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Adenovirus Type 2 mRNA in Transformed Cells: Map Positions and Difference in Transport Time

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Adenovirus type 2 rat transformed cells produced two polyadenylic acid-terminated mRNA's with approximate coordinates 1.5–4.4 and 4.4–11.0 on the physical map of the adenovirus type 2 genome. These mRNA's were also formed early during lytic infection in addition to one or more smaller mRNA's from the 4.4–11.0 region. In transformed cells, the 1.5–4.4 mRNA appeared in the cell cytoplasm without detectable lag, whereas the 4.4–11.0 mRNA required at least 20 to 30 min for the maximal rate of accumulation.

Several experimental designs for locating the site of origin of mRNA's by hybridization to restriction fragments of DNA (17) have been used to map the adenovirus type 2 (Ad2) genome (see reference 9 for review; 1–3, 5, 7, 13, 14, 18, 19, 25, 26). The hybridization of labeled size-fractionated RNA to excess amounts of DNA fragments (1, 5, 7, 24, 25) is the best method to measure the time and order of cytoplasmic appearance of various new mRNA species as well as to explore the stability of the new molecules in the cytoplasm. As a preliminary to studying the metabolism of virus-specific mRNA's in Ad2-transformed cells, we have used a variety of restriction fragments to locate more precisely the map positions of the 19S and 14S Ad2 mRNA molecules previously identified in transformed cells (3, 26). The 14S Ad2-specific mRNA is complementary to DNA sequences between 0 and 4.4 and the 19S complementary to sequences between 4.4 and 10.7 on the conventional physical map. (The physical map [see reference 9] of the linear Ad2 DNA consists of 100 units each representing 1% of the genome; the "left" end is so designated because RNA transcripts 5′ → 3′ begin at the "left" and to to the "right." The "left" end is taken to be 0 on the map.) We have also found that a [^{3}H]uridine label accumulates first in the 14S species of Ad2 mRNA. Finally, we have compared the results on mRNA's in transformed cells to those on the mRNA from the same genome region early in the infectious cycle.

MATERIALS AND METHODS

Cells and virus. The growth of HeLa cells and the 8617 strain of rat cells, as well as the preparation of RNA from these cells, has been described (1, 8a, 16). The growth and purification of adenovirus and its DNA and of restriction fragments were described (1, 25).

The polyadenylic acid [poly(A)]-terminated mRNA was selected by polyuridylic acid-Sepharose chromatography (16) and ethanol precipitated. The RNA was dissolved in buffer containing 0.01 M Tris (pH 7.4), 0.01 M EDTA, and 0.2% sodium dodecyl sulfate and heated at 85°C for 2 min before sucrose gradient sedimentation in 15 to 30% sucrose in the same buffer containing 0.05 M NaCl. The RNA was centrifuged in the SW40 rotor at 40,000 rpm for 8.5 h.

Hybridization was carried out to DNA bound to nitrocellulose filters (12) as described (26). Hybridization was carried out in 0.3 M NaCl–10 mM TES–10 mM EDTA–0.2% sodium dodecyl sulfate for 30 to 40 h with 2- to 5-μg equivalents of Ad2 DNA fragments, as described in the figure legends. Under these conditions greater than 80% of the complementary RNA was hybridized, as monitored by successive hybridization of the incubation mix to Ad2 DNA fragments. Before hybridization to Ad2 fragment filters, the RNA was alkali broken to ~200 nucleotide lengths in 0.2 N NaOH for 25 min at 0°C and neutralized with HEPES buffer.

RESULTS

Polyadenylated cytoplasmic RNA of Ad2-transformed 8617 cells. The left-hand 11% of the Ad2 genome is most important and probably sufficient for transforming cells (10, 11, 21). The frequently used Ad2-transformed rat embryo cell line, strain 8617, is known to contain 19S and 14S mRNA molecules complementary to DNA from the 0–11 region of Ad2 (1 unit = 1% of the genome = 0.35 kilobase [1–3, 5, 7]). The exact map location of the 19S and 14S RNAs within this region of the genome and, in fact, whether there were only two molecules have not been established. Detailed cleavage maps of the left-hand 11% of the Ad2 genome,

are, however, known from the extensive work of Sharp, Mulder, Roberts and their colleagues (see 9 and 17 for review). Thus, dissection of the genome can be carried out as specified in Table 1 and Fig. 1 (see Materials and Methods) to give fragments with map coordinates 0–2.9; 2.9–7.5; 7.5–11.0; 0–4.4 and 4.4–25.5; and 0–58.5. Filters bearing DNA fragments were prepared, and poly(A)-terminated [^{3}H]uridine-labeled mRNA was extracted and selected from 8617 cells and sedimented through sucrose gradients to achieve satisfactory separation of the presumed two species, 14S and 19S (Fig. 1). Two virus-specific peaks of RNA were indeed detected by hybridization to the *Eco*RI-A fragment, 0–58.5. Samples of RNA from the appropriate fractions were then hybridized to various other DNA fragments.

TABLE 1. *DNA restriction fragments generated from the left-hand end of the Ad2 genome*[a]

Map coordinates	Enzyme(s) used	Fragment
0–2.9	*Sma* I	*Sma* I-J
0–4.4	*Hpa* I	*Hpa* I-E
0–58.5	*Eco*RI	*Eco*RI-A
2.9–7.5	*Sma* I/*Hin*dIII	*Sma* I-E/*Hin*dIII-G
2.9–11.0	*Sma* I	*Sma* I-E
4.4–7.5	*Hpa* I/*Hin*dIII	*Hpa* I-C/*Hin*dIII-G
4.4–25.5	*Hpa* I	*Hpa* I-C
7.5–11.0	*Sma* I/*Hin*dIII	*Sma* I-E/*Hin*dIII-C
11.6–18.2	*Sma* I	*Sma* I-F

[a] Map coordinates of *Hpa* I, *Eco*RI, and *Hin*dIII 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 coordinates are from Carl Mulder (personal communication).

The smallest RNA, 14S, hybridized to fragments 0–2.9, 2.9–7.5, and 0–4.4, but not to 7.5–11.0 or 4.4–25.5. This mRNA, which is known to be read in a rightward direction (9, 21), should have a chain length of about 1 kilobase or about 3 map units, based on sedimentation size (24). The 14S mRNA hybridized about 40% to the 0–2.9 fragment and 60% to the 2.9–7.5 fragment. By this determination the ~3 map units covered by the 14S would extend from about ~1.5 to 4.5 or almost entirely within the

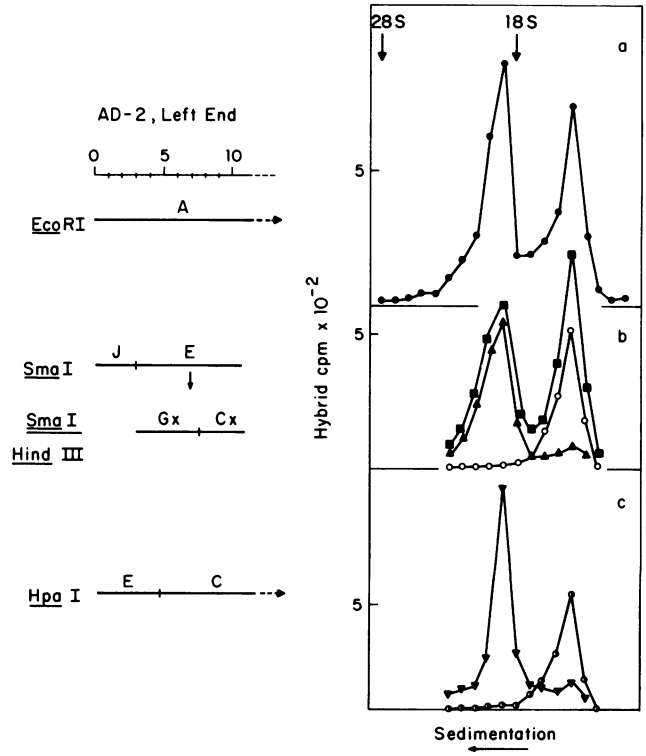

FIG. 1. *Mapping of Ad2-transformed cell mRNA. The map of the left-hand portion of Ad2 genome as determined by restriction digestion with EcoRI, Hpa I, and HindIII is diagrammed on the left. On the right are given results of hybridization to various DNA fragments of poly(A)-terminated RNA from Ad2-transformed cells (strain 8617) labeled for 3 h with [^{3}H]uridine (100 μCi/ml). (a) Hybrids to EcoRI-A. (b) Hybrids to Sma I-J (○); Sma I-E/HindIII-G (■); and Sma I-E/HindIII-C (▲). (c) Hybrids to Hpa I-E (◑) and Hpa I-C (▼). The filters contained an amount of each DNA fragment that would be contained in 2 μg (2-μg equivalents) of total Ad2 DNA; the RNA from 5 × 10^7 to 10^8 cells was used from each gradient.*

Hpa I-E, 0–4.4 fragment. This agrees with the result that very little 14S RNA hybridized to the 4.4–25.5 fragment.

The 19S RNA, which should be about 2 to 2.2 kilobases or 6 map units long, hybridized to the 4.4–25.5 fragment but showed no hybridization to the 0–4.4 fragment. Less than 2% of the Ad2-specific poly(A)-containing RNA from the 8617 cells hybridized to the 11.6–18.2 fragment (data not shown). Thus the 19S mRNA would appear to be complementary to almost the entire 4.4–11.0 fragment.

The mapping studies on the transformed cell mRNA's used RNA prepared from cells that had been labeled for 3 to 4 h. In such preparations there was always between 1.5 and 2 times as much total radioactivity in the 19S mRNA as in the 14S mRNA. Such a distribution of radioactivity is compatible with equal numbers of molecules and approximately equal stability for the two molecules. To investigate whether these two mRNA's that derive from contiguous regions in the DNA were synthesized, processed, and delivered to the cytoplasm in an equimolar fashion, RNA from cells that had been labeled for a variety of pulse lengths was sedimented and hybridized to the fragment 0–58.5 (*Eco*RI-A). The smaller mRNA was detectable in cells labeled for only 10 min and was still the predominant labeled species after a 40-min labeling period (Fig. 2). In various experiments, from 1.5 to 3 times as much 14S mRNA as 19S mRNA was present after a 40- to 60-min label time. Only after 90 min of labeling were the 14S and 19S equal in total radioactivity, indicating that approximately twice as many 14S mRNA molecules had entered the cytoplasm during this period.

Nuclear RNA was examined after a 10-min label period to determine whether the shorter mRNA could be detected as a discrete species in the nucleus. Even with so short a labeling period very little labeled RNA complementary to the 0–2.9 region could be observed (Fig. 3), whereas a large amount of RNA sedimenting between ~17–21S was found to hybridize to the 2.9–11 fragment.

Thus the time of appearance of labeled molecules of these two mRNA's in the cytoplasm is quite different even though both molecules are poly(A) terminated and derived from neighboring sites on the Ad2 genome. We do not know whether both mRNA's are part of the same transcription unit in transformed cells, but the entire region may form one transcription unit early in adenovirus infection (8a).

Cytoplasmic RNA from the *Eco*RI-C region. Although our primary interest in studying the 8617 transformed cell line has been the two

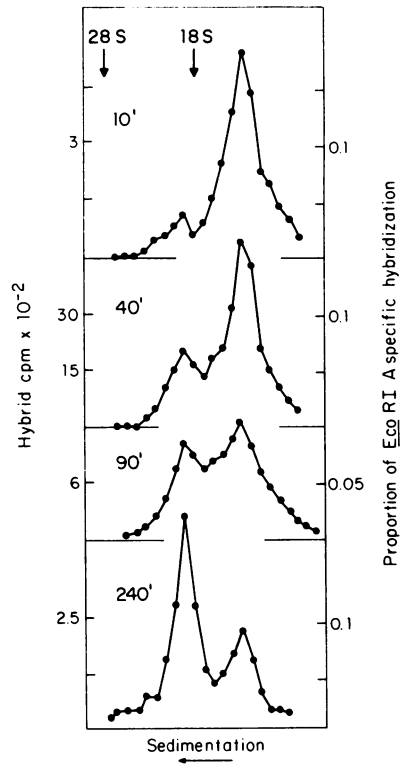

FIG. 2. *Time course of appearance of labeled Ad2-specific mRNA's. Poly(A)-terminated cytoplasmic RNA was prepared from 8617 cells after the indicated times of label, denatured, and subjected to sucrose gradient analysis. Portions of each gradient were hybridized to EcoRI-A (0–58.5) DNA (2-μg equivalents of DNA/filter); total RNA in each sample was equivalent to that from ~10⁶ cells.*

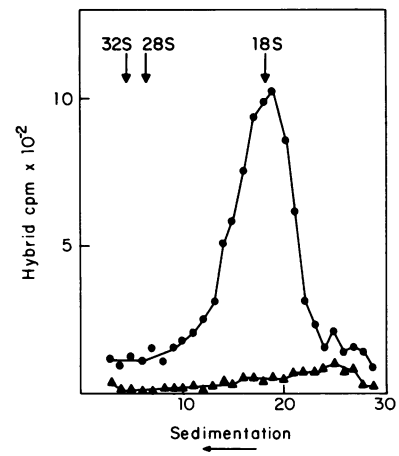

FIG. 3. *Nuclear RNA after 10-min label. Nuclear RNA was prepared from 3 × 10⁸ 8617 cells after a 10-min exposure to [³H]uridine (200 μCi/ml). The RNA was denatured and sedimented in the SW27 rotor (24,000 rpm, 18 h) and hybridized to Sma I-J (▲) and Sma I-E (●) Ad2 DNA fragments.*

mRNA's identified in the 0–11 region, it is well established that this line carries additional portions of the virus genome and that some cytoplasmic RNA complementary to the *Eco*RI-C region was detected by complementarity to ^{32}P-labeled DNA (10, 11, 22). We therefore tested for the presence of discrete species of poly(A)-terminated cytoplasmic RNA from this region. The *Eco*RI-C fragment (89.7–100) is known to encode several mRNA species that are produced early in infection (2, 7, 9, 21). In the transformed cells, poly(A)-terminated, cytoplasmic RNA of ~18–20S that hybridized to *Eco*RI-C was also detected (Fig. 4). The *Eco*RI-C-specific material was considerably broader in sedimentation, indicating that more than one *Eco*RI-C-specific mRNA molecule might be present.

Early mRNA from the 0–11 region. To define more precisely the map positions of the early lytic mRNA, hybridization of ^{3}H-labeled early RNA was carried out to the same fragments as were used to map transformed cell mRNA (see Fig. 5). The results suggest that the same mRNA's found in transformed cells plus additional smaller mRNA's from the 4.4–11 region are present early in the lytic cycle.

First, 14S mRNA was found to hybridize to both the 0–2.9 and 0–4.4 regions. Second, the only fragments to hybridize 19S RNA included regions to the right of 4.4. As had been found in the transformed cell, no significant hybridization (less than 3% of the amount hybridized to fragments 0–4.4 and 4.4–11.0) was found to the Ad2 fragment extending from 11.6–18.2. This suggests that during early infection and in the transformed cell the same mRNA coding regions at the left-hand end of the Ad2 genome are utilized. The difference between the transformed cell mRNA and early lytic mRNA was the presence of ~14–15S mRNA, which hybridized about equally to the 4.4–7.5 and 7.5–11.0 regions.

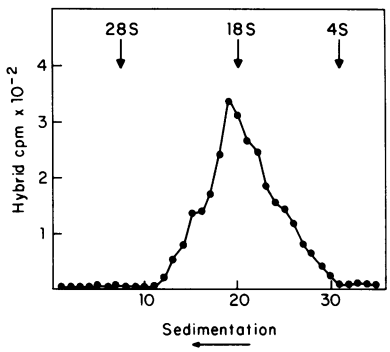

FIG. 4. *Presence of mRNA from EcoRI-C fragment (89.7–100) in cytoplasm. Cytoplasmic poly(A)-terminated RNA prepared as in Fig. 1 and 2 was hybridized to the EcoRI-C (89.7–100) fragment.*

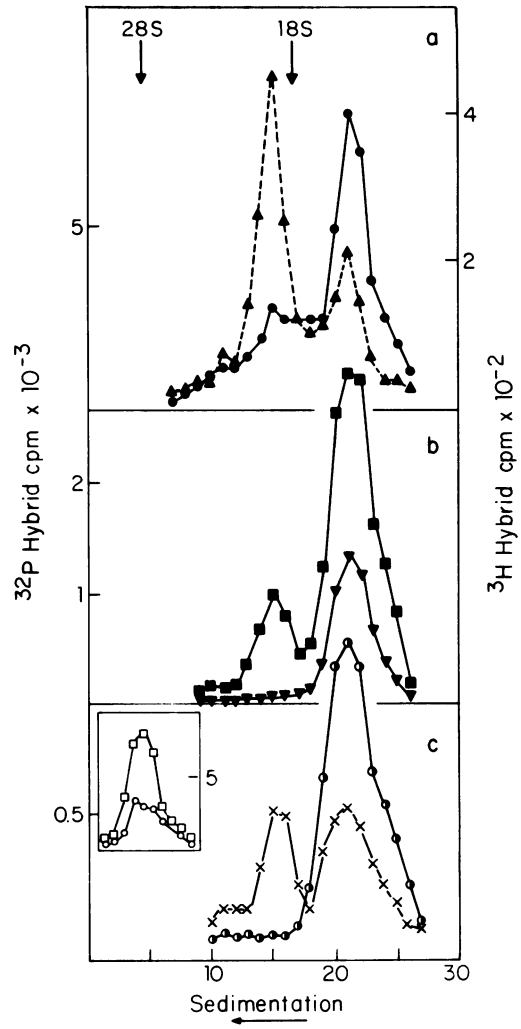

FIG. 5. *Ad2-specific RNA from HeLa cells early in infection. HeLa cells were infected with 2,000 particles of Ad2 per cell. After 1 h, 25 μg of cycloheximide per ml was added, and cells were labeled for 3 h with $^{32}PO_4$; poly(A)-terminated cytoplasmic RNA was prepared, sedimented, and hybridized to various DNA fragments as described in the legends to Fig. 1 and 2. (a) ^{32}P-labeled poly(A)$^+$ mRNA of Ad2 early, lytically infected HeLa cells was cosedimented with [^{3}H]uridine-labeled poly(A)$^+$ mRNA from 8617 cells and hybridized to the EcoRI-A fragment (0–58.5). Symbols: ^{32}P (●); ^{3}H (▲). (b and c) ^{32}P-labeled early Ad2 lytic RNA hybridized to Sma I-J (▼), Sma I-E (■), Hpa I-E (◑), and Hpa I-C (×) fragments (Insert) ^{3}H-labeled poly(A)$^+$-containing RNA was prepared from lytically infected cells as described above, and the 14S mRNA region was hybridized to fragments Hpa I-C/HindIII-G (□) and Sma I-E/HindIII-C (○)*

From the data it is not possible to judge between two similar sized molecules: one from each of those regions or one molecule that lies equally in the two regions.

Effect of cycloheximide on labeling of various early mRNA's. In the experiments reported in the previous section the infected cells were treated with 25 μg of cycloheximide per ml during the labeling, a treatment previously documented to increase the labeling of Ad2-specific mRNA (6, 18). Table 2 confirms the fact that cycloheximide does indeed increase Ad2-specific mRNA and adds the additional information that (i) the labeling of total poly(A)-containing mRNA was not increased but, in fact, declined slightly, (ii) the labeling of all Ad2 mRNA's was increased, and (iii) the mRNA's in the 2.9–11.0 region were increased somewhat more than that in the 0–2.9 region.

To determine more specifically the effect of cycloheximide on the relative proportions of the 19S and 14S mRNA's encoded within the 0–11 region of the Ad2 genome, the poly(A)-containing RNA was fractionated on sucrose gradients and hybridized to Ad2 fragments 0–4.4 and 4.4–25.5. Figure 6 shows that the addition of cycloheximide 1 h before and during the labeling clearly alters the relative amounts of labeled Ad2-specific mRNA's between these two regions. The labeling of the 14S mRNA complementary to the 4.4–25.5 fragment is preferentially increased about 13-fold, whereas the labeled 19S mRNA hybridizing to the same DNA fragment is increased only 3.5-fold. The labeled 0–4.4 mRNA is increased under these conditions by five- or sixfold. These results demonstrate that the accumulation of labeled molecules of these specific mRNA's can be differentially modified but that all of the various mRNA species observed in the presence of cycloheximide are also present without the drug.

Time course of appearance of early mRNA's. The final experiment performed with cells early in infection was an examination of the time course of labeling of the 0–4.4 and 4.4–11 mRNA's in the presence of cycloheximide (Fig. 7). As was the case with transformed cells, the 0–4.4 14S mRNA was the dominant species after a 10-min labeling time. After a 45-min labeling time an approximately equal amount of radioactivity was present in the RNA complementary to the 0–4.4 and 4.4–11.0 fragments. The majority of the 4.4–11-specific RNA sedimented at 14S. Finally, after 3 h of labeling, the majority of the labeled mRNA from the 0–11 region was complementary to the 4.4–25.5 fragment, with both 14S and 19S mRNA's detectable. It might seem possible, therefore, that the initial poly(A)-terminated nuclear products in both lytically infected and transformed cells are the 0–4.4-specific 14S mRNA and the 4.4–11.0-specific 19S mRNA. The larger molecule either might undergo cleavage or a different processing might occur to RNA from that region in the lytic cycle. The 4.4–11-specific 14S mRNA cannot, however, be simply the poly(A)-containing half of the 19S mRNA, i.e., a cytoplasmic degradation product, for this 14S mRNA is polyadenylated and hybridizes to both the right (4.4–7.5)- and left (7.5–11.0)-hand ends of the 4.4–11.0 region of the Ad2 genome (Fig. 5c, in-

TABLE 2. *Effect of cycloheximide on Ad2-specific RNA synthesis early in lytic infection*[a]

Ad2 DNA fragment	cpm hybridized		
	Control, no cycloheximide (4.8 × 10⁶)[b]	+ Cycloheximide (25 μg/ml) (4.15 × 10⁶)[b]	+ Cycloheximide/control
Sma I-J, 0–2.9	546	2,880	5.3
Sma I-E, 2.9–11.0	786	5,754	7.3
*Eco*RI-B, 58.5–70.7	531	6,417	12.1
*Eco*RI-D, 75.9–83.4	674	5,294	7.8
*Eco*RI-C, 89.7–100	767	11,313	14.8

[a] Total cytoplasmic poly(A)⁺ mRNA was prepared from Ad2-infected HeLa cells in the presence or absence of 25 μg of cycloheximide per ml, as described in the legend to Fig. 6. Ten percent of the poly(A)⁺ mRNA of each sample was hybridized to 5-μg-equivalent fragments of the indicated DNA fragments.

[b] Total poly(A)⁺ mRNA.

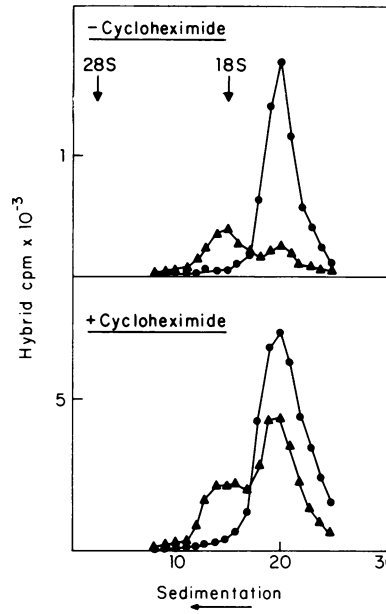

FIG. 6. *Effect of cycloheximide on Ad2-specific mRNA labeled early in infection. Cells prepared as in Fig. 5 were either treated with 25 μg of cycloheximide per ml between 2 and 3 h after infection or not treated. Both cultures were then labeled for 45 min with [³H]uridine, and cytoplasmic poly(A)-terminated RNA was selected, sedimented, and hybridized to the DNA fragments: Hpa I-E (●); Hpa I-C (▲).*

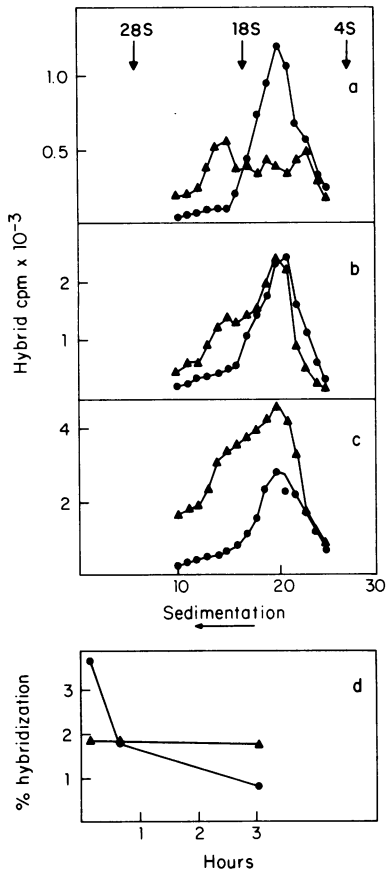

FIG. 7. *Time course of Ad2-specific mRNA labeled early in infection. Cytoplasmic poly(A)-terminated RNA was prepared from HeLa cells 3 h postinfection after labeling for (a) 10 min, (b) 40 min, and (c) 3 h, as described in the legend to Fig. 5. Cycloheximide was present 1 h before and during the labeling to prevent late Ad2 mRNA synthesis. The RNA was sedimented and hybridized to Ad2 DNA fragments. (d) Summary of the labeling. Symbols: Hpa I-E (●); Hpa I-C (▲).*

sert). Thus it may be that in the lytic infection in HeLa cells some additional nuclear processing pathway(s) allows formation of mRNA's from areas of the 0–11 region different from those in transformed cells.

DISCUSSION

A major point established by this study is that in Ad2-transformed cells (strain 8617), the 0–11 region of the virus genome encodes two mRNA's, one of which maps from about 1.5–4.4 and one of which maps from 4.4–11.0. Earlier data established that all the mRNA in this region is read in a rightward direction (9). From the present data it is not possible to tell whether the 3′ and 5′ ends of the two mRNA's might be separated by a short distance or even overlap for 50 to 100 nucleotides as do some of the simian virus 40 mRNA's (S. Weissman, personal communication). Second, both mRNA's are poly(A) terminated and are presumed to have cap structures at their 5′ ends, since all mRNA's except the picornaviruses do have such a 5′ cap structure (see 23 for review). Therefore, it was surprising to find that the 14S, 0–4.4 mRNA was detected in such a short time in the cytoplasm, whereas the 19S, 4.4–11 mRNA required approximately 20 to 30 min to enter the cytoplasm. This latter time has been assumed to be the average time taken for an mRNA to enter the cytoplasm (8). Recent studies on labeling total HeLa cell mRNA with adenosine, however, have shown quite clearly that labeled nuclear-derived poly(A) can enter the cytoplasm within 5 to 10 min after labeling (20). In agreement with those results, the present studies show that a discrete mRNA deriving from an integrated piece of viral DNA can enter the cytoplasm within a few minutes after synthesis.

A third point of interest raised by these studies is the possible difference in the turnover rates of the two transformed cell mRNA's. Since in the cytoplasm the smaller mRNA is labeled predominately in a 10-min pulse, but is a minority species after longer labels, it is possible that the 14S mRNA has a faster turnover time than the 19S mRNA. Thus the two mRNA's from a contiguous region of the DNA may be both processed and transported differentially from the nucleus in addition to having a different metabolic fate in the cytoplasm. These results possibly afford an opportunity to examine the differential metabolism of poly(A) of two discrete mRNA's with different half-lives.

A final circumstance that recommends these mRNA's for metabolic studies is their existence in both the lytic and transformed cells. It is clear that the same mRNA's are made early in infection, although more than one product may be delivered into the cytoplasm from the 4.4–11 region. From the data in Fig. 7 it would appear that at least the more rapid transport of 14S mRNA found in transformed cells may also be true in lytic infection. This might mean that the rapid exit of the 14S is a result of its primary structure. The more general question of whether differential metabolic behavior of different mRNA's is intrinsic to the mRNA or is determined by the cellular milieu might therefore be answered by further work with these Ad2 mRNA's.

ACKNOWLEDGMENTS

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ADDENDUM

After submission of this manuscript for publication, Chow et al. (4) reported map coordinates similar to those presented here for the 19S and 14S early Ad2 mRNA's complementary to the left-hand end of the Ad2 genome.

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