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Groups of Adenovirus Type 2 mRNA's Derived from a Large Primary Transcript: Probable Nuclear Origin and Possible Common 3' Ends

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Late in adenovirus type 2 infection, a number of mRNA's apparently arise by processing a large nuclear transcript that represents the right-hand 85% of the genome (summarized in Evans et al., Cell 12:733-739, 1977). Hybridization of labeled late mRNA to a series of DNA restriction fragments representing the right-hand 70% of the genome demonstrates at least 12 discrete mRNA's that appear to fall into five groups, each possibly containing a common 3' terminus. The processing necessary to generate these mRNA's apparently occurs in the nucleus. All the mRNA's appear to contain a sequence of ~100 nucleotides complementary to a fragment with coordinates 25.5-27.9. This fragment contains one of the regions found by Berget et al. (Proc. Natl. Acad. Sci. U.S.A. 74:3171-3175, 1977), Chow et al. (Cell 12:1-8, 1977), and Klessig (Cell 12:9-22, 1977) to be "spliced" onto the 5' end of late adenovirus type 2 mRNA's. Because the sequences to be spliced exist only once per large transcript, any of the mRNA-specific regions might only be preserved from a small fraction of the transcripts. Measurement of the transport efficiency of regions of the large nuclear transcript, in fact, shows that only 15 to 25% of any particular region is transported to the cytoplasm. The overall conclusion of these experiments is that the large late nuclear transcript can be processed in the nucleus to yield any one of many (~12) mRNA's; the unused portions of the primary transcript then accumulate in the nucleus or are destroyed.

Information about the synthesis of virus-specific mRNA in adenovirus-infected cells has progressed very rapidly since restriction endonucleases became available to divide the virus DNA into a reproducible, ordered set of fragments (29, 31). The approximate sites on the genome that correspond to specific early and late mRNA's (32, 34), the determination of which strand of the DNA is transcribed, and the direction of transcription of early and late mRNA's were all determined by hybridization of unlabeled RNA to fragmented labeled DNA (for a review, see reference 9). More recently (6, 26), a detailed map of mRNA sites in specific regions of adenovirus type 2 (Ad2) DNA was obtained by observing "R loop" formation—R loops (40) are RNA-DNA hybrids in which one strand of the DNA duplex is displaced by the mRNA molecule—in specific regions of the genome.

In parallel with the mapping of mRNA sites on the Ad2 genome, biochemical studies of mRNA formation revealed that Ad2 mRNA, in common with cellular mRNA, requires a number of nuclear post-transcriptional modifications of the mRNA. These include the addition of polyadenylic acid [poly(A)] to the 3' terminus (32) and specific methylations (28, 37); internal adenylate residues are methylated to give m^6A approximately once every 200 A's, in addition to the formation of a 5'-terminal methylated structure, termed a "cap" (35). The primary RNA transcripts that undergo this nuclear processing are not simply the same length as the mRNA itself. For example, late in infection a single large transcriptional unit from ~16 to 100 on the rightward-reading strand is copied into a primary transcript ~28,000 nucleotides long (the convention [9] for the description of the Ad2 genome divides into 100 units the double-stranded DNA, each ~350 nucleotide pairs or 0.35 kilobase [kb] long. The rightward transcribed *r* strand has 0 as its left end and 100 at its right, with the reverse true for the leftward strand *l*. The coordinates of particular regions of the genome for either restriction fragments or mRNA coordinates is given by two numbers, the percentages from left to right; for example, the 36.7-40.5 fragment or the 50-62 mRNA) (2, 8, 14, 43). Over 95% of the synthesis of RNA from the regions of the genome encoding the major

late proteins (8, 9, 21), the major core, penton, hexon, "100K," and fiber polypeptides, occurs as part of this long RNA molecule. Moreover, labeling of mRNA that originates in this region is equally sensitive to UV irradiation as the formation of the large nuclear transcript itself, thereby identifying the large transcript as the obligatory precursor to the mRNA lying within its boundaries (13, 14). Thus, the processing procedure appears to include site-specific cleavage of a large transcript as well as addition of cap and poly(A) to the mRNA.

In studying the origin of the 5′ ends of late mRNA's in the 50–100 region, Berget et al. (4), Chow et al. (5), Gelinas and Roberts (12), and Klessig (19) have discovered a most surprising fact. The 5′-terminal 150–200 nucleotides of a number of the late Ad2 mRNA's do not come from regions of the Ad2 genome contiguous to the remainder of the mRNA but from three distant sites. Because the large nuclear Ad2 primary transcript appears to be the mRNA precursor for mRNA molecules from this region, they conclude that 50 to 100 nucleotides from each of three sites, 16.7, 19.7, and 26.0, are cleaved out separately and ligated onto the 5′ ends of each mRNA molecule derived from the large transcript. This astounding development has prompted us to map as accurately as possible the number and relative amounts of various mRNA molecules deriving from the large transcript and to determine whether the formation of all of the relevant mRNA's appears to be a nuclear event. To satisfy these goals, mRNA mapping by hybridization of labeled RNA is necessary because of the difficulty of obtaining quantitative and kinetic data on mRNA distribution by using electron microscopic techniques. Our conclusions are that at least 12 different mRNA's can be derived from nuclear processing of the 16 to 100 transcript. These RNAs fall into groups where two to four mRNA's may share the same 3′ terminus. The map positions assigned by hybridizing labeled molecules agree well with the electron microscopic mapping (5, 6, 26). A measurement of the conservation of various parts of the large nuclear transcript is described that suggests that each long RNA transcript possibly gives rise to only one mRNA, presumably because each transcript contains only one set of 5′ spliced sequences necessary to form the mRNA.

MATERIALS AND METHODS

Cell and virus growth as well as the purification of adenovirus have been described previously (42).

Labeling and extraction of RNA. HeLa cells were infected at multiplicities of 2,000 particles per cell. At the appropriate time after infection, the infected cells were concentrated to 10^6 cells per ml in fresh medium, and [^{3}H]uridine was added (10 to 20 μCi/ml, 20 mCi/μmol). After labeling, the cells were chilled and collected by centrifugation. Cytoplasmic and nuclear extracts were prepared as described previously. The extraction of RNA as well as the preparation of poly(A)-containing RNA by polyuridylic acid Sepharose chromatography has been described (27). Single-stranded RNA was precipitated from 2 M LiCl overnight at 4°C.

Electrophoresis of RNA. ^{3}H-labeled RNA samples (including ^{32}P -28S and -18S rRNA markers) were dissolved in ETS (0.01 M Tris–0.01 M EDTA–0.2% sodium dodecyl sulfate [pH 7.4]) and made 50% (vol/vol) formamide. After heating to 65°C for 2 min, bromophenol blue and glycerol were added, and the sample was applied to a 2.7% acrylamide–0.14% bisacrylamide gel (0.6 by 10 cm) prepared in Loening buffer (23). Electrophoresis was at 6 mA/gel for 3 to 4 h. Gels were sliced into 2-mm fractions, and the RNA was eluted in 0.5 ml of TESS buffer [0.01 M *N*-tris(hydroxymethyl)methyl-2-aminomethane-sulfonic acid (pH 7.4)–0.3 M NaCl–0.01 M EDTA–0.2% sodium dodecyl sulfate] at 65°C overnight. RNA was fragmented with alkali as described (16).

Electrophoresis in 98% formamide-containing gels was performed as described by Duesberg and Vogt (7).

Hybridizations. Hybridizations of RNA to filter-bound Ad2 DNA fragments were carried out as described previously (2). The RNA from each 10^7 cells was exposed to DNA fragments equivalent to 1 μg of total Ad2 DNA. Excess of DNA was proven by rehybridizing supernatant RNA to fresh filters revealing at least 80% of the hybridizable RNA to be removed in the initial exposure.

RESULTS

Experimental design. Late in Ad2 infection, the majority of Ad2-specific cytoplasmic RNA is poly(A) terminated [poly(A)+; 22, 33]. Moreover, almost all (>90%) of the labeled poly(A)+ RNA that reaches the cytoplasm is Ad2 specific (22, 32). Thus, by collecting the labeled poly(A)+ RNA late in infection, the total virus-specific mRNA can be examined without further purification. Because the first step in the design of the present work was to separate Ad2-specific molecules on the basis of size, the electrophoretic migration of the total poly(A)+ RNA late in infection was compared under two conditions: (i) electrophoresis of denatured RNA in 98% formamide and (ii) denaturation followed by electrophoresis through gels not containing formamide. With both procedures (Fig. 1), discrete size classes of labeled poly(A)+ RNA migrating between 18S and 28S rRNA were observed as previously reported for Ad2-infected cells (22, 30, 39). The approximate distribution of labeled RNA compared with 28S and 18S rRNA markers was similar, but the resolution was considerably better in the gels without the formamide. This technique was therefore adopted for the present work.

The experiments to be described were designed to map the various mRNA's in different regions of the genome by first separating the mRNA according to size, followed by hybridization of the labeled RNA to a sufficient array of Ad2 DNA restriction fragments (in DNA excess) so that most mRNA would hybridize across a boundary between two fragments (Table 1). The percentage of labeled RNA of a specific size that hybridized to each DNA fragment would then allow assignment of the position of the 5′ and 3′ termini of the mRNA exclusive of post-transcriptionally added sequences. In some cases an mRNA species might span an entire fragment and hybridize to both right-hand and left-hand neighboring fragments. Such a situation would offer a measure of the length of mRNA independent of its electrophoretic migration because the size of the fragment totally spanned by the mRNA would be known from the DNA restriction fragment maps. The accuracy of the map assignments made with these techniques would depend on the error in assaying the RNA:DNA hybridization, the size determination of RNA by gel analysis, and the accuracy of the restriction fragment map of the Ad2 genome. An overall accuracy of at least 1 to 2% on the physical map would be expected for the relative positioning of the various mRNA's, especially when reviewed in light of the positioning of termini reported from microscopic techniques (5, 6). The technique of hybridizing labeled mRNA molecules, of course, also allows a means of studying the metabolism of specific mRNA's and a quantitation of the relative amounts of each mRNA that reach the cytoplasm. As pointed out below, nucleotide analysis of RNA and DNA is finally necessary to locate exactly the region of DNA corresponding to a particular RNA.

TABLE 1. *DNA restriction fragments of Ad2 used in mapping mRNA locations*

Map coordinates[a]	Enzyme(s) used	Name of fragment
25.5–27.9	*Hpa* I	*Hpa* F
29.1–36.7	*Sma* I/*Bam* I	*Sma* B/*Bam* D
36.7–40.5	*Sma* I	*Sma* I
40.5–52.6	*Sma* I	*Sma* D
40.5–42.7	*Sma* I/*Kpn*	*Sma* D/*Kpn* H
42.7–47.4	*Kpn*	*Kpn* I
47.4–52.6	*Sma* I/*Kpn*	*Sma* D/*Kpn* D
52.6–56.9	*Sma* I	*Sma* H
56.9–58.5	*Sma* I/*Eco*RI	*Sma* A/*Eco* A
58.5–70.7	*Eco*RI	*Eco* B
70.7–75.9	*Eco*RI	*Eco* R
75.9–83.4	*Eco*RI	*Eco* D
83.4–89.7	*Eco*RI	*Eco* E
89.7–100	*Eco*RI	*Eco* C

[a] Map coordinates of *Hpa* I, *Bam* I, and *Eco*RI cleavage sites are taken from data collected by Marc Zabeau and distributed at the 1976 Cold Spring Harbor Workshop on DNA tumor viruses. The *Sma* I cleavage sites are taken from Carel Mulder (personal communication). The *Kpn* cleavage sites within the *Sma* D fragment were determined from the size of the fragments produced as well as their sensitivity to *Sal* I digestion.

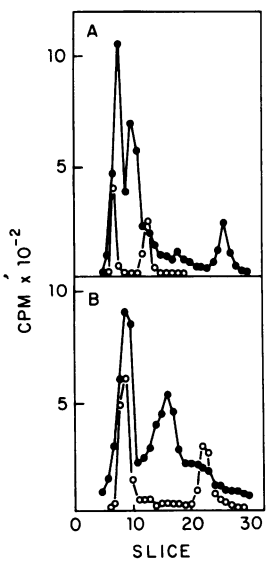

FIG. 1. *Electropherograms of [³H]uridine-labeled late Ad2 RNA (●) together with ³²P-rRNA (○) in 98% formamide-containing polyacrylamide gel (A) and acrylamide gel prepared in aqueous buffer (B). Electrophoresis was from left to right. A portion (20 μl) of each eluted gel slice was counted in triton scintillation fluid.*

General results. The data from several such RNA:DNA hybridizations of electrophoretically separated RNA from cells labeled from 15 to 18 h after infection are shown in Fig. 2, 3, and 4. The general results to note in examining the data of Fig. 2 and 3 are as follows. (i) mRNA hybridizations were carried out to many different DNA fragments from 36.7 to 89.7, and the size distribution of RNA that hybridized to each DNA fragment was distinctive; many different specific mRNA's from approximately 10S to 28S (about ~0.8 to 5 kb) were clearly present. (ii) The reproducibility of the electrophoretic pattern is quite good; for example, compare Fig. 2A and 3A, which represent two different RNA preparations hybridized to two different preparations of the DNA fragment 36.7–40.5. (iii) Some DNA fragments hybridize two or three mRNA's, each of which is greater than the length of the fragment. (Note that above the mRNA was broken with alkali to chain sizes ~0.2 to 0.3 kb and that all the DNA fragments exceeded 0.7 kb and were present in excess; thus, any single DNA molecule would actually hybrid-

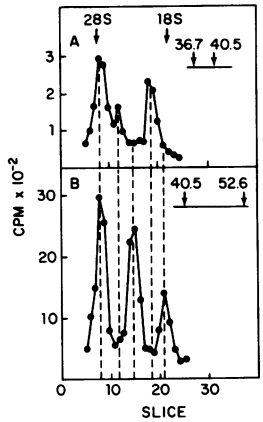

FIG. 2. *Electropherograms of late [³H]uridine-labeled Ad2 RNA hybridized to (A) Sma I (36.7–40.5) filters and to (B) Sma D (40.5–52.6) filters. Electrophoresis was from left to right, with the arrows indicating the positions of 28S and 18S HeLa rRNA's.*

ize less than its full length of RNA, a necessity in filter hybridization experiments if the DNA is not to be released from the filter [15].) For example, Fig. 3B shows that fragment 42.7–47.4, which is 1.64 kb long, hybridized three mRNA's, 27S, 23S, and 19S RNA, all larger than 18S rRNA, which is 2.1 kb long. Clearly, several different discrete mRNA's share at least a part of their sequences. This proved to be true in virtually every region of the large transcript that gives rise to mRNA molecules. (iv) Each one of the eight fragments to the left of 83.4 hybridized large RNA migrating together with 28S rRNA, which is 5 kb long. Because the DNA fragments used in the present hybridization work represent about 18 to 20 kb in total length, at least three to four such large mRNA's must exist. (v) The DNA fragment that hybridized the largest amount of labeled ~27S to 28S mRNA was in the 52.6–56.9 region where the hexon mRNA is known to map (9, 21). This finding is consistent with the earlier observation that hexon protein is the most prominently synthesized protein late in infection (45). (vi) Although total cytoplasmic poly(A)+ RNA was used for the analyses of Fig. 2 and 3, poly(A)+ RNA from polysomes yielded patterns after hybridization identical to those shown in Fig. 3 (data not shown).

Sorting out the 27S to 28S mRNA's. The large 27S to 28S mRNA's that hybridized to virtually every DNA fragment (Fig. 2 and 3) were positioned as follows.

(i) The 38–50 mRNA. A 27S mRNA hybridized both to the 36.7–40.5 region and to the 40.5–52.6 region. It did not, however, hybridize to the 29.1–36.7 region (data not shown), placing its 5′ end in the 36.7–40.5 region. About three

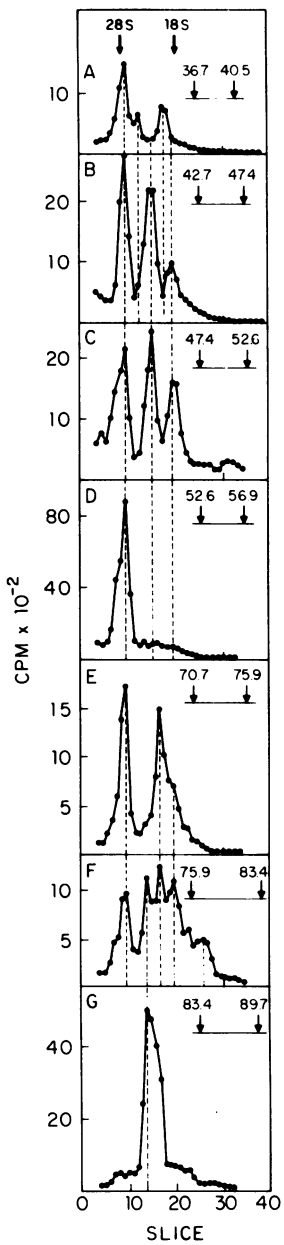

FIG. 3. *Electropherograms of late [³H]uridine-labeled Ad2 RNA hybridized to (A) Sma I (36.7–40.5), (B) Kpn I (42.7–47.4), (C) Sma D/Kpn D (47.4–52.6), (D) Sma H (52.6–56.9), (E) Eco F (70.7–75.9), (F) Eco D (75.9–83.4), and (G) Eco E (83.4–89.7) DNA filters. Electrophoresis was from left to right.*

times as much of this labeled 27S mRNA hybridized to the 42.7–47.4 region as to the 36.7–40.5 region. Because 42.7–47.4 is 1.64 kb long and is entirely traversed by the large mRNA, then ~0.5 to 0.6 kb of the long mRNA

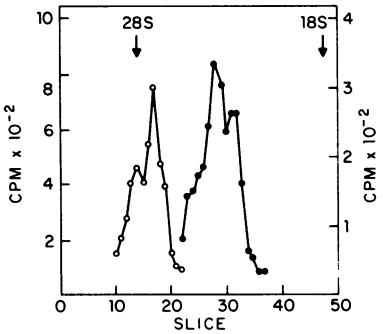

FIG. 4. *Electropherogram of late [³H]uridine-labeled Ad2 RNA hybridized to Sma H (52.6–56.9)* (○) *and to Eco E (83.4–89.7)* (●) *DNA filters. Electrophoresis was from left to right; arrows indicate positions of HeLa 28S and 18S rRNA's. In this experiment the gel was fractionated into 1-mm slices rather than 2-mm slices to afford higher resolution.*

should lie to the left of 40.5 in the 36.7–40.5 region (Table 2). The 5′ end of this RNA (exclusive of the spliced sequences) is thus placed at about 38 to 39. If this mRNA is 4.5 to 5.0 kb long and contains ~0.3 kb of 5′ and 3′ sequences added during processing, then it would occupy about 4.2 to 4.7 kb, or 12 to 13% of the genome. Its encoded 3′ end would then lie around 50–52.

(ii) The 50–62 mRNA and the 68–80 mRNA. The next possible 4.5 to 5.0 kb RNA to the right of ~50–52, without extensive overlap with the previous large mRNA, would begin around 50 and extend to around 62. Hybridization to DNA fragments in this region, in fact, identified such an mRNA. The 52.6–56.9 fragment from the hexon coding region (9, 21) hybridized a very large amount of mRNA in the 27S to 28S size range as previously noted. In addition to the very prominent 27S peak in the hybridization pattern to the 52.6–56.9 region, a shoulder of 28S was noted. Further analysis under conditions of higher resolution demonstrated a separation of two RNAs nominally a 27S and a 28S species, with a predominance of label in the 27S species (Fig. 4). These prominently labeled mRNA's extend through 56.9–58.5 (a short fragment for which hybridization data is not shown in Fig. 3, but is given in Table 2). In addition, the neighboring rightward fragment 58.5–70.7 also hybridized a large amount of RNA in the 27S to 28S size range. Thus, it appears that these two mRNA's begin around 50–52, span the 52.6–58.5 region, and terminate in the left portion of the 58.5–70.7 fragment. The proportion of the radioactive RNA hybridized to 58.5–70.7 that was to the 50–62 mRNA's (and therefore the number of bases covered in the 58.6–70.7 region) could not

TABLE 2. *Hybridization of [³H]uridine-labeled late RNA to Ad2 restriction fragments*

RNA species	Expt	Hybridization (cpm) to fragment									
		36.7–40.5	40.5–42.7	42.7–47.4	47.4–52.6	52.6–56.9	56.9–58.5	58.5–70.7[a]	70.7–75.9	75.9–83.4	83.4–89.7	89.7–100
27S	1	4,200		7,500								
	2		415	891								
23S	1			7,850	7,200							
	2		16	1,902	1,862							
19S	1			3,200	6,000							
	2		13	702	1,003							
27S to 28S	1					24,300		19,200				
	3					17,200	8,700					
27S	1								4,900	3,400		
23S to 24S	4									1,570	12,087	4,085

[a] Hybridized radioactivity in 58.5–70.7 represents 50–62 RNA as well as 67–80 RNA. The contribution due to 68–80 RNA was calculated from the size of the RNA and hybridized radioactivity to two neighboring rightward fragments to be 3,200 cpm of the total of 19,200 hybridized to the 58.5–70.7 fragment.

be simply calculated (see Table 2), however, because the next fragment to the right, 70.7–75.9, also hybridized an mRNA of approximately 27S. However, the 70.7–75.9 fragment is only 2.1 kb long itself, so a long 27S mRNA must project on either or both sides. The 75.9–83.4 fragment did indeed hybridize a 27S RNA but a lesser amount of radioactivity than the 70.7–75.9 fragment (Table 2). From the proportion of ~27S RNA hybridized to 70.7–75.9 and 75.9–83.4, it could be concluded that at least 2 kb of the long mRNA spanning the 70.7–75.9 region must lie before the 70.5–75.9 region, that is, in the 58.5–70.7 region. Therefore, two different long mRNA's derive sequences from either end of the 58.5–70.7 region.

In summary, we can identify four large ~4.5- to 5-kb mRNA's, one from 38–50, two of very similar size in the 50–62 region, and one from 68–80.

Common 3′ termini: the 36.7–62 region. As pointed out above, several regions of the Ad2 genome appear to encode multiple mRNA's that share some of the same sequences. Such a group of mRNA's might be arranged in any of the following ways: (i) code for the same or a part of the same polypeptide and share either 5′ or 3′ termini; (ii) share a terminus, but the longer mRNA might be translated only over a portion of its total length to produce a different peptide from the shorter mRNA (reminiscent of Sindbis virus [36]); or (iii) exist as "overlapping genes" that might be translated in different-reading frames as with φX174 (3) and simian virus 40 (S. Weissman, personal communication). An effort was made, therefore, to calculate terminal positions of such overlapping mRNA's from the size estimate afforded by electrophoretic migration plus a determination of the fraction of such mRNA's that hybridized to contiguous DNA fragments.

The data suggest a number of regions within which from two to four mRNA's seem to terminate. In one of these regions, ~50 on the genome, our conclusions are greatly strengthened by the data of Ziff and Fraser (46) that the oligonucleotides of three different-sized mRNA's are indistinguishable. Because we reached the same conclusion on the basis of the proportion of hybridization of various sized mRNA's to DNA fragments between 40 and 52, we feel more confident that we can place 3′ ends reasonably accurately. In addition, our data agree with the assignment of 3′ ends by electron microscopic data (5, 6, 26). Nevertheless, in the absence of fingerprint data on various-sized mRNA's from each putative 3′-coterminal site, we cannot firmly conclude 3′ identity.

We will first consider the mRNA's complementary to fragments between positions 36.7 and 52.6. There are three species of RNA, 27S, 25S, and 20S, complementary to 36.7–40.5, a fragment that is itself only 1.33 kb long. Thus, each of these three mRNA's should also hybridize to DNA on one or the other side of the 36.7–40.5 region. The examination of labeled RNA complementary to the neighboring right-hand fragment, 40.5–52.6, showed that three distinctive RNA peaks were also observed, 27S, 23S, and 19S, only the longest of which coincided with the mRNA's complementary to 36.7–40.5. Because all the mRNA's in the entire region (16–100) are transcribed to the right (5′→3′), the two smaller mRNA hybridized to the 36.7–40.5, therefore, must derive sequences to the left of 36.7–40.4, i.e., from the 29.1–36.7 region, and both must have their 3′ ends within 36.7–40.5. A separate preparation of RNA was prepared and hybridized to the 29.1–36.7 fragment. No 28S RNA hybridized, but both a 25S and 20S RNA were detected. Thus, these two mRNA's, the 25S and 20S, have sequences in the 29.1–36.7 region and end in the 36.7–40.5, but we cannot determine precisely their 3′ termini. The large 27S mRNA begins within 36.7–40.5 and continues into 40.5–52.6.

To determine more accurately the map positions of the three RNAs in the 40.5–52.6 region, further subdivision of this DNA fragment with another restriction enzyme (*Kpn*) was required. DNA fragments with coordinates 40.5–42.7, 42.7–47.4, and 47.4–52.6 were obtained, and the proportion of radioactivity hybridized from each of the 27S, 23S, and 19S mRNA's to each of these fragments was determined (Fig. 3B and C, Table 2). The 23S and 19S mRNA's failed to hybridize to the 40.5–42.7 fragment (Table 2), but both mRNA's as well as the 27S RNA hybridized to 42.7–47.4 and 47.4–52.6 fragments (Fig. 3B and C). However, no hybridization of 23S and 19S mRNA's occurred to the 52.6–56.9 fragment, demonstrating that the encoded 3′ ends of these molecules must lie within the 47.7–52.6 region. If a 27S RNA does begin in the 36.7–40.5 as concluded above, it also could not extend beyond approximately 50–52 on the genome. Thus, it appears likely that three mRNA's, 27S, 23S, and 19S, end near the 50–52 region.

Ziff and Fraser (46) have identified three mRNA's of approximately the same lengths (27S, 23S, and 19S) as described here whose 3′ terminal 100 nucleotides, i.e., the nucleotides next to poly(A), hybridize to the 40.5–52.6 DNA fragment. As mentioned previously, all three of these 3′ ends have the same oligonucleotide composition; moreover, these distinctive oligonucleotides are also present in *Escherichia coli* RNA

polymerase transcripts of the 47.4 to 52.6 region. Thus, we can conclude that these three mRNA's (27S, 23S, and 19S) all terminate very close to the same site.

Common termini: the 50-62, 68-80, and 83-92 groups. Finally, it appears that three other groups of mRNA that, within a group, may share common 3'-terminal regions exist in the 50–62, 67–83, and 83–92 regions of the genome. As discussed above, two large RNAs, a 28S and 27S species, can be resolved by high-resolution gel analysis in the 50–62 region (Fig. 4). As discussed later, if these two mRNA's have nonoverlapping 5' regions that might be necessary for each to be translated into different proteins, they may have common 3' termini.

Next, both the 70.7–75.9 and 75.9–83.4 fragments (Fig. 3E, F, and G) hybridized a 27S, 22S, 19S mRNA group. A small ~1.5 kb mRNA may also hybridize to the 75.9–83.4 fragment. By estimating the proportions of these various mRNA's hybridizing to each of these two fragments coupled with the gel estimates of the mRNA sizes, it seems likely that all four of these mRNA's have 3'-terminal sequences in the region of 79–80. All would likely terminate before the beginning of yet another mRNA around 83 that continues across into the neighboring 83.4–89.7 region.

The 80–90 region has been shown by a variety of experiments to encode the fiber protein (21, 25). A prominent 24S peak of RNA lying mainly in the 83.4–89.7 region was resolved into two mRNA's by high-resolution analysis (Fig. 4). These mRNA's extend into the 89.7–100 fragment, a distance equal to about one-fifth of that which lies in the 83.4–89.7 region (Table 2). The larger RNA of the two may begin in the 75.9–83.4 fragment because a small amount of labeled RNA of the correct length hybridized to both this fragment and the 83.4–89.7 fragment (Fig. 3F and G). Possibly, it is the smaller RNA that encodes the fiber protein because the region of from ~80 to 85 is nonessential for virus growth (9), whereas the fiber protein is most likely an essential gene product.

Summary of mRNA detected. The techniques used for these hybridization studies distinguish a very large number of mRNA's, probably as many as 12, that are distinct in size and in map position. The earlier data on protein synthesis programmed by Ad2 mRNA has also identified at least nine specific proteins encoded in this area (9, 20, 21). In addition the in vitro protein synthesis studies identified the size of various mRNA's that gave maximal programming for particular proteins. The present work indicates many overlapping mRNA's, and it seems possible that the 5' most section of each Ad2 mRNA is utilized in translation as, in fact, is the case for togaviruses (18, 36) and RNA tumor virus (17, 38) mRNA's. These RNAs contain two or more potential entry sites for ribosomes, but only the 5' site is active. By making the assumption of 5' regions being the translated portions of overlapping mRNA's and using all the available data, the map assignments shown in Fig. 5 were made. Table 3 gives a summary of the calculations employing gel-estimated lengths and proportions of RNA hybridizing to specific regions that have been used to determine the map positions presented in Fig. 5. We wish to again emphasize that only in the region of 50 is there oligonucleotide evidence of exact 3'-coterminal mRNA's, but the data on this region obtained by hybridization predicted the coterminal nature of these mRNA's in that region. Our best estimate would be, therefore, that the other groups of mRNA's may also terminate at common 3' poly(A) addition sites.

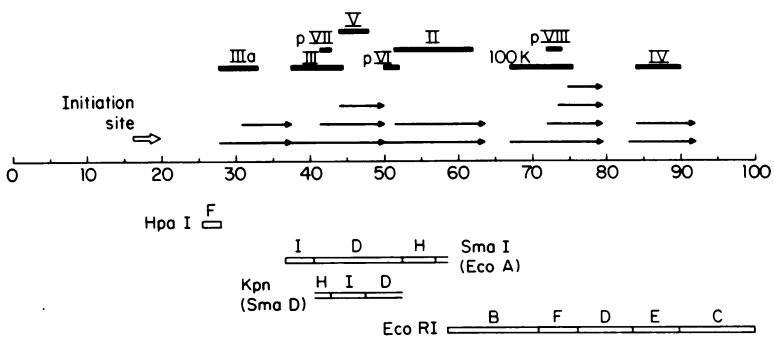

FIG. 5. *Schematic representation of late adenovirus RNAs generated in region of 27–92 from the adeno genome. Positions of RNAs were derived from data presented in Fig. 2 through 4 and Table 3. Solid bars above RNAs indicate possible map location of late proteins, based on approximate genome locations and sizes of mRNA's encoding respective proteins (20). Initiation site at 16 for the major late transcription unit is from data of Evans et al. (8).*

TABLE 3. *Sizes and coordinates of late adenovirus RNAs*

Fragment	Approximate S value[a]	Estimated length (kb)[b]	Coded size (kb)[c]	Genome (%)[d]	Coordinates	
					5′	3′
Sma I	25	3.8	3.5	10.0	28.5[e]	38.5[f]
	20	2.7	2.4	7.0	31.5[e]	38.5[f]
Sma D	27	4.7	4.4	12.5	38.5 (±0.5)[g]	51.0[e]
	23	3.3	3.0	8.5	42.5[e]	51.0 (±0.9)[g]
	19	2.4	2.1	6.0	45.0 (±0.4)[e,g]	51.0 (±0.9)[g]
Sma H	28	5.1	4.8	13.5	49.5[e]	63.0 (±1.7)[g]
	27	4.6	4.4	12.0	51.0[e]	63.0 (±1.7)[g]
Eco F	27	4.7	4.4	12.5	67.0[e]	79.5[g]
	22	3.0	2.7	7.5	72.0	79.5
	19	2.4	2.1	6.0	73.5	79.5
Eco D	13	1.5	1.2	3.5	76–79.5	80–83.5[h]
Eco E	24	3.4	3.1	8.5	83.0[e]	91.5 (±0.2)[g]
	23	3.1	2.8	8.0	83.5[e]	91.5 (±0.2)[g]

[a] Nominal S value determined from gel analysis by comparison to 28S and 18S to be the same S value relative to rRNA in sucrose gradients for hexon mRNA (*Sma* H specific) and fiber mRNA (*Eco* E specific).

[b] Estimated lengths given by Loening (23), assuming 28S and 18S to be 5.1 and 2.1 kb, respectively (44).

[c] Size from footnote *b* minus 150 nucleotides for the poly(A) segment and 150 nucleotides for the 5′ noncoded leader sequence.

[d] Value in footnote *c* divided by 35,500 and recorded to the nearest 0.5%.

[e] Length of RNA from gel analysis and one terminus calculated as in footnote *e* allowed calculation of second terminus.

[f] Assumption has been made that where an RNA terminates and a new RNA begins, no gap or overlap exists between the two.

[g] Determined on basis of overlap of RNA in two adjacent fragments (see Table 2).

[h] Because this particular RNA lies entirely within the *Eco* D (75.9–83.4) fragment and no overlaps were obtained, the exact coordinates are unknown.

Presence of RNA sequences from 25.5–27.9 in various mRNA's. Because each mRNA deriving mainly from sequences to the right of 30 is thought to contain three ligated oligonucleotide regions (4, 5, 12, 19), labeled RNA was hybridized to one small DNA fragment that contains one of these regions. The 25.5–27.9 DNA fragment, itself only about 0.8 kb long, hybridized labeled RNA from every size class between 2 and 5 kb, with broad peaks corresponding to the major size classes of mRNA (Fig. 6). The total amount of labeled mRNA hybridized to the 25.5–27.9 DNA compared to the total of all other fragments to the right of 30 was small (~2 to 4%) and distinctly higher in the 2- to 3-kb range than in the 5-kb range. Thus, the amounts hybridized to 25.5–27.9 are in agreement with the possibility of approximately 100 nucleotides from 25.5–27.9 existing in every mRNA (Table 4).

In addition a small (8S) species of RNA was found hybridizing to the 25.5–27.9 fragment (slices 33 to 35 in both total mRNA and RNA hybridizing to 25.5–27.9) that was not found to hybridize to any fragment in the region from 29–92. The possibility that a specific mRNA exists that is encoded by the DNA in this region of the genome is under further investigation.

Cellular location of processing event. Aloni et al. (1) have described results late in simian virus 40 infection that support a possible cytoplasmic processing of a 19S into a 16S mRNA. Because so many of the mRNA's described in this report also overlap in sequence, as do the simian virus 40 mRNA's, the possibility of cytoplasmic processing might also be raised for Ad2 mRNA formation. Infected cells

TABLE 4. *Hpa F (25.5–27.9) hybridization*[a]

RNA species	25.5–27.9 hybridization (cpm)	30 to 100 hybridization (cpm)	% in 25.5–27.9	Nucleotides in 25.5–27.9
27S to 28S	860	37,650	2.3	104
22S to 24S	330	11,200	2.9	84
	300	10,900	2.8	
19S to 20S	250	6,800	3.7	81
8S	120			

[a] One preparation of poly(A)+ RNA was separated by gel electrophoresis and was used to obtain data in Fig. 3 and 6. The counts per minute hybridized to the *Hpa* F DNA fragment (25.5–27.9) are compared in this table to the total hybridized to all other DNA fragments between 30 and 100. Slice 10 represents 27S to 28S RNA, 15 and 16 represent 22S to 24S RNA, and 19 represents 19S to 20S RNA. The sizes of the mRNA's [poly(A) and 5′ leader] were taken from gel migration to be 4,500, 2,900, and 2,200.

were labeled for 45 min (in contrast to 3 or 4 h used for all previous experiments); one-half of the culture was harvested, and the remainder was treated ("chased") with actinomycin D for an additional 3 h to suspend further mRNA labeling. The mRNA was analyzed as before by hybridization to the 40.5–52.6 and the 70.7–75.9 regions from which several apparently 3'-coterminal mRNA's are derived (Fig. 7). All species of mRNA were present after the 45-min label time in approximately the same ratios as observed for the 3-h label time (see Fig. 3 and 4), and no significant change was observed in the absence of further RNA synthesis for 3 h. Furthermore, the polysomal RNA from cells labeled continuously from 12 to 28 h of infection contained virtually identical proportions of the various mRNA's in each region (data not shown).

Next, cells were labeled for 12 min, and nuclear, poly(A)+ RNA was isolated and subjected to gel electrophoresis and hybridization to DNA in the 40.5–52.6 region (Fig. 8). All the species previously detected in the cytoplasm, the 27S, 23S, and 19S mRNA's, were also present in the nucleus. It appears, therefore, that the processing of RNA to yield mRNA from the Ad2-infected cells occurs in the nucleus.

Efficiency of mRNA formation from primary transcripts. One of the prime reasons for obtaining detailed information on the map position of specific Ad2 mRNA's was to allow a study of the efficiency of utilization as mRNA of different segments of the large late primary nuclear RNA transcript. Suppose that a particular mRNA portion of a primary nuclear transcript was all converted to cytoplasmic mRNA within 15 to 20 min and this particular cytoplasmic mRNA was relatively stable. The increase in that fraction of labeled nuclear RNA corresponding to the sequence present in the partic-

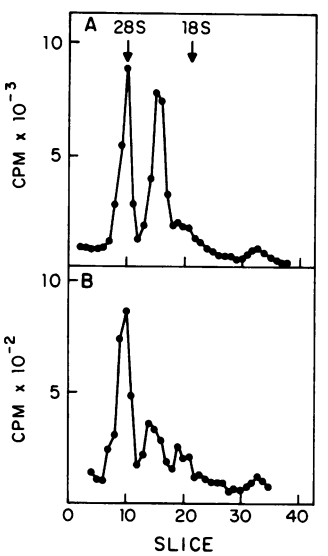

FIG. 6. *Electropherograms of total unhybridized late [³H]uridine-labeled Ad2 RNA (A) and RNA hybridized to Hpa F (25.7–27.9) DNA filters (B). Electrophoresis was from left to right. A portion (10 μl) of each eluted gel slice was counted to obtain the profile in (A).*

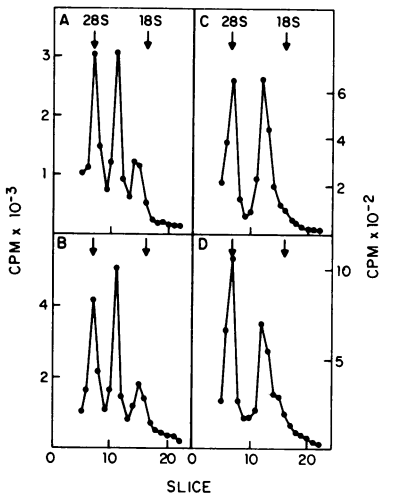

FIG. 7. *Electropherograms of late [³H]uridine-labeled Ad2 RNA hybridized to Sma D (40.5–52.6) DNA filters (A and B) and to Eco F (70.7–75.9) DNA filters (C and D). (A) and (C) represent RNA labeled during a 45 min pulse, and (B) and (D) represent RNA from cells labeled for 45 min and exposed to actinomycin D (5 μg/ml) for an additional 3 h.*

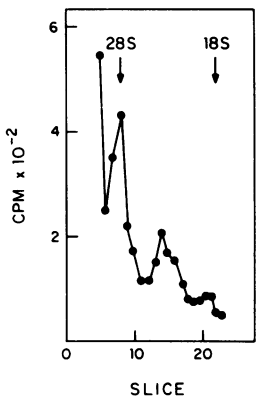

FIG. 8. *Electropherogram of late [³H]uridine-labeled poly(A)-containing nuclear Ad2 RNA hybridized to Sma D (40.5–52.6) DNA filters. RNA was labeled for 12 min at 15 h postinfection and prepared as described in the text. Electrophoresis was from left to right. Arrows indicate positions of HeLa 28S and 18S rRNA's. The nuclei in this preparation were detergent washed to remove all cytoplasm.*

ular mRNA would be followed in 15 to 20 min by a parallel increase in this particular labeled mRNA. A time course of incorporation of [^{3}H]-uridine into nuclear and cytoplasmic RNA was first determined for RNA complementary to Ad2 DNA fragments 52.6–56.9, 70.5–75.9, and 83.4–89.7; all of these DNA regions are either entirely or mostly represented in mRNA. The curve of labeled nuclear RNA for each fragment rose rapidly, as expected, reaching a plateau after about 40 to 60 min (Fig. 9). The labeled mRNA began to appear approximately linearly after about 20 min. The rate of labeling of nuclear and cytoplasmic RNA specific for the three regions was estimated from the maximal slope of increase and is shown in Table 5. From the maximum rates of labeling of the same sequences in the nucleus and then the cytoplasm, the utilization of the nuclear RNA for any of the three regions was calculated; about 20% of the 52.6–56.9 region and about 15% of each of the other two regions were conserved. Because the nuclei were not stripped of cytoplasm by detergent treatment in this experiment, at least 15% of the cytoplasmic RNA might remain in the nuclear fraction (41). Therefore, the poly(A)+ nuclear RNA of the correct size for mRNA was measured by hybridization to 52.6–56.9 and 83.4–89.7. In each case, an amount of labeled RNA equal to about 20 to 30% of that already detected in the cytoplasm was found. If this poly(A)+ RNA is added to that in the free cytoplasm, the maximum numbers for the transport efficiency would be between 20 and 26% for these three sections of the Ad2 genome. An

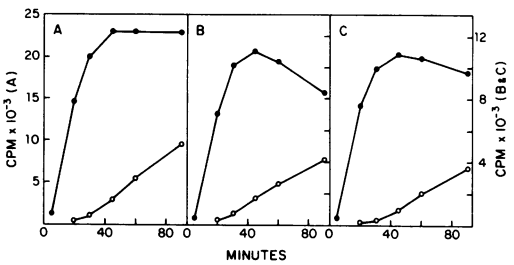

FIG. 9. *Kinetics of synthesis of adenovirus nuclear RNA and the appearance of labeled RNA in the cytoplasm specific for DNA fragments 52.6–56.9 (A), 70.7–75.9 (B), and 83.4–89.7 (C). HeLa cells infected for 15 h with adenovirus were concentrated to 10^6 cells per ml, and [^{3}H]uridine was added to 100 μCi/ml. Samples of 2×10^7 cells were removed at indicated intervals, and the nuclear (●) and cytoplasmic (○) RNAs were prepared. Each RNA sample was precipitated with LiCl to remove viral DNA. Hybridizations were performed with filters containing DNA fragments equivalent to an amount present in 5 μg of adeno DNA.*

TABLE 5. *Efficiency of transport of late Ad2 RNAs*

DNA fragment	% Transport[a]
36.7–40.5	5
40.5–42.7	4
42.7–47.5	19
52.6–56.9	17
70.7–75.9	13
83.4–89.7	13

[a] Determined from rate of labeling of cytoplasmic RNA between 45 and 90 min (counts per minute increase per 45 min) divided by the rate of labeling of nuclear RNA between 5 and 20 min (counts per minute increase per 15 min). The increase in radioactivity was approximately linear during the time chosen for the estimation (see Fig. 9).

analysis was also made of regions of the DNA genome where multiple mRNA's arise, namely, 40.5–52.6. In such regions where the primary transcript appears to be used for more than one type of mRNA, the transport efficiency should vary for RNA complementary to contiguous DNA fragments, being higher where sequences were shared by all mRNA's and lower for fragments that provide sequences exclusively for one of the group of mRNA's. The efficiency of transport of nuclear RNA from the 40.5–42.7 fragment that hybridizes the 27S but not the 23S and 19S mRNA's (see Fig. 3) was compared with the efficiency of transport of the nuclear RNA from the 42.7–47.4 region that is completely included in two and partially included in a third mRNA. Only 4% of the 40.5–42.7 region was transported compared with 19% of the 42.7–47.4 region. Another region, 36.7–40.5, which provides sequences for several mRNA's, including a long 27S mRNA with several shorter 3'-coterminal mRNA's, also showed a transport of only 5%.

These data are most compatible with the interpretation that each primary transcript gives rise to a limited number of mRNA's, possibly one; thus, any one of the potential mRNA regions exists with only a 5 to 30% success rate.

DISCUSSION

The major facts established by this work are shown in Fig. 5—a large number, perhaps 12 or more, mRNA species of distinct size (electrophoretic migration) and genome position can be distinguished from 29.1 through 100. McGrogan and Raskas (24) also have recently demonstrated multiple species of RNA in this region. These mRNA's are synthesized (labeled) late in infection, and all appear to derive from one primary nuclear RNA transcript (2, 8, 13, 14, 43). All the mRNA's contain a 3' poly(A), several have been definitely demonstrated to contain a

5′ cap (12), and from the average content of caps in the total late Ad2 mRNA's, most molecules must contain a cap (37). In each specific case so far examined, the nuclear processing also apparently involves breakage and reunion at 16.7, 19.7 and 26 in the primary transcript to produce an mRNA (4, 5, 12, 19). Additional supporting evidence is offered on this point by the datum reported here than DNA from 25–27 hybridizes an amount of labeled RNA equivalent to ~100 nucleotide portions of every size class of Ad2 mRNA's.

If this picture of multiple recombinational possibilities for the late 16–100 primary transcript is correct, many intriguing questions are posed.

(i) Do all of these events occur in the nucleus? At least a preliminary answer to this question is afforded in Fig. 8, where it is shown that all of a group of overlapping mRNA's observable after long labels in the cytoplasm are labeled within 12 min in the nucleus. Moreover, the proportions of these mRNA's in the cytoplasm appears to remain fixed in the absence of further RNA synthesis; the processing events, at least for some of the Ad2 mRNA's, would appear to be nuclear.

(ii) Can a primary transcript give rise to only one mRNA? If the putative ligation sites at 16.9, 19.7, and 26.0 are not repetitive at those sites (and this can only be told by sequence analysis), then one transcript could donate the 5′ 150 to 200 nucleotides to only one mRNA. Such a mechanism would allow any specific mRNA region to exit to the cytoplasm with a frequency sustantially less than one. An attempt to measure transport efficiency of several regions of the primary transcript known to encode major mRNA species shows approximately 15 to 20% conservation for three of the four and 20 to 30% conservation for one (the hexon region). These results are in accord, but clearly do not establish the proposal that one transcript can be converted into only one mRNA.

(iii) What locates the primary cleavage sites? The most striking general fact about the distribution of cleavage sites (or mRNA termini) concerns the 3′ termini. There may be only five different 3′ regions that exist for 12 different mRNA's—one group of mRNA's (ending at ~50) share the exact same 3′ regions (46), and there may be four other such common 3′ sites. However, each of the 12 mRNA's may have different sequences in its 5′ portions, excluding, of course, the terminal ~200 nucleotides that are presumably common to all mRNA's.

The meaning of this arrangement of presumed coding regions may possibly parallel the mode of polypeptide synthesis in certain togaviruses (18, 36), the retroviruses (17, 38), and the papovaviruses (S. Weissman, personal communication). For example, with Sindbis virus (35) and Rous sarcoma virus (38), mRNA's of various lengths exist that are translated into a single polypeptide. Thus, we might expect the 28S mRNA from 50–62 to encode the small pVI protein from its 5′ region and the 27S mRNA, a slightly shorter molecule, to lack the pVI sequence and encode the hexon protein. In this case no overlapping coding sequences would occur. Lewis et al. (21) suggested such a possibility several years ago but did not attempt to effect a complete separation of the two mRNA's to prove the point.

In the region that encodes the penton protein, 40.5–52.6, the overlapping mRNA's and the proteins encoded by each present greater difficulties. The penton protein is ~70,000 daltons, which requires at least 2,300 nucleotides or about 7.0% of the genome. The overlapping mRNA's in this region appear not to leave that much space free at the 5′ end of any of the three mRNA's in the region. Thus, these mRNA's might be read in a different frame as is the case for certain regions in ϕX174 (3) and simian virus 40 (Weissman, personal communication). Alternatively, these mRNA's might encode polypeptides that were identical at their COOH-terminal segments but possessed different NH_2 termini.

(iv) What are the relative proportions of the various mRNA's and how are these decisions mediated?

The one clear fact is that the mRNA's from different regions neither exit nor exist at steady state in equal concentration in the cytoplasm; e.g., there is much more labeled hexon mRNA than any other mRNA. However, Flint and Sharp have shown mRNA from 70–76 to be the most populous at steady state (10). Does this mean that the cleavage schedule favors certain regions over others? Not necessarily. If there were five equally probable initial cleavages, say to create five distinct 3′ termini, followed by a subdivision within these groups based on the number of mRNA's within each section, then no primary cleavage mechanism would be required to divide the primary transcript unequally. Such a model of processing accords with, but by no means is proved by, the present data. In four different regions, RNA appears to be conserved with about 20% efficiency. The greater accumulation of hexon might then be explained by the existence of only two mRNA's from this region. Clearly this discussion of these complicated events should only be taken as an introduction to the problem. For example, no mention has yet been made of cytoplasmic stability of different mRNA's, possibly a most important point in

controlling relative concentrations of the different mRNA's. The point worth making is that even though a remarkably complex set of post-transcriptional events is involved in producing the welter of Ad2 mRNA's from the large transcript, there is as yet no need to hypothesize that these are coordinated into a regulatory function. Such steps may be necessary and deliver different amounts of primary transcript into different mRNA's, but this may occur with a fixed probability and not be a regulated set of events capable of responding to change. Only more experiments can settle this issue.

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