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Two regions of the adenovirus 2 genome specify families of late polysomal RNAs containing common sequences

(adenovirus RNA/transcriptional mapping/RNA·DNA hybridization)

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ABSTRACT **Late cytoplasmic RNAs specified by two regions of the adenovirus 2 genome (39.3–51.8 and 70.7–83.4 map units) were analyzed by size fractionation of poly(A)-[^{3}H]RNA and subsequent hybridization to DNA fragments. Both regions encode RNAs whose sequence content exceeds the coding capacity of the region. These multiple transcripts are likely to function as mRNAs, because they are present on polyribosomes. The DNA segment 39.3–51.8 specifies 27S, 22S, and 18S RNAs. The genome sites specifying these three size classes were determined by hybridizations with seven different DNA fragments from this region of the genome. The 3′ termini of all three size classes are specified by sequences near a common site, position 50.1. The 27S RNA includes sequences beginning near 39.3, the 22S RNA contains sequences from 41.0, and the 18S RNA includes sequences from approximately 45.3 on the unit genome. A second family of four RNAs is transcribed from 70.7–83.4. These 28S, 22S, 18S, and 16S RNAs have a relationship similar to the RNAs transcribed from 39.3–51.8. Sequences near the 5′ ends of these four size classes are specified by different genome sites. However, the 3′ termini of all four size classes were localized near map position 80.4. The synthesis of families of RNA may allow the translation of multiple polypeptides from a genome segment that has only one terminator site for mRNA synthesis.**

The availability of specific DNA fragments has enabled the formation of transcriptional maps for adenovirus 2 RNAs synthesized both early and late in productive infection. By late times, after the onset of viral DNA replication, greater than 90% of the coding capacity of the virus is represented in cytoplasmic transcripts (1, 2). The viral mRNAs synthesized at typical late times, 18 hr after infection, are derived more than 99% from the *r* strand of the genome (3), the strand transcribed in the rightward direction on the conventional map (1). Transcriptional maps have been derived from two types of experiments: The number of nucleotides transcribed into mRNA has been determined by hybridization of nonradioactive RNAs with separated strands of radioactive DNA fragments (1, 2). mRNA species transcribed from various regions of the genome have been identified by hybridization analysis of radioactive RNAs separated by size (4–6).

Mapping experiments with cytoplasmic viral RNAs synthesized late in infection revealed an unexpected size distribution for the RNAs transcribed from two separate regions of the viral genome (6). Each region specified a series of cytoplasmic RNAs whose collective sequence content exceeded the coding capacity of the region at least 2-fold. These two regions included the sequences contained in map positions 39.3–51.8 (*Sma* I-D DNA fragment) and 70.7–83.4 (*Eco*RI-F and -D DNA fragments). The region 39.3–51.8 appears to code for several virion proteins, penton (85,000 daltons) pVII (20,000 daltons) and V (48,400 daltons) (7). The late proteins identified as products of the second region, 70.7–83.4, are pVIII (26,000 daltons) and a 100,000-dalton polypeptide.

To better define the relationship between the series of RNAs transcribed from each of these regions, it was necessary to establish the presence of each of these RNAs in polyribosomes and to map in greater detail the sequences specifying each species. The mapping data presented here establish that, within each set of RNAs, sequences towards the 3′ ends are shared, and the longer the molecule, the further it extends in the 5′ direction.

MATERIALS AND METHODS

Virus Infection, Labeling of Cultures, and RNA Purification. Maintenance of KB cell suspension cultures and infections with adenovirus type 2 were performed as described previously (4, 6). Cytoplasmic RNA was purified from cultures labeled with [^{3}H]uridine (40 Ci/mmol; New England Nuclear at 50 μCi/ml. [^{3}H]RNA labeled 12–14 hr after infection was utilized in mapping the cytoplasmic RNAs from the *Eco*RI-F,D region, and [^{3}H]RNA labeled from 17–19 hr was used in analyzing the *Sma* I-D transcripts.

Polyribosomes were isolated by sedimentation through a 7–47% sucrose gradient. An aliquot of the polysomal fraction was dialyzed against 0.01 M Tris·HCl, pH 7.5/0.01 M NaCl and treated with 0.02 M EDTA before sedimentation on a second sucrose gradient (8).

Cytoplasmic or polysomal samples were adjusted to 0.01 M Tris at pH 7.5, 0.1 M NaCl, 0.002 M EDTA, and 0.2% sodium dodecyl sulfate, and RNA was extracted as previously described (6). Poly(A)-RNA was selected by oligo(dT) cellulose chromatography (9). [^{3}H]RNA and [^{14}C]rRNA markers were denatured and fractionated on 3.5% polyacrylamide gels containing 98% formamide (6).

Preparation of DNA Fragments. Adenovirus 2 DNA from virions was purified according to the procedure of Tibbetts *et al.* (10). Preparation of *Eco*RI and *Sma* I fragments of adenovirus 2 DNA was described previously (6). *Sma* I-D, *Eco*RI-F, and *Eco*RI-D fragments were purified by hydroxylapatite chromatography (6) and subcleaved with endo R·*Hin*dIII under standard digestion conditions: 0.01 M Tris at pH 7.5, 0.01 M $MgCl_2$, 0.005 M 2-mercaptoethanol, and 0.15 M KCl (11). Subcleavage with endo R·*Bgl* II was performed in 0.05 M Tris at pH 7.9, 0.05 M KCl, 0.01 M $MgCl_2$, and 0.005 M 2-mercaptoethanol (M. Zabeau, personal communication).

RNA·DNA Hybridization. Aliquots (100 μl) of [^{3}H]RNA eluted from gel slices were annealed to viral DNA as described previously (6). Amounts of DNA fragments were defined as microgram equivalents; a 1-μg equivalent of a DNA fragment

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Table 1. Subcleavage fragments used for mapping studies

Enzyme used for subcleavage	Map position of subcleavage product from primary DNA fragment		
	Sma I-D*	*Eco*RI-F†	*Eco*RI-D†
Endo R-*Hin*dIII	39.3–41.0	70.7–72.9	75.9–79.6
	50.1–51.8	72.9–75.9	80.4–83.4
Endo R-*Bgl* II	39.3–45.3		
	45.3–51.8		

Primary fragments were purified as described in the text and subcleaved with the appropriate enzyme. The products were isolated and size was estimated by electrophoresis on agarose gels. In order to establish their origin, the resulting fragments were hybridized to either *Hin*dIII fragments, for subcleavages generated with endo R-*Hin*dIII, or to *Bgl* II fragments, for subcleavages obtained with endo R-*Bgl* II (unpublished data). In certain figure legends, DNA fragments generated by cleavage with two different restriction enzymes are identified by designating the two fragments whose common sequences define the subcleavage fragment. For these designations, the fragment produced in the primary cleavage is listed first, followed by the fragment that would be produced by the second enzyme.

* Map positions are given in map units, with 0 map units being the left end of the genome and 100 map units corresponding to the right end. Map positions for *Hin*dIII subcleavages of *Sma* I-D fragment were calculated relative to the *Hin*dIII sites determined by R. Roberts (personal communication), and the position of the *Bgl* II site was determined by M. Zabeau (personal communication).

† The map position for the *Hin*dIII subcleavages of *Eco*RI-F and *Eco*RI-D were calculated relative to the *Eco*RI sites reported by Mulder *et al.* (12).

is the amount of DNA derived from 1 μg of whole adenovirus DNA. To ensure purity, all fragments used for hybridization were repurified by a second electrophoresis.

RESULTS

For the mapping studies described below, a subcleavage map was prepared for the relevant regions of the genome (unpublished data). The various DNA fragments used in these experiments and the region of the genome contained in each fragment are summarized in Table 1. To facilitate presentation of the data, DNA fragments will often be identified simply by genome map units.

Multiple Adenovirus Transcripts in Polysomal RNA. To determine if the cytoplasmic adenovirus transcripts from map positions 39.3–51.8 and 70.7–83.4 are in polysome structures, polyribosomes were isolated from cultures labeled 12–14 hr after infection. Late in adenovirus infection most of the labeled polysomal RNA is virus specified (13, 14). To assay for contamination with nuclear ribonucleoprotein, a portion of the pooled polysomal fractions was treated with EDTA before sedimentation on a second sucrose gradient. Greater than 95% of the labeled RNA was released by EDTA treatment and therefore can be considered part of functioning polyribosomes.

Poly(A) polysomal RNA was fractionated by electrophoresis on polyacrylamide gels containing 98% formamide. RNA eluted from each gel slice was hybridized to relevant DNA fragments bound to membranes. The hybridization profiles obtained for genome regions encoding multiple transcripts are shown in Fig. 1. The 27S, 22S, and 18S RNAs encoded by *Sma* I-D fragment (39.3–51.8) in cytoplasmic RNA were also found to be present in polysomal RNA (Fig. 1*A*). Likewise, the *Eco*RI-F,D region gave exactly the same profile as previously found for cytoplasmic RNA (Fig. 1 *B* and *C*). *Eco*RI-F gave major peaks of hybridization designated as 28S and 22S RNAs,

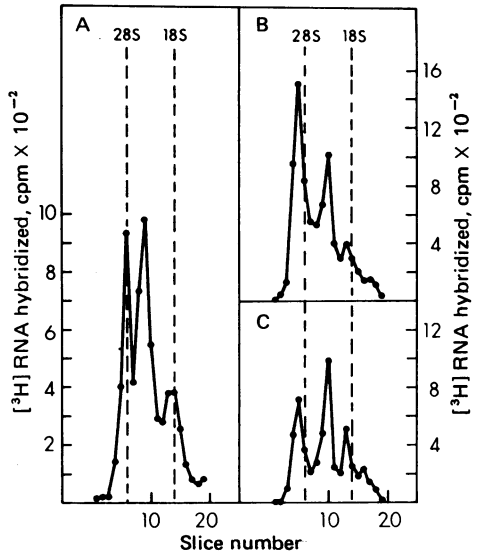

FIG. 1. Size distribution of polysomal [^{3}H]RNAs specified by DNA sequences in *Sma* I-D (39.3–51.8), *Eco*RI-F (70.7–75.7), and *Eco*RI-D (75.7–83.4). RNA was labeled from 12 to 14 hr after infection and isolated from the polysomal fraction. Poly(A)-containing [^{3}H]-RNA (3×10^5 cpm) was subjected to electrophoresis, and the RNA was eluted from each 1-mm slice by incubation in 200 μl of 0.90 M NaCl/0.090 M sodium citrate at pH 7.0 (6× SSC) containing 0.1% sodium dodecyl sulfate at 66° for 24 hr. Samples (150 μl) of each eluted RNA fraction were annealed simultaneously on filters containing 1-μg equivalents of (*A*) *Sma* D, (*B*) *Eco*RI-F, and (*C*) D fragments at 66° for 20 hr. The dashed lines indicated the positions of 28S and 18S rRNA markers.

and a minor 18S component. *Eco*RI-D detected RNAs migrating at 28S, 22S, and 18S, and a minor peak of 16S RNA.

Detailed Mapping of the RNAs Transcribed from the Region 39.3–51.8. *The Sma* I-D fragment, which specifies 27S, 22S, and 18S RNAs, contains 12.5% of the genome, approximately 4500 nucleotide base pairs. In comparison, the three RNAs transcribed from this fragment contain approximately 4500, 2900, and 1800 nucleotides, more than twice as many nucleotides as the fragment. To investigate the sequence relationship between these RNAs, and to determine the region within *Sma* I-D fragment that codes for each of these molecules, a detailed analysis was performed using DNA fragments obtained by subcleavage of *Sma* I-D (see Table 1).

The hybridization of poly(A) cytoplasmic RNA to the subcleavage fragments is presented in Fig. 2. In addition to the hybridization data, each panel contains a bar diagram that indicates the genome origin and map coordinates of the particular fragment used. Of the four fragments used for this experiment, the left-most fragment (39.3–41.0; Fig. 2*A*) annealed only the 27S species. The 39.3–45.3 fragment annealed a prominent 27S molecule and smaller amounts of 22S RNA (Fig. 2*B*). The right half of *Sma* I-D, the 45.3–51.8 fragment, hybridized all three size classes (Fig. 2*C*). In contrast, the 50.1–51.8 fragment, the right 15% of *Sma* I-D, did not hybridize significantly to any of these transcripts (Fig. 2*D*). These hybridizations provide the following conclusions:

(*i*) The 3′ termini of all three of these RNAs map between the *Hin*dIII cleavage site at 50.1 and the *Bgl* II site at 45.3.

(*ii*) All or nearly all the sequences encoding the 18S RNA are found in the right half of this region, map positions 45.3–50.1.

(*iii*) The 5′ portion of the 22S RNA is encoded within the region 41.0–45.3.

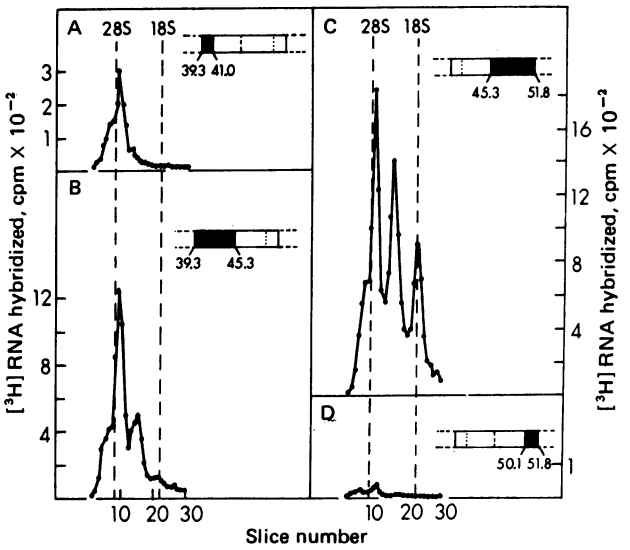

FIG. 2. Size distribution of cytoplasmic RNAs specified by segments of *Sma* I-D (39.3–51.8). Poly(A) cytoplasmic RNA (1×10^6 cpm) was fractionated by electrophoresis in formamide gels and the eluted RNA was hybridized to 1-μg DNA equivalents of fragments generated by the cleavage of *Sma* I-D fragment with either endo R·*Hin*dIII or endo R·*Bgl* II. (*A*) *Sma*-D/*Hin*-J fragment (39.3–41.0); (*B*) *Sma*-D/*Bgl*-A fragment (39.3–45.5); (*C*) *Sma*-D/*Bgl*-D fragment (45.3–51.8); (*D*) *Sma*-D/*Hin*-A fragment (50.1–51.8). The shaded area on the map in each panel indicates the particular subcleavage of *Sma*-D fragment utilized as hybridization probe. Solid vertical lines on the map are *Sma* I cleavage sites. Dotted lines are *Hin*dIII cleavage sites, and the dashed line is the endo R·*Bgl* II site.

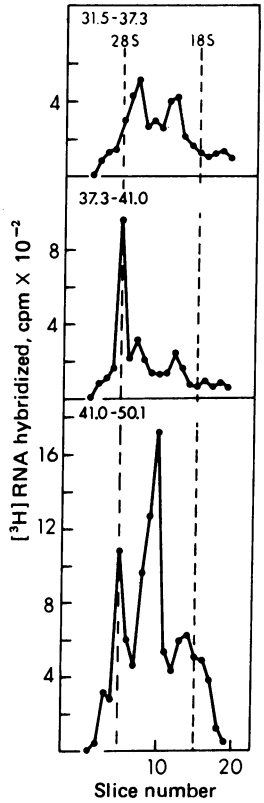

FIG. 3. Hybridization analysis with *Hin*dIII fragments representing map positions 31.5–50.1. Poly(A) cytoplasmic RNA (5×10^5 cpm) was fractionated by electrophoresis and the eluted RNA was hybridized as described in Fig. 1. Each RNA fraction was hybridized on filters to 1.5 μg equivalent of *Hin*dIII DNA fragments I, J, and D containing the sequences in map positions 31.5–37.3, 37.3–41.0, and 41.0–50.1, respectively. The hybridization profiles are presented in genomic order of the fragments, and the positions of 28S and 18S rRNA markers are depicted by dashed lines.

To localize sequences specifying nucleotides near the 5′ end of the 27S RNA, hybridization experiments were performed with fragments that contain sequences to the left of 39.3 (Fig. 3). These experiments used three endo R·*Hin*dIII fragments, 31.5–37.3, 37.3–41.0, and 41.0–50.1. Previous hybridizations had detected 26S and 21S RNAs encoded by fragments to the left of 39.3 (6). These RNAs were detected by hybridization with the 31.5–37.3 fragment (Fig. 3*A*). The 37.3–41.0 fragment annealed significant amounts of 27S RNA in addition to small amounts of the 26S and 21S RNAs (Fig. 3*B*). The 41.0–50.1 fragment annealed the three RNA size classes (Fig. 3*C*), detected with *Sma* I-D. Thus sequences near the 5′ terminus of the 27S RNA are most likely specified by nucleotides within 37.3–41.0 on the genome.

Detailed Mapping of the Multiple RNAs Transcribed from the Region 70.7–83.4. The four RNA size classes (28S, 22S, 18S, and 16S) encoded by the sequences in *Eco*RI fragments F and D contain 4900, 2900, 1800, and 1400 nucleotides, compared to 4500 base pairs in the *Eco*RI-F and -D fragments.

Hybridization results with subcleavage fragments are presented in Fig. 4. The left-most fragment from this region (70.7–72.9) hybridized primarily the 28S RNA and in addition small amounts of the 22S size class (Fig. 4*A*). The next segment, 72.9–75.9, detected the 28S, 22S, and 18S size classes and perhaps small amounts of the 16S RNA (Fig. 4*B*). The 75.9–79.6 fragment produced by subcleavage of *Eco*RI-D clearly contains sequences present in all four RNA size classes (Fig. 4*C*). The fragment 80.4–83.4 did not anneal to any of these RNAs. These hybridizations provide the following conclusions:

(*i*) Sequences specifying the 3′ termini of all four RNAs are between 79.6 and 80.4.

(*ii*) Of the four RNAs, only the 28S and 22S size classes contain transcripts of sequences to the left of 72.9.

(*iii*) The 5′ portion of the 18S RNA is encoded within the region 72.9–75.9.

To obtain further information regarding the 5′ portions of the 28S and 22S RNAs, *Eco*RI-B was subcleaved with endo R·*Bgl* II to give three fragments: 58.5–60.2, 60.2–63.2, and 63.6–70.7. The 22S RNA did not anneal to any of these fragments. Significant hybridization of the 28S size class was detected only with the 63.6–70.7 probe (data not shown). Therefore the 22S RNA is encoded by sequences to the right of 70.7, whereas the 5′ portion of the 28S RNA is encoded by sequences between 63.6 and 70.7.

DISCUSSION

On the basis of their presence in polyribosomes, the multiple RNA size classes synthesized late in adenovirus 2 infection are likely to function as mRNAs. Mapping studies of these RNAs have been performed in this study and are summarized in Fig. 5. Although analysis of the mapping experiments utilized RNA molecular weights that were deduced solely from migration rates in polyacrylamide gels, sequential mapping with smaller fragments led to the formulation of transcriptional maps that correlate with the size estimations. Our interpretation of the data is based upon the hybridization of each RNA species to the series of restriction fragments that encodes it. For example, a 27S molecule containing approximately 4500 nucleotides is transcribed primarily from the 39.3–51.8 segment, which contains 4500 base pairs. This RNA size class anneals as expected

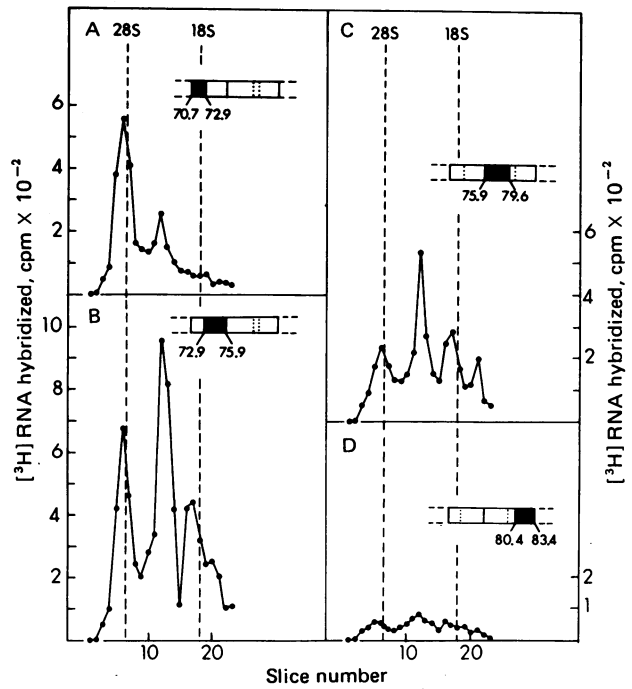

FIG. 4. Size distribution of RNAs hybridizing to the endo R·*Hin*dIII subcleavage of *Eco*RI-F and -D fragments. Poly(A) cytoplasmic RNA (2×10^6 cpm) was prepared and fractionated as described above. [^{3}H]RNA eluted from gel fractions was hybridized on filters containing 1 μg equivalent of DNA generated by the digestion of purified *Eco*RI-F and -D fragments with endo R·*Hin*dIII. (*A*) RI-F/*Hin*-A fragment (70.7–72.9); (*B*) RI-F/*Hin*-H fragment (72.9–75.9); (*C*) RI-D/*Hin*-H fragment (75.9–79.6); (*D*) RI-D/*Hin*-E fragment (80.4–83.4). The shaded area on the map in each panel indicates the particular *Hin*dIII subcleavage that was utilized to produce the hybridization profile. The solid vertical lines on the map represent *Eco*RI cleavage sites, and the dotted lines correspond to *Hin*dIII sites.

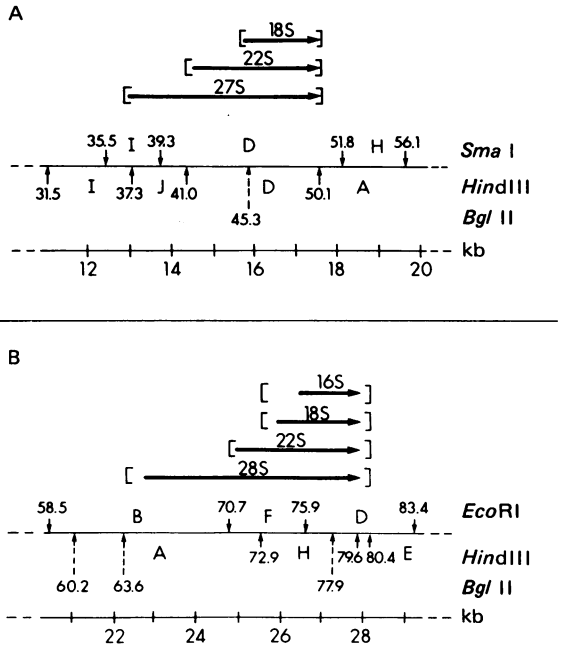

FIG. 5. (*A*) The family of mRNAs synthesized from map positions 37.3–50.1. (*B*) The family of mRNA synthesized from map positions 63.6–80.4. For both panels the lower line provides reference positions represented by vertical bars in increments of 1000 base pairs (1 kb) in the genome. The cleavage maps for endo R·*Bgl* II (M. Zabeau, personal communication), endo R·*Hin*dIII (R. Roberts, personal communication), and endo R·*Sma* I (6) are depicted by the indicated lines. The locations of cleavage sites are shown as small vertical arrows, and the pertinent fragments are identified with letters. The horizontal arrows represent the RNA species identified in this study. The direction of the arrow indicates the direction of transcription from the *r* strand of the viral genome, and the length is proportionate to the molecular weight of the RNA. The brackets around a species indicate the extent of uncertainty in assigning genome sites.

to three subcleavage fragments (Fig. 2). The 600 base pair fragments from 39.3–41.0 anneal approximately one-fourth as much 27S RNA as the 2100 nucleotide segment from 39.3–45.3. Likewise the 39.3–45.3 fragment hybridizes about three-fourths as much 27S RNA as the 2300 base pair segment from 45.3–51.8. Although not quantitative, such data support the notion that the 27S size class is transcribed in approximately equal proportions from each of these fragments, as expected from the model shown in Fig. 5. Similar conclusions are reached by analysis of the other RNA size classes.

For both RNA families, the sequences in common map in the 3′ portion of the RNAs. From calculations such as those reviewed above, our mapping studies fix the 3′ sites for each mRNA to within approximately 2.5 map units or approximately 850 nucleotides. The results of electron microscopic studies of adenovirus RNA hybridized to viral DNA fragments are compatible with our conclusions (15). In addition, fingerprint analyses have been performed with late RNAs selected by specific DNA fragments (E. Ziff, personal communication). The 3′-terminal oligonucleotides encoded by map positions 47.4–51.8 were found in RNAs of three different size classes, comparable to those we describe. We conclude that the 3′ ends of the RNAs in the 39.3–51.8 family are coterminal.

The two regions analyzed in the present study have different roles in the temporal expression of the viral genome. The segment 39.3–51.8 is part of a large block of *r* strand transcripts that are expressed as RNA only at late times (1, 2). In contrast, the RNAs specified by 70.7–80.4 are encoded by a complex region. This region is expressed at both early and late times as *r* strand transcripts (1, 2). The major early RNA is a 20S molecule (4). Although the early 20S RNA is apparently different in size from the four late RNAs, all four of the late size classes contain sequences that are transcribed at early times (6).

Families of mRNA may have functions in both the synthesis and transition of mRNA. If indeed the 3′ termini of the RNAs in each size class are identical, families of mRNA may provide a mechanism for generating multiple polypeptides from a genome region having only one site for terminating mRNA chains. Translation studies will distinguish between the possible models for expression of sequences in the mRNA families. It may be that only the 5′ sequences unique to each mRNA are translated, as has been found in the simian virus 40 system (16, 17). A different example of the multiple use of a set of DNA sequences is found early in adenovirus infection. The transforming region of the genome specifies several small mRNAs that do not overlap each other but do share sequences with a single large mRNA (5, 18). In bacteriophage systems, multiple molecular forms have been found for T7 lysozyme mRNA (19). A striking example is the overlapping genes in bacteriophage φX174 (20). In this instance, two polypeptides are translated from the same sequence but in different reading frames.

In vitro translation of late mRNA selected by the 39.3–51.8 region of the genome has resolved three products: penton (85,000 daltons), V (48,500 daltons), and pVII (20,000 daltons) (7). These polypeptides appear to account for the entire coding capacity of this region, approximately 4500 nucleotides. Two

late proteins, a 100,000-dalton polypeptide and pVIII (26,000 daltons), have been identified to date as a product of the 70.7–80.4 region (7). These peptides require at least 4000 nucleotides of mRNA. Thus either a 16S or 18S RNA could specify pVIII and a 22S or 28S RNA could encode the 100,000-dalton peptide. Because translation studies with fragment-specific RNAs demonstrated that sequences from *Eco*RI-B are present in the mRNA for the 100,000-dalton protein, the 28S RNA is a more likely candidate for this mRNA.

A possible mechanism for the synthesis of these families of mRNA is suggested by the recent identification of a 150 nucleotide leader sequence that is sommon to many late adenovirus mRNAs (21, 22). This leader sequence is encoded by a region suggested to be the major late promoter (23). From these findings it has been proposed that each primary transcript may give rise to a single mRNA, with the leader being fused to mRNA sequences during processing. The presence of leader sequences in the two RNA families described here has been confirmed by electron microscopy (22). However, further studies are necessary to determine if each RNA species is processed to completion in the nucleus; alternatively, some of the large species may be cleaved in the cytoplasm to generate smaller RNAs.

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