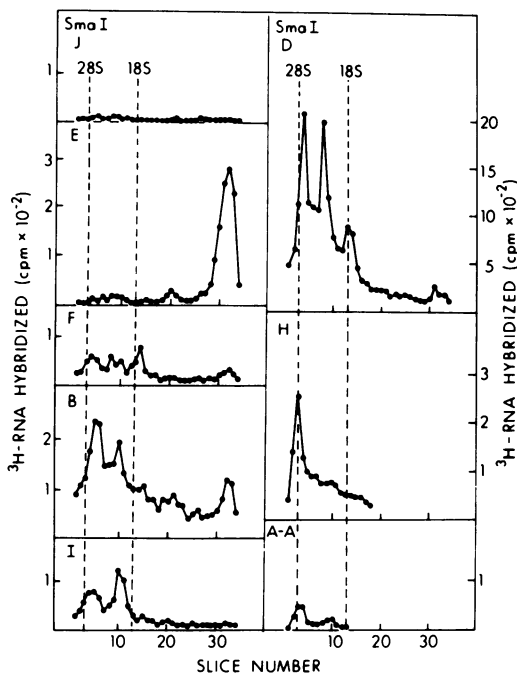
FIG. 2. Hybridization of ³H-labeled cytoplasmic RNA to SmaI fragments derived from the left-hand 58% of the viral genome. Poly(A)-containing RNA was isolated from the cytoplasm of cultures labeled with [³H]uridine from 17 to 19 h after infection. [³H]RNA (10⁶ cpm) was fractionated on 3% polyacrylamide gels cast in 98% formamide. Electrophoresis was for 5.5 h at 2 mA/gel. RNA was eluted from 1-mm gel slices in 200 μl of 6 × SSC containing 0.1% sodium dodecyl sulfate (see text). The eluted RNA was hybridized for 24 h at 66°C to eight SmaI fragments obtained by cleaving EcoRI-A DNA. Each membrane contained a 1-μg equivalent of fragment DNA (the amount of DNA derived from 0.58 μg of EcoRI-A). The results are arranged to correspond to the linear order of the fragments: J, E, F, B, I, D, H, and A-A (see Fig. 1). To provide 28S and 18S reference markers, a parallel gel contained 2 × 10⁵ cpm of ³H-labeled late RNA plus 2 × 10⁴ cpm of uniformly labeled ¹⁴C-cytoplasmic RNA isolated from uninfected KB cells.

Cytoplasmic RNAs transcribed from the right-hand 42% of the adenovirus 2 genome.

³H-labeled cytoplasmic RNA containing poly(A) was fractionated by electrophoresis in formamide gels, and the eluted RNA was hybridized simultaneously to the five EcoRI fragments that constitute the right-hand 42% of the adenovirus 2 genome (Fig. 3). These five fragments have the genome order B,F,D,E,C (see Fig. 1 for cleavage map). The estimated molecular weights of the polyadenylated RNAs transcribed from these fragments and the coding capacity of these fragments are summarized in Table 2.

Improved resolution of the RNAs encoded by

EcoRI-F and EcoRI-D fragments was achieved by examining RNAs synthesized at 12 h after infection (Fig. 4). The RNA size classes synthesized at 12 h were essentially the same as those synthesized at 18 h (data not shown). However, the 16S and 28S RNAs specified by EcoRI-D and the 18S RNA specified by EcoRI-F were detected in relatively greater amounts.

As already noted for the RNA size classes transcribed from the left-hand 58% of the genome, many of the fragments from the right-hand 42% also specify RNAs whose total molecular weight is greater than the respective coding capacity (see Table 2).

Detection of early RNA sequences in RNA size classes synthesized at late times. Some of the cytoplasmic viral RNAs synthesized at late times include sequences that are also synthesized early (42, 44). These early RNAs are probably examples of the class II early RNAs, se-

TABLE 1. Size classes of polyadenylated RNA transcribed from the left-hand 58% of the genome[a]

SmaI fragment	Transcriptional capacity (mol wt, × 10⁻⁶)	Late RNAs	Mol wt of late RNAs (× 10⁻⁶)	Total mol wt of RNAs transcribed from each fragment (× 10⁻⁶)
J	0.34			
E	0.90	13S	0.29	0.29
F	0.78	?		
B	1.95	13S	0.29	
		21S	0.88	
		26S	1.40	2.57
I	0.37	21S	0.88	
		26S	1.40	2.28
D	1.44	18S	0.62	
		22S	0.97	
		27S	1.52	3.11
H	0.58	28S	1.65	1.65
A-A	0.28	28S	1.65	1.65

[a] The transcriptional capacity of the SmaI DNA fragments derived from cleavage of EcoRI-A DNA was determined from the molecular weight of each of the SmaI fragments (Mulder, personal communication). The S value for each RNA size class was estimated from the mobility in formamide gels, using KB and E. coli rRNA's as standards (41). Molecular weights were calculated from the S value using the Spirin equation (molecular weight = 1,550S²·¹) (40). The molecular weight of genome-specified RNA was assumed to be the molecular weight less 50,000 for the poly(A) segment. The RNA size classes were identified by the experiment shown in Fig. 2.
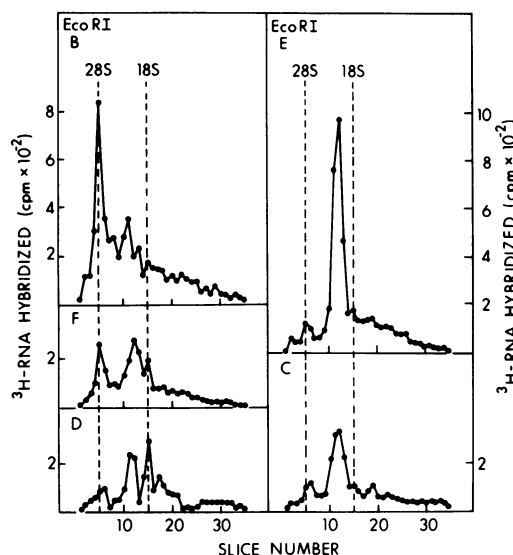
FIG. 3. *Hybridization of ³H-labeled cytoplasmic RNA to EcoRI fragments representing the right-hand 42% of the viral genome. Poly(A)-containing cytoplasmic RNA was prepared and gel electrophoresis was performed as described in the legend to Fig. 2. RNA was eluted in 200 μl of 6× SSC, 0.1% sodium dodecyl sulfate, and each fraction was hybridized simultaneously to 0.5-μg equivalents of the five EcoRI fragments, B, F, D, E, and C. The hybridization profiles are presented in the genomic order of the fragments. Dashed lines indicate the position of the 28S and 18S rRNA markers.*

quences whose cytoplasmic transcripts are in relatively high concentrations at late times (10). The early sequences present in cytoplasmic RNAs synthesized at 18 h postinfection can be detected by hybridization-inhibition experiments (10, 23, 42).

To determine which RNA species synthesized at 18 h contain early sequences, hybridization-inhibition experiments were performed with RNAs fractionated by size (Fig. 5). Initial control experiments were performed to determine the concentration of RNA required to give maximal inhibition. Membranes containing EcoRI-B,F,D,E,C, SmaI-E, and SmaI-B fragment DNAs were presaturated with excess amounts of one of three unlabeled RNAs. In the second step presaturated filters were annealed to aliquots of late ³H-labeled cytoplasmic RNA eluted from gel slices. One aliquot was incubated with a set of fragment filters presaturated with RNA from uninfected cells, the second aliquot with fragments prehybridized with early inhibitor, and the final aliquot with fragments prehybridized with late RNA.

As expected, the hybridization to filters presaturated with RNA from uninfected cells revealed the same RNA size classes described

above. Hybridization with filters prehybridized with late cytoplasmic RNA reduced the hybridization to all fragments 80 to 90%. Using early RNA as inhibitor, the hybridization profiles obtained with EcoRI-B,F,E,C and SmaI-B fragments were not altered. Thus, the late RNA species transcribed from these fragments do not contain early sequences. Hybridization of the 13S RNA encoded by SmaI-E was reduced 80% by early RNA. This region of the genome has previously been shown to specify a 13S early RNA, which continues to be synthesized at late times (8). In control experiments, hybridization of total ³H-labeled late RNA to the EcoRI-D fragment was inhibited 60% by early RNA (data now shown). Surprisingly, hybridization of all of the EcoRI-D size classes synthesized at 18 h was inhibited to the same extent, approximately 60% (Fig. 5). This result demonstrates that all the size classes transcribed late from EcoRI-D contain sequences that are also expressed at early times.

DISCUSSION

The primary goals of these studies were the identification of late viral mRNA species and the genome sites that specify those species. The

TABLE 2. *Size classes of polyadenylated RNA transcribed from the right-hand 42% of the genome[a]*

EcoRI fragment	Transcriptional capacity (mol wt, × 10⁻⁶)	Late RNAs	Mol wt of late RNAs (× 10⁻⁶)	Total mol wt of RNAs from each fragment (× 10⁻⁶)
B	1.41	28S	1.65	1.65
F	0.60	28S	1.65	
		22S	0.97	
		18S	0.62	3.24
D	0.87	28S	1.65	
		22S	0.97	
		18S	0.62	
		16S	0.47	3.71
E	0.73	22S	0.97	0.97
C	1.19	22S	0.97	0.97

[a] The transcriptional capacity was determined from the molecular weight of each of the EcoRI DNA fragments (27). The S value for each RNA size class was estimated from the mobility in formamide gels as compared to KB and E. coli rRNA's (41). The molecular weight of the RNA species was calculated from the S value using the Spirin equation (molecular weight = 1550S^{2.1}) (40). The molecular weight of genome-specified RNA was assumed to be the molecular weight less 50,000 for the poly(A) segment. The RNA size classes were identified from the experiments shown in Fig. 3.
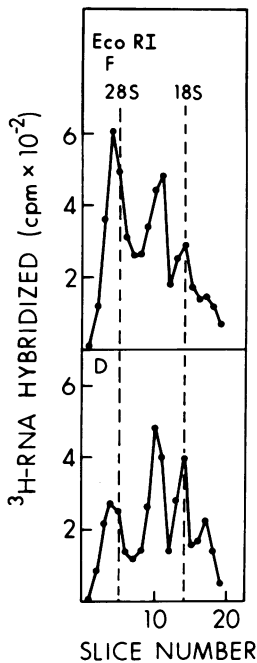
FIG. 4. *Polyadenylated RNAs specified by EcoRI fragments F and D: hybridization of ³H-cytoplasmic RNA labeled from 12 to 14 h after infection. Poly(A)-containing RNA was isolated from the cytoplasm of cultures labeled with [³H]uridine from 12 to 14 h after infection. [³H]RNA (5 × 10⁵ cpm) was fractionated on a 3.5% polyacrylamide-formamide gel by electrophoresis for 6 h at 2 mA/gel. Eluted [³H]RNA was hybridized to filters containing 1-µg equivalents of either EcoRI-F or EcoRI-D DNA. To obtain the positions of 28S and 18S rRNA's, a small sample of [³H]RNA was mixed with ¹⁴C-labeled cytoplasmic RNA from uninfected cells, and the mixture was fractionated on a parallel gel.*

FIG. 5. *Hybridization-inhibition studies with ³H-labeled late cytoplasmic RNA fractionated by size. Cytoplasmic RNA was prepared from cultures labeled 17 to 20 after infection with [³H]uridine. Polyadenylated RNA (1.2 × 10⁶ cpm) was fractionated on 3% polyacrylamide-formamide gels, and the eluted RNA was divided into three aliquots. Three separate hybridization-inhibition experiments were performed using 0.5-µg equivalents of SmaI fragments E and B and EcoRI fragments B, F, D, E, and C (see text). The first set of DNA fragment filters was hybridized with 1 mg of cytoplasmic RNA per ml from cultures harvested at 18 h after infection (○); the second set was prehybridized with 1.5 mg of early cytoplasmic RNA per ml purified from cycloheximide-treated cultures harvested at 6 h after infection (△); the third set was prehybridized with 1 mg of cytoplasmic RNA per ml from uninfected KB cells (●). Each set of prehybridized filters was incubated with aliquots of RNA eluted from gel slices, and the hybridized counts per minute were determined. The arrows indicate the positions of the 28S and 18S rRNA markers.*

identification of a viral RNA size class as an mRNA species requires that the molecule be homogeneous in size, that it contain a unique base sequence, and that it function as mRNA. In principle, the hybridization of an RNA size class to a DNA fragment of comparable size can provide adequate evidence for physical identification of a species. For some of the size classes we have studied, this criterion appears to be fulfilled.

Although the details of the present studies are discussed below, two important observations should be noted:

(i) First, there appear to be at least several regions (for example, 0.40 to 0.50 and 0.70 to 0.83 on the unit genome) that specify RNAs whose sequence content is greater than the coding capacity of the DNA segment. These families of RNAs share common sequences and are present in polyribosomes (M. Mcgrogan and H. J. Raskas, manuscript in
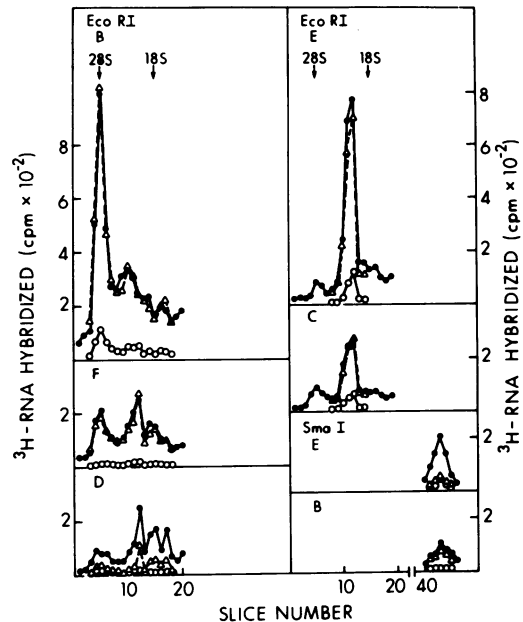
preparation). A similar structural relationship exists between 19S and 16S late RNAs transcribed from simian virus 40 (1, 19). Although the translation products of these adenovirus RNAs have not yet been examined, it may be that only limited portions of the RNAs, for example, sequences at the 5' ends, are actually translated. This possibility is consistent with the observation that in in vitro systems, six virus-specific polypeptides were translated from RNAs much larger than expected for the size of the polypeptides (20).

(ii) A second observation pertains to sequences contained in the *EcoRI-D* fragment. This fragment specifies 20S and 13S early

mRNA's (43). However, late in infection, the early sequences appear to be part of RNA species that are different in size from the species synthesized before the onset of DNA synthesis.

Preliminary mapping experiments with late cytoplasmic RNA were performed by Tal et al. (43). This study established that 27S, 24S, and 19S RNAs were transcribed from the *Eco*RI-A fragment, and that *Eco*RI-E specified a 24S RNA. To attain the sensitivity and resolution required to perform a detailed analysis of late RNA, we found it necessary to fractionate RNA on formamide gels and to insure that each hybridization was carried out in DNA excess. The size classes we have identified by this procedure may not represent a complete catalog but do include all size classes that we found to be reproducible, and at least threefold above hybridization background.

From our interpretation of the results, we suggest that there are at least 13 cytoplasmic RNA species synthesized late in infection. These viral RNAs have been organized into a transcription map (Fig. 6). Transcription maps have been prepared previously from the results of liquid hybridizations between separated strands of radioactive DNA fragments and cytoplasmic RNAs (16, 32, 33, 38, 46). Such studies identify blocks of early and late sequences but do not allow species identification. The most recent of these maps is presented in Fig. 6 for comparison to the species map proposed here. Since greater than 95% of the cytoplasmic viral RNA synthesized late in infection is specified by the *r* strand (30), we have assumed for the present that the RNA size classes we detected are specified by this strand.

Proposed map. For each region of the genome the following data are the basis for the map of late viral RNA:

0.00 to 0.11 on the unit genome. At early times in infection, the *r* strand of the left end of the genome is transcribed into cytoplasmic RNA (8, 16, 32). The transcripts from this region include two 13S RNAs, 11S RNA, and a 23S species (7, 8; F. Eggerding and H. J. Raskas, manuscript in preparation). One of the 13S RNAs is transcribed from a terminal region (0.00 to 0.075) defined by the *Hin*dIII-G fragment, and the second RNA is specified by the region 0.075 to 0.11. At late times we have detected only a 13S RNA, which annealed to *Sma*I-E but not to *Sma*I-J (Fig. 2). Hybridization-inhibition studies demonstrated that this 13S RNA is composed of sequences that are transcribed into cytoplasmic RNA at early times (Fig. 5) (8). Therefore, the 13S RNA detected at 18 h may be identical to the early 13S RNA specified by the 0.075 to 0.11 region of the
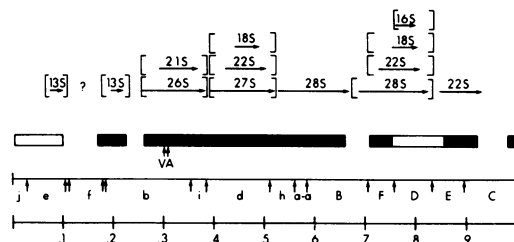

FIG. 6. *Map of cytoplasmic viral RNA species synthesized late in infection. The lower line provides reference positions for sites on the genome. The space between each pair of vertical lines represents 0.10 genome unit having a coding capacity of 1.15 × 10⁶ daltons of RNA. The second line from the bottom reviews the cleavage sites relevant to this study. Fragments produced by cleavage with endo R · EcoRI are identified with uppercase letters and those produced by endo R · SmaI by lowercase letters. The blocks shown on the third line from the bottom are the regions of the genome transcribed from the r strand based on liquid hybridization studies (32). The open blocks are regions transcribed at early times, and the shaded blocks are late specific. Regions for which the bar is omitted are regions from which mRNA is coded by the l strand. The upper portion of the figure is based on the studies reported in the Discussion. The map includes the RNA size classes synthesized at both 12 and 18 h after infection and assumes that all these size classes are r strand transcripts. The brackets around a species indicate the extent of uncertainty in assigning the genome site. The line representing each size class is proportional to its molecular weight as deduced from migration during electrophoresis (Tables 1 and 2). The arrows indicate the direction of transcription.*

genome.

0.11 to 0.39 on the unit genome. Liquid hybridization studies have shown that this segment contains late cytoplasmic RNA transcribed from the *l* strand (38, 46). These *l* strand transcripts are probably divided into two different blocks of genes (32). Since all other late sequences are specified by the *r* strand, the metabolism of these RNAs may be different from that of other late RNAs. These cytoplasmic RNAs transcribed from the *l* strand may be synthesized earlier than 12 h, may be relatively unstable as cytoplasmic species, or may be inefficiently transported to the cytoplasm. Hybridizations of [³H]RNA with *Sma*I-F DNA (0.113 to 0.18) have not identified a discrete RNA species. One of the RNAs detected in hybridizations with *Sma*I-B DNA is a 13S RNA. The proposed *r* strand region (0.18 to 0.22) (32) between the two blocks of *l* strand transcripts is large enough to specify this RNA.

The region of the genome from 0.26 to 0.39 is represented by sequences in *Sma*I fragments B and I. These two segments specify 26S and 21S

RNA size classes. Although part of the mRNA transcribed from SmaI-B is encoded by the l strand, for the present we have assumed that the RNAs synthesized at late times use the r strand as template. It should be noted that the low-molecular-weight VA RNA maps at 0.30 on the r strand of the unit genome (25, 31, 49). Thus, the sequences in VA RNA may also be in the 26S species.

0.39 to 0.51 on the unit genome. DNA fragment SmaI-D encodes a complex set of cytoplasmic RNAs. SmaI-D specifies 27S, 22S, and 18S RNAs, which have a collective molecular weight of 3.26×10^6, more than twice the transcriptional capacity of this fragment. As described above, further experiments have confirmed that the three RNAs specified by SmaI-D are present in polyribosomes and that these RNAs contain common sequences (McGrogand and Raskas, in preparation).

0.51 to 0.71 on the unit genome. Three contiguous fragments all specify 28S RNA, SmaI-H, SmaI-A-A, and EcoRI-B. This 28S size class is different from the 27S RNA specified by SmaI-D; it has a different migration rate in gel electrophoresis, and RNA selected by SmaI-D does not rehybridize to EcoRI-B (data not shown). Fragment H, together with A-A and the portion of EcoRI-B that specifies r strand RNA (0.585 to 0.65) (32), are sufficient to specify a single 28S species.

0.71 to 0.83 on the unit genome. This region includes EcoRI fragments F and D. Hybridization with these two fragments yielded a result similar to that described earlier for the SmaI-D fragment: the RNA size classes identified exceed the coding capacity of the fragments. RNAs synthesized at 12 and 18 h postinfection contain four size classes from this region, 28S, 22S, 18S, and 16S RNAs (Fig. 4). All four RNAs were detected in hybridizations with EcoRI-D; only the three larger RNAs annealed to EcoRI-F DNA. The simplest interpretation of these results is that each of three RNAs contains sequences from both fragments, F and D. Although quantitative inferences from our hybridization data cannot be precise, it appears that the 28S, 22S, and 18S RNAs contain progressively fewer sequences from EcoRI-F. This model is supported by subsequent hybridization experiments with smaller DNA fragments generated by subcleaving EcoRI-F and EcoRI-D (McGrogan and Raskas, in preparation). Thus, if all four of these RNAs contain some sequences in common, it is likely that the shared sequences are in the 3' half of the molecule. It is significant that fragments F and D are not large enough to specify a 28S RNA. Therefore, we suggest that this species is

encoded in part by EcoRI-B. This model is consistent with observations that the only late polypeptide identified as a product of this region, a 100K protein, could be translated with RNAs selected by EcoRI fragments B, F, and D (20).

A particularly interesting finding regarding these RNAs was obtained from hybridization-inhibition experiments (Fig. 5). All four of these RNAs were shown to contain EcoRI-D sequences that are transcribed into early mRNA. In contrast, the sequences from EcoRI-F were not related to any early RNAs. Previous studies have shown that the early RNAs specified by EcoRI-D migrate as 20S and 13S RNAs (11, 43).

0.83 to 1.00 on the unit genome. Both EcoRI-C and EcoRI-E specify a 22S RNA (Fig. 3), and rehybridization of RNA selected by EcoRI-E (unpublished data) demonstrated that sequences transcribed from these fragments are covalently linked. The 22S RNA corresponds to the block of contiguous late specific sequences transcribed from the r strand of these two fragments (32). An RNA small enough to be specified from the terminal region of EcoRI-C (0.98 to 1.00) has not been detected in samples labeled at various times after infection (unpublished data of D. Carlson).

Relationship of RNA size classes to viral proteins. A partial genetic map for adenovirus 2 peptides has been constructed from in vitro translation studies of fragment-specified mRNA (3, 20, 21) and by studying the DNA of recombinants between adenovirus 2 ND1 and adenovirus 5 (26, 37). This map suggests correlations between some of the viral RNA size classes we have observed and the viral polypeptides synthesized at late times. The data suggest that the SmaI-D region (0.40 to 0.50 on the genome) may code for the core proteins p-VI (27K), p-VIII (20K), and p-V (48.5K). The gene for the major virion protein, hexon (120K), has been shown by both translation and recombinant studies to be located in the region 0.50 to 0.60, sequences included in SmaI-H, SmaI-A-A, and EcoRI-B fragments. We suggest that a 28S mRNA is transcribed from sequences found in these fragments (see Fig. 6). The 100K polypeptide was the only late protein found to be specified by the second region, which specifies a family of mRNA's, 0.70 to 0.80 (EcoRI-B,F,D). It has been established by both translation and physical mapping studies that the fiber gene (IV, 62K) is located in the EcoRI-E,C region (0.85 to 0.95). The 22S RNA transcribed from this region has a coding capacity of 97K daltons of protein, considerably more than the molecular weight of the fiber polypeptide. For further

correlations of mRNA's with polypeptides, it is apparent that RNAs fractionated by size and selected by fragment hybridization must be used in in vitro translation studies.

Origin of the late mRNA's. The present study has established the identity of cytoplasmic viral mRNA's synthesized late in infection. Although these RNAs are probably transcribed by RNA polymerase II (50), other details of their origin are not clear at present. Several observations suggest that the cytoplasmic molecules are not primary transcripts: nuclear RNAs synthesized at late times include sequences that are not in the cytoplasm (33, 38, 48), and nuclei contain RNAs that are apparently larger than the cytoplasmic species (5, 29, 48). One possibility is that there is only one major promoter for late transcription from the r strand and that most mRNA's are derived by processing a single large precursor (5). Studies of nuclear RNA synthesized early in infection have identified polyadenylated nuclear RNAs whose structural features are compatible with a precursor role (11); however, these putative precursors represent a transcript of no more than 15% of the length of the viral genome. The notion of a limited number of promoters for late transcription must be balanced by the observation that transcripts from different late genes accumulate in strikingly different quantities in the cytoplasm (17). In our study the transcripts from SmaI-D accumulated at nearly a 10-fold-higher rate than RNAs from other regions of the genome (see Fig. 2). Such differential accumulation of cytoplasmic RNAs may reflect a variety of physiological features, differences in transcription, processing, and transport, or stability in the cytoplasm. Further analyses of individual mRNA's are necessary to resolve these alternatives.

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ADDENDUM IN PROOF

In two recent studies, adenovirus 2 cytoplasmic RNAs synthesized late in infection have been localized to genome sites by R-loop mapping (L. T. Chow, J. M. Roberts, J. B. Lewis, and T. R. Broker, Cell, in press; J. Meyer, P. D. Neuwald, S.-P. Lai, J. V. Maizel, Jr., and H. Westphal, J. Virol. 21:1010-1018, 1977).

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