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Adenovirus Transcription

II. RNA Sequences Complementary to Simian Virus 40 and Adenovirus 2 DNA in Ad2$^+$ND$_1$- and Ad2$^+$ND$_3$-Infected Cells

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The genomes of the two nondefective adenovirus 2/simian virus 40 (Ad2/SV40) hybrid viruses, nondefective Ad2/SV40 hybrid virus 1 (Ad2$^+$ND$_1$) and nondefective hybrid virus 3 (Ad2$^+$ND$_3$), were formed by a deletion of about 5% of Ad2 DNA and insertion of part of the SV40 genome. We have compared the cytoplasmic RNA synthesized during both the early and late stages of lytic infection of human cells by these hybrid viruses to that expressed in Ad2-infected and SV40-infected cells. Separated strands of the six fragments of ^{32}P-labeled Ad2 DNA produced by cleavage with the restriction endonuclease *Eco*RI (isolated from *Escherichia coli*) and the four fragments of ^{32}P-labeled SV40 DNA produced by cleavage with both a restriction nuclease isolated from *Haemophilus parainfluenzae*, *Hpa*1, and *Eco*RI were prepared by electrophoresis of denatured DNA in agarose gels. The fraction of each fragment strand expressed as cytoplasmic RNA was determined by annealing fragmented ^{32}P-labeled strands to an excess of cellular RNA extracted from infected cells. The segment of Ad2 DNA deleted from both hybrid virus genomes is transcribed into cytoplasmic mRNA during the early phase of Ad2 infection. Hence, we suggest that Ad2 codes for at least one "early" gene product which is nonessential for virus growth in cell culture. In both early Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$-infected cells, 1,000 bases of Ad2 DNA adjacent to the integrated SV40 sequences are expressed as cytoplasmic RNA but are not similarly expressed in early Ad2-infected cells. The 3′ termini of this early hybrid virus RNA maps in the vicinity of 0.18 on the conventional SV40 map and probably terminates at the same position as early lytic SV40 cytoplasmic RNA. Therefore, the base sequence in this region of SV40 DNA specifies the 3′ termini of early messenger RNA present in both hybrid virus and SV40-infected cells.

The genome of adenovirus 2 (Ad2) can be considered as a eukaryotic chromosome. The linear viral DNA, coding for some 20 to 40 proteins, is transcribed in nuclei of infected mammalian cells, and viral mRNA is subsequently translated on the cells' polyribosomes.

Recently, specific fragments of Ad2 DNA produced by cleavage with restriction endonucleases have been used to study the extent of transcription in both lytically infected (30, 32, 33; S. J. Flint, P. H. Gallimore, and P. A. Sharp, J. Mol. Biol., in press) and transformed cells (Flint et al., J. Mol. Biol., in press). Early in the lytic cycle, preceding viral DNA replication, cytoplasmic RNA complementary to a total of 23% of the viral DNA is transcribed from four discrete regions of the viral genome. By contrast, all, or nearly all, of the Ad2 genome is transcribed into cytoplasmic RNA after the onset of viral DNA replication, at late times during infection; RNA complementary to the equivalent of one strand of the viral DNA is then present on polyribosomes. A map of these cytoplasmic viral sequences has been constructed using the 5′ to 3′ polarity of the two strands of Ad2 DNA (30; Flint et al., J. Mol. Biol., in press).

Five nondefective Ad2/simian virus 40 (SV40) hybrid viruses have been isolated (15, 16). Their genomes, which comprise partly Ad2 and partly SV40 sequences, have been studied in detail by electron microscopy (8), DNA-RNA hybridization (6) and base sequence techniques (3). The data for two of these viruses, nondefective Ad2/SV40 hybrid virus 1 (Ad2$^+$ND$_1$) and nondefective Ad2/SV40 hybrid 3 (Ad2$^+$ND$_3$), summarized from this literature, are shown in Fig. 1 and compared with the corresponding

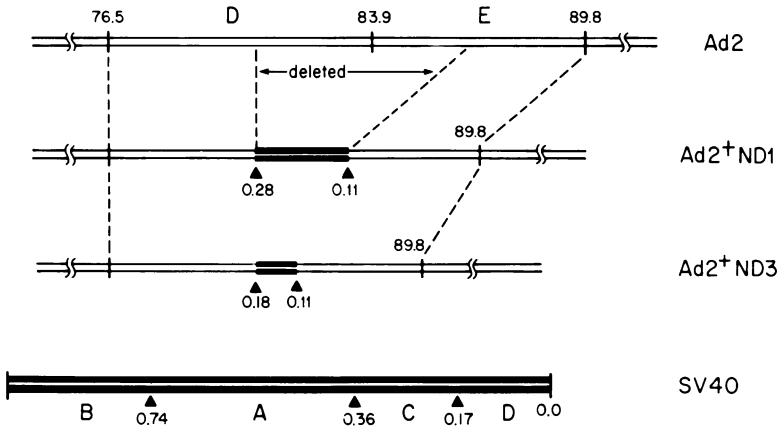

FIG. 1. *The position in Ad2 map units of EcoRI-Ad2 fragments D and E are shown on the top line and the modifications of this region in Ad2+ND$_1$ and Ad2+ND$_3$ DNA are drawn below. In all cases, SV40 and Ad2 map positions are given below and above the line, respectively. The substituted SV40 DNA segment is depicted with thick lines and the parental Ad2 sequences as thin lines. Arrows (▲) denote the position of HpaI cleavage sites in SV40 DNA and a vertical line drawn through both strands shows the position of EcoRI cleavage sites. The positions of the four SV40 fragments produced by cleavage with EcoRI and HpaI endonucleases are shown below the SV40 genome map.*

regions of the parental SV40 and Ad2 genomes. Both hybrid DNAs were formed by a deletion of a segment (some 5%) of the Ad2 genome, and, although the position of the deletion may be different in the two hybrids, the restriction endonuclease isolated from *Escherichia coli* (*Eco*RI) cleavage site at position 0.839 on Ad2 DNA is missing in both Ad2+ND$_1$ and Ad2+ND$_3$ DNA. Thus these two hybrid viruses contain only part of the sequences of *Eco*RI Ad2 fragments D and E. Sequential cleavage of SV40 DNA by *Eco*RI at position 0.0 (18, 20) and by a restriction endonuclease isolated from *Haemophilus parainfluenzae* (*Hpa*I) at positions 0.17, 0.36, and 0.74 (2, 31) yields four fragments, SV40 A, B, C and D. The SV40 DNA sequences substituted in place of deleted Ad2 DNA in these hybrid viruses begin at 0.11 on the SV40 genome in both viruses and end at 0.28 and 0.18 in Ad2+ND$_1$ and Ad2+ND$_3$, respectively. Both viruses contain the *Hpa*I cleavage site at 0.17 on the SV40 genome; Ad2+ND$_1$ thus contains sequences homologous to 55 and 35% of SV40 C and SV40 D fragments, respectively, whereas 5 and 35% of SV40 C and SV40 D, respectively, are present in Ad2+ND$_3$ DNA.

Both hydrid viruses undergo a normal lytic cycle in human cells, but only Ad2+ND$_1$ plaques efficiently on African green monkey kidney cells (16). Presumably, a gene product from the SV40 segment in Ad2+ND$_1$ is responsible for the lytic growth on monkey cells because co-infection of monkey cells with SV40 and Ad2 results in an enhancement of the Ad2 virus yield from the infected cell (27). An "early" SV40-specific antigen, the U antigen, has been detected in Ad2+ND$_1$-infected cells, but not in Ad2+ND$_3$-infected cells (16, 17). Moreover, Oxman et al. (22) have shown that Ad2+ND$_1$-infected cells contain RNA sequences that compete with SV40 early RNA sequences in RNA hybridization to SV40 DNA immobilized on a filter.

In the early stages of SV40 infection of monkey cells, mRNA whose 5′ end maps at about position 0.67 and whose 3′ end maps at approximately position 0.18 is found in the cytoplasm (3, 9, 10, 28) (see Discussion for elaboration of this point). This early mRNA is transcribed from left to right using the E strand as template (12, 29). Reason dictates that the base sequences near position 0.18 specify the 3′ end of the mRNA, either by termination of transcription or by an RNA processing mechanism. Both Ad2+ND$_1$ and Ad2+ND$_3$ contain this region of the SV40 genome (Fig. 1), suggesting that SV40 RNA sequences expressed during lytic infection by Ad2+ND$_1$ or Ad2+ND$_3$ might also terminate around 0.18. Previous studies have shown that RNA complementary to most of the *l* strand of Ad2 DNA is transcribed during the late stages of Ad2 infection (25, 30) and that one of the early mRNA species appears to be transcribed from the segment of the Ad2 genome deleted in these hybrid viruses (30; Flint et al., J. Mol. Biol., in press). We have therefore examined the effects of these rearrangements of viral DNA on the expression of mRNA. The cytoplasmic RNA expressed in Ad2+ND$_1$- and Ad2+ND$_3$-infected cells at both

early and late times has been mapped by annealing cellular RNA to the ^{32}P-labeled, separated strands of restriction endonuclease fragments of both Ad2 and SV40 DNA. The data are discussed in terms of models of mRNA production.

MATERIALS AND METHODS

Cells. Human cells, either KB or HeLa cell lines, were grown in suspension cultures containing minimal essential medium (Joklik modified) purchased from GIBCO and supplemented with 5% calf serum. CV-1 cells (obtained from Janet Mertz, Stanford University) and HeLa monolayer cells (obtained from J. F. Williams, Institute of Virology, Glasgow) were cultured in plastic dishes in Dulbecco's modification of Eagle medium (D medium) supplemented with 10% fetal calf serum.

Viruses. A high titer stock of a four times plaque-purified SV40 strain 777 was used (28).

Ad2^{+}ND$_1$ stock obtained from Andrew Lewis, National Institutes of Health, was grown in suspension KB cells and assayed by plaque formation on monolayers of HeLa cells (35). The preparation of viral DNA has been described previously (21, 31).

Ad2^{+}ND$_3$ virus seed stock has been described previously (16) and was propagated by passage on primary human embryonic kidney cells.

Ad2, originally obtained from J. Rose, National Institutes of Health, Bethesda, Md., was propagated in suspension cultures of KB cells (26). This virus stock was also used in previous studies (24; Flint et al., J. Mol. Biol., in press).

DNA. ^{32}P-labeled SV40 DNA (specific activity 3×10^6 counts/min per μg) was prepared from CV-1 cells 48 h after infection with approximately 10 PFU/cell by Hirt extraction (7) as described previously (1).

^{32}P-labeled Ad2 DNA (specific activity 4×10^6 counts/min per μg) was prepared as described previously (30; Flint et al., J. Mol. Biol., in press).

Separation of the strands of viral fragments. SV40 fragment strands were separated by a method similar to that described for Ad2 fragment strands (30; Flint et al., J. Mol. Biol., in press). ^{32}P-labeled fragments of SV40 DNA were produced by sequential digestion with both *Hpa*1, isolated from *H. parainfluenzae* (31), and *Eco*RI, isolated from *E. coli*, RY13 (R. N. Yoshionori, Ph.D. thesis, Univ. of California, San Francisco, 1971). The four SV40 fragments were resolved by electrophoresis in 2.2% acrylamide, 0.7% agarose gels and the DNA from each band eluted by electrophoresis (24). Eluted fragments of SV40 DNA were extracted with buffered phenol and precipitated with ethanol. Approximately 0.5 μg of a purified fragment DNA was dissolved in 10 μl of 0.01 M Tris-hydrochloride buffer, pH 8.5, containing 1.0 mM EDTA. Fragment DNA was denatured by treatment at room temperature for 10 min with 0.2 M NaOH, 2 μl of 0.5% bromophenol blue and 60% sucrose solution were added, and the sample was immediately layered on to a 1.4% agarose gel (0.8 by 15 cm) and a voltage gradient of 2 V/cm was applied. The electrophoresis buffer was 0.03 M Tris, 0.036 M NaH_2PO_4, and 0.001 M EDTA, pH 7.7 (5). After 8 h, the gels were stained by immersion in 0.5 μg of ethidium bromide per ml and the fluorescence of the bound dye was photographed during excitation with an ultraviolet lamp (31).

Recovery of the SV40 fragment strands from the agarose gel and further purification were as described for Ad2 fragment strands (30; Flint et al., J. Mol. Biol., in press). Briefly, a section of agarose gel containing a DNA band was dissolved by boiling for 5 min in 0.5 ml of 0.1 M phosphate buffer, pH 6.8, containing 1.0 M NaCl, 0.3 M NaOH, and 0.4% sodium dodecyl sulfate (SDS). After neutralization of the base with HCl, each sample was incubated at 68 C for 10 to 24 h to allow renaturation of all contaminating complementary strand DNA sequences with the strand in excess. Single-strand DNA was selected by collecting the ^{32}P-labeled DNA that would not bind to a hydroxylapatite column at 60 C in 0.125 M phosphate buffer containing 0.4% SDS. Generally, about 75% of the radioactivity extracted from the gels was recovered as single-stranded DNA.

^{32}P-labeled strands of the *Eco*RI fragments of Ad2 DNA were prepared as described previously (30; Flint et al., J. Mol. Biol., in press).

Extraction of RNA. KB cells were infected with 10 PFU/cell of Ad2 at a cell density of 3×10^6 cells/ml. After 30 min, the cells were diluted 10-fold with medium: this is considered zero time of infection. For early RNA preparation, cytosine arabinoside (20 μg/ml) was added to the culture medium and cells were harvested 8 h later. "Late" RNA samples were extracted from cells 18 h after infection. In all cases, nuclear and cytoplasmic fractions were prepared by treatment with the nonionic detergent NP40 (14). Nuclear and cytoplasmic RNA were prepared from these fractions as described previously (30; Flint et al. J. Mol. Biol. in press). All RNA samples were digested with DNase I (Worthington Biochemical Corp., Freehold, N. J.) and oligonucleotides were removed by chromatography on G-75 Sephadex (28).

Hybridization conditions. All hybridization reactions were carried out at 68 C in 0.10 to 0.25 ml of 1.0 M NaCl buffered with 0.10 M phosphate buffer, pH 6.8 (13). After incubation for 24 h, samples were diluted 10-fold in 0.125 M phosphate buffer, pH 6.8, containing 0.4% SDS and assayed by chromatography on hydroxylapatite columns (28).

RESULTS

SV40 strand separation. The four specific fragments of SV40 DNA produced by cleavage with the restriction endonucleases *Eco*RI and *Hpa*1 have been used for mapping viral RNA sequences by annealing separated, fragmented ^{32}P-labeled strands of each fragment to excess quantities of cellular RNA (28). Assuming that (i) the fragment is uniformly labeled by ^{32}P, (ii) the fragment strand is fragmented to a length of 200 to 300 bases, and (iii) sufficient cellular RNA is added to ensure saturation of all the complementary DNA sequences, then the fraction of ^{32}P-labeled DNA entering RNA-DNA hybrid as assayed by chromatography on hy-

droxylapatite columns is a measure of the region of DNA expressed in cellular RNA.

Previously, separated strands of the four restriction endonuclease fragments of SV40 DNA described above have been prepared by annealing denatured ^{32}P-labeled fragment strands with excess asymmetric SV40 complementary RNA obtained by transcribing viral DNA in vitro with *E. coli* RNA polymerase (9, 28). SV40 complementary RNA anneals to the *E* strand of SV40 DNA, the in vivo template for early mRNA, but not to the *L* strand, the in vivo template for late mRNA (34). This method of strand separation is expensive in terms of the requirement for *E. coli* polymerase and template SV40 DNA to prepare the necessary 20-fold molar excess of cRNA to anneal to ^{32}P-labeled fragment DNA. Agarose gel electrophoresis for strand separation of DNA was first described by Hayward (5) and has since been used to separate the strands of all six fragments of Ad2 DNA produced by cleavage with the restriction endonuclease *Eco*RI (30; Flint et al., J. Mol. Biol., in press). We therefore separated the strands of each of the four specific fragments of SV40 DNA by electrophoresis. Figure 2 shows an example of four such gels, electrophoresis of denatured DNA of SV40 fragments A, B, C and D. Each gel contains three bands of DNA, the two faster migrating bands being the separated fragment strands, whereas the slowest migrating band comprises renatured material. After purification, each fragment strand was annealed to excess asymmetric SV40 complementary RNA (34) to prove that the two strands of a fragment of SV40 DNA were separated by electrophoresis. The slower migrating strands of SV40 A and C fragments and the faster migrating strands of SV40 B and D fragments anneal to SV40 complementary RNA and are part of the *E* strand, whereas the other strand of each fragment does not renature with SV40 complementary RNA and is therefore part of the *L* strand (Table 1). The reason for the separation of complementary strands of DNA by electrophoresis is unknown; however, the SV40 genome is similar to the Ad2 genome in that it is asymmetric in the unknown property producing different mobilities during electrophoresis.

Patch et al. (23) have previously shown that the Ad2$^+$ND$_1$ DNA strand annealed to poly (U, G) banding at a lower density in a CsCl equilibrium centrifugation density gradient contained part of the *E* SV40 strand. Therefore, the *l* or light strand of *Eco*RI-Ad2 fragments D and E are covalently linked to the *E* strand of SV40 fragments C and D in the hybrid viruses (30; Flint et al., J. Mol. Biol., in press).

Mapping of Ad2-specific RNA sequences in Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$ early RNA. The separated strands of *Eco*RI cleavage fragments of ^{32}P-labeled Ad2 DNA isolated by gel electrophoresis were annealed at 68 C in 0.125 M phosphate buffer, pH 6.8, and 1 M NaCl for 24 h with different concentrations of RNA isolated from cells at early times after Ad2$^+$ND$_3$ infection. The fraction of ^{32}P-labeled DNA entering hybrid was assayed by chromatography on hydroxylapatite, with the results shown in Fig. 3. The saturation values obtained for the annealing of early Ad2$^+$ND$_3$ RNA to the strands of *Eco*RI cleavage fragments of Ad2 DNA, A, B, C, and F are identical to those observed with early Ad2 RNA (see Table 2): that is, 38 and 52% of the *h* strand of *Eco*RI-Ad2 fragments B and C, respectively, and 12% of the *l* strand of *Eco*RI-Ad2 fragment A annealed to early hybrid RNA. As expected, early Ad2$^+$ND$_3$ cytoplasmic RNA annealed to a very small extent, if at all, with either strand of the *Eco*RI-Ad2 fragment E but surprisingly did form hybrid with 40% of the *l* strand of *Eco*RI-Ad2 fragment D. When early cytoplasmic RNA extracted from Ad2$^+$ND$_1$-infected cells was annealed to the separated strands of *Eco*RI Ad2 DNA fragments, similar saturation values were observed (Table 2). Data for the pertinent *Eco*RI-Ad2 fragments, D and E, are shown in Fig. 3g and h, respectively.

A comparison of early cytoplasmic RNA from Ad2- Ad2$^+$ND$_1$- and Ad2$^+$ND$_3$-infected cells. We have previously shown that early during the lytic cycle of Ad2 infection 40% and 45% of the *l* strand of *Eco*RI fragment D and fragment E, respectively, are expressed as cytoplasmic mRNA (30; Flint et al., J. Mol. Biol., in press). The data shown in Fig. 4a and b confirm those observations with the same DNA samples used above for mapping early hybrid RNA sequences. If the early Ad2 RNA sequences that annealed to the *l* strand of adjacent *Eco*RI-Ad2 fragments D and E are part of one continous chain, then these RNA sequences should not be present in cells infected with either Ad2$^+$ND$_1$ or Ad2$^+$ND$_3$ because the complementary DNA sequences are deleted from the hybrid virus genome (see Fig. 1). As predicted, cells infected with either hybrid virus do not contain early cytoplasmic RNA sequences complementary to the *l* strand of *Eco*RI fragment E. However, contrary to our expectations, the early RNA extracted from cells infected with Ad2$^+$ND$_1$ or Ad2$^+$ND$_3$ formed hybrid with 40% of the sequences of the *l* strand of *Eco*RI-Ad2 fragment D (Fig. 3d and g). We therefore have compared directly the early RNA sequences expressed in Ad2- and hybrid virus-infected cells by summa-

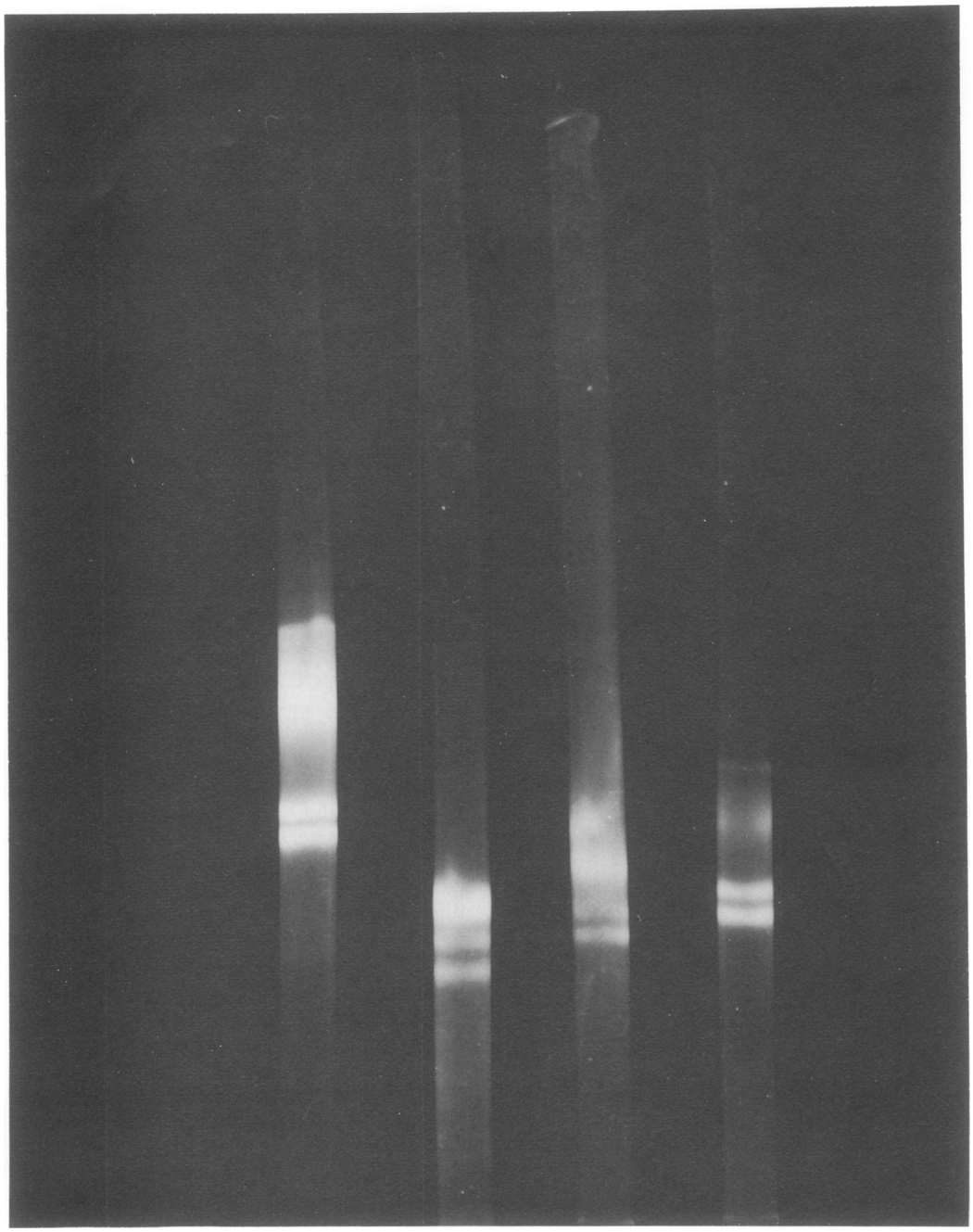

FIG. 2. *Strand separation of the four fragments produced by cleavage of SV40 DNA with EcoRI and Hpa1. Ten micrograms of* ^{32}*P-labeled, covalently closed SV40 DNA was digested with EcoRI and Hpa1 restriction endonuclease. The digestion mixture was layered onto a 2.2% acrylamide, 0.7% agarose gel, and the four fragments were resolved by electrophoresis (31). All gels were stained by immersion in buffer containing 0.5 μg of ethidium bromide per ml and DNA bands were located by excitation of bound dye with ultraviolet light. Fragment DNA was eluted from each band by electrophoresis into dialysis tubing (24) and concentrated by ethanol precipitation. Approximately 0.5 μg of each fragment DNA was dissolved in 10 μl of 0.01 M Tris, 0.001 M EDTA, pH 8.5, and 2 μl of 1.0 M NaOH was added to denature the DNA. After addition of 3 μl of 2% bromophenol blue and 60% sucrose solution, the mixture was layered onto a 1.4% agarose gel (0.8 by 15 cm) and a 2 V/cm electrical potential was applied for 8 h. The agarose gels were stained by immersion in 0.5 μg of ethidium bromide per ml and fluorescence of dye bound to DNA was photographed during excitation with an ultraviolet lamp (31).*

TABLE 1. *Hybridization of SV40 fragment strand with asymmetric SV40 complementary RNA*[a]

SV40 fragment	Relative mobility of strand	% ^{32}P DNA hybrid	SV40 strand assignment
A	Fst	0.4	*L*
A	Slow	89.5	*E*
B	Fast	92.2	*E*
B	Slow	0.0	*L*
C	Fast	0.7	*L*
C	Slow	90.2	*E*
D	Fast	92.3	*E*
D	Slow	0.8	*L*

[a] About 200 counts/min (specific activity 10^6 counts/min per μg) of each ^{32}P-labeled fragment strand and 0.045 μg of SV40 complementary RNA, prepared as previously described (28), were incubated in 0.15 ml of 0.10 M phosphate buffer, pH 6.8, and 1.0 M NaCl for 24 h at 68 C. The fraction of ^{32}P-labeled DNA entering hybrid was assayed by chromatography on hydroxylapatite columns. The amount of SV40 complementary RNA added to the reaction mixture was saturating.

tion experiments using ^{32}P-labeled *l* strand DNA of *Eco*RI-Ad2 fragment D. Figure 4a shows the fraction of ^{32}P-labeled *l* strand of *Eco*RI-Ad2 fragment D entering hybrid when labeled DNA was added to incubation mixtures containing increasing amounts of early Ad2$^+$ND$_1$ RNA either alone or, in a parallel set of reaction mixtures, with a saturating amount of early Ad2 RNA. Whereas only 40 and 45% of the labeled DNA enters hybrid when incubated with excess early Ad2$^+$ND$_1$ and Ad2 RNA, respectively, a mixture of the two RNA samples is complementary to 82 to 84% of the sequences of the *l* strand of *Eco*RI-Ad2 fragment D. Figure 4c shows that the same results are obtained when Ad2$^+$ND$_3$ and Ad2 early RNA are compared in a similar experiment; that is, the saturation values of the independent samples are summed when a mixture of the two RNAs is annealed to the labeled DNA. Therefore, those early Ad2 RNA sequences complementary to 45% of the D fragment of Ad2 DNA are not present in cells infected with either hybrid virus. Instead, a different set of sequences, comprising 40% of the same fragment (approximately 1,000 bases), is expressed as early cytoplasmic RNA. Proof that the same region of the *l* strand of *Eco*RI-Ad2 fragment D is expressed in early RNA from cells infected with either Ad2$^+$ND$_1$ or Ad2$^+$ND$_3$ is provided by the data shown in Fig. 4d. Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$ early RNA were mixed and annealed to ^{32}P-labeled DNA of the *l* strand of *Eco*RI fragment D, when only 40% of the DNA formed hybrid with RNA.

Mapping of SV40-specific RNA sequences in Ad2$^+$ND$_3$ and Ad2$^+$ND$_1$ early RNA. Separated strands of the four fragments of SV40 DNA produced by cleavage with *Eco*RI and *Hpa*1 were annealed under standard conditions to increasing amounts of early Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$ RNA. Early Ad2$^+$ND$_1$ RNA contained RNA sequences complementary to only 46% of the *E* strand of SV40 fragment C (Fig. 5). Ad2$^+$ND$_1$ DNA is not homologous to any DNA sequences of SV40 fragments A or B DNA and the lack of hybrid formation with these fragments during incubation with RNA extracted from Ad2$^+$ND$_1$- and Ad2$^+$ND$_3$-infected cells was therefore expected. The DNA sequences homologous to 35% of SV40 fragment D found in Ad2$^+$ND$_1$ apparently are not expressed as cytoplasmic mRNA before the replication of hybrid virus DNA.

In comparable hybridization experiments between Ad2$^+$ND$_3$ early RNA and separated strands of SV40 fragments C and D no detectable RNA-DNA hybrid formation was observed, although Ad2$^+$ND$_3$ contains DNA sequences homologous to approximately 35 and 5% of the SV40 fragments D and C, respectively. RNA sequences homologous to this 50-base segment of SV40 fragment C probably could not have been detected by these methods (see Discussion). Early Ad2$^+$ND$_3$ RNA, like early Ad2$^+$ND$_1$ RNA, does not appear to contain sequences complementary to SV40 fragment D.

Late cytoplasmic viral RNA sequences in Ad2$^+$ND$_3$- and Ad2$^+$ND$_3$-infected cells. Late cytoplasmic RNA, prepared as described, was annealed to ^{32}P-labeled strands of *Eco*RI-Ad2 fragments. Saturation values similar to those found using late Ad2 RNA (30; Flint and Sharp, Brookhaven Symp., in press) were observed with the separated strands of fragments A, B, C and F (data not shown). However, only 60% of the *l* strand of *Eco*RI-Ad2 fragments D and E annealed to late Ad2$^+$ND$_3$ (Fig. 6a and b). Identical results were obtained with late Ad2$^+$ND$_1$ RNA (data not shown). Because 56 and 61% of the DNA sequences of *Eco*RI-Ad2 fragments D and E, respectively, are present in either hybrid virus DNA, all of the Ad2 DNA sequences in these strands must be expressed as late mRNA during infection by both Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$. Viral RNA complementary to all of the *l* strand of *Eco*RI Ad2 fragments D and E is also found in the cytoplasm late after Ad2 infection.

When late Ad2$^+$ND$_3$ cytoplasmic RNA was annealed to the separated strands of the SV40 C and D fragments, sequences complementary to only 15% of the *E* strand of the SV40 D frag-

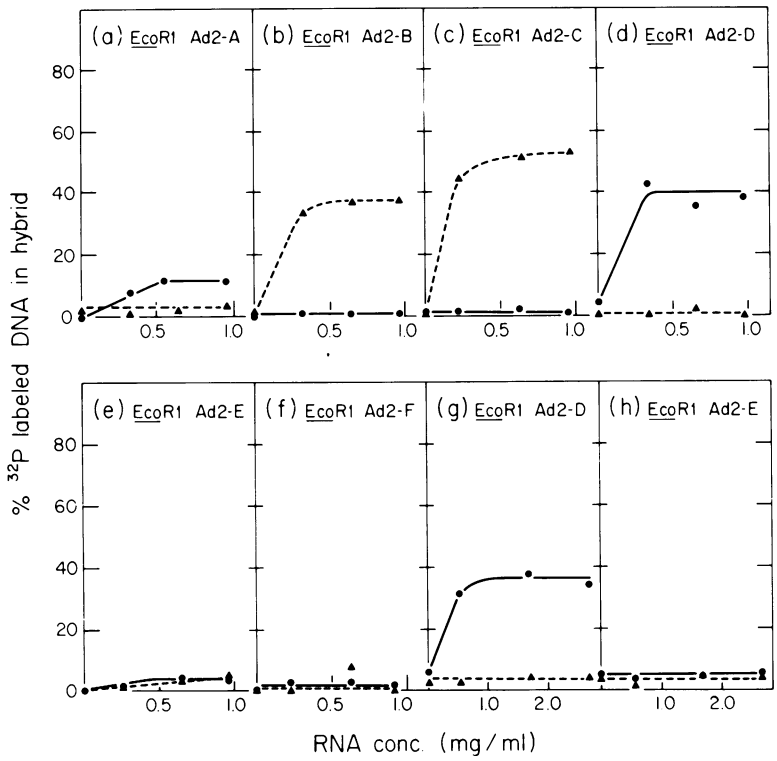

FIG. 3. *Hybridization of early Ad2⁺ND₃ and Ad2⁺ND₁ RNA to separated strands of EcoRI cleavage fragments of* ^{32}*P-labeled Ad2 DNA.* ^{32}*P-labeled separated strands of the EcoRI cleavage fragments of Ad2 DNA were prepared as described previously (30; Flint et al., J. Mol. Biol., in press). About 200 counts/min of* ^{32}*P-labeled DNA was incrubated in 0.15 ml of reaction mixture with increasing amounts of early cytoplasmic RNA prepared as described. The reaction mixture, containing 0.125 M phosphate buffer, pH 6.8, and 1.0 M NaCl, was annealed for 24 h at 68 C and* ^{32}*P-labeled DNA entering hybrid was assayed by chromatography on hydroxylapatite. When the same RNA samples were treated with 0.3 M NaOH for 16 h at 37 C and then incubated with the* ^{32}*P-labeled strands, no detectable hybrid was found. The percentage of the h strand (▲) and l strand (●) of the six EcoRI fragments of Ad2 DNA entering hybrid after incubation with Ad2⁺ND₃ early RNA is shown in panels (a) to (f). Panels (g) and (h) show the hybridization of early Ad2⁺ND₁ RNA to separated strands of EcoRI fragments D and E.*

TABLE 2. *Ad2 RNA sequences complementary to Ad2, Ad2⁺Nl, and Ad2⁺ND₃ early RNA*[a]

Early RNA	% ^{32}P-labeled DNA in hybrid											
	A		B		C		D		E		F	
	h	*l*	*h*	*l*	*h*	*l*	*h*	*l*	*h*		*h*	*l*
Ad2	0	15	30	0	50	0	0	40	0	40	0	0
Ad2⁺ND₁	0	15	35	0	50	0	0	37	0	0	(5)	0
Ad2⁺ND₃	0	12	38	0	52	0	0	40	0	(4)	0	0

[a] The fraction of ^{32}P-labeled strand entering hybrid with an excess of each early cytoplasmic RNA is given for each *Eco*RI fragment and strand combination. The saturation values for Ad2 early have been previously published (Flint et al., J. Mol. Biol., in press); those for Ad2⁺ND₃ RNA are taken from Fig. 3. Saturation values for Ad2⁺ND₁ early RNA are also taken from Fig. 3 (*Eco*RI fragments D and E) and from additional experiments, not shown. Brackets indicate nonsaturating values.

ment were detected (Fig. 6c and d). Ad2⁺ND₃ DNA contains sequences homologous to 35% of SV40 fragment D DNA; thus, only a half of these sequences appear to be expressed as late cytoplasmic RNA. By contrast, late nuclear RNA extracted from the same Ad2⁺ND₃-infected cells contained RNA complementary to all 35% of the SV40 fragment D. Late AD2⁺ND₁

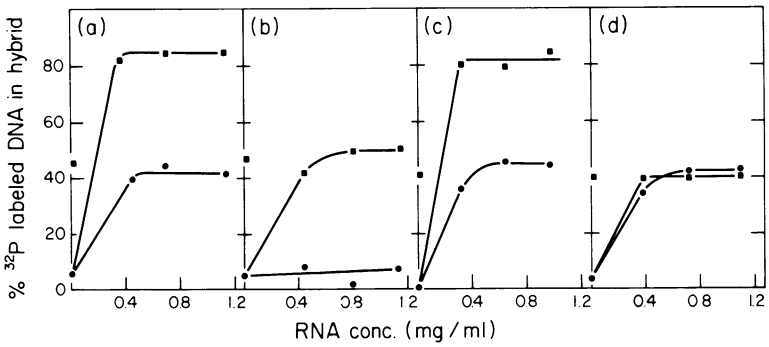

FIG. 4. *Summation experiments performed by annealing mixtures of early Ad2, Ad2⁺ND₁ and Ad2⁺ND₃ RNA to ³²P-labeled strands of EcoRI-Ad2 fragments D and E. Two-hundred counts/min of ³²P-labeled strand was added to each of two reaction mixtures containing the concentration of early hybrid virus RNA shown on the abscissa. To one of the reaction mixtures, saturating amounts of a second early RNA sample was added. The two hybridization mixtures, volume 0.15 ml, were incubated for 24 h in 1.0 M NaCl and 0.125 M phosphate buffer, pH 6.8, at 68 C. The fraction of DNA entering hybrid was assayed by chromatography on hydroxylapatite columns as described. Panel (a) shows annealing of increasing concentrations of early Ad2⁺ND₁ RNA to ³²P-labeled 1 strand of EcoRI-Ad2 fragment D either alone (●) or in the presence of 1.15 mg of early Ad2 cytoplasmic RNA (■). Forty-five percent of EcoRI fragment D 1 strand entered hybrid when incubated with 1.15 mg of early Ad2 cytoplasmic RNA per ml alone ([■] on the ordinate). Panel (b) shows the annealing of ³²P-labeled l strand of EcoRI-Ad2 fragment E to early Ad2⁺ND₁ TNA either alone (●) or in a mixture with 1.15 mg of early Ad2 RNA per ml (■). This concentration of early Ad2 RNA saturates 45% of the ³²P-labeled DNA ([■] on the ordinate). Panel (c) is similar to panel (a) except that early Ad2⁺ND₃ RNA was annealed to the l strand of EcoRI-Ad2 fragment D either alone (●) or in the presence of 0.33 mg/ml of early Ad2 RNA (■). Panel (d) shows the results of annealing increasing amounts of early Ad2⁺ND₁ RNA, either alone (●) or in the presence of 0.49 mg of early Ad2⁺ND₃ RNA per ml (■), to ³²P-labeled l strand of EcoRI-Ad2 fragment D.*

cytoplasmic RNA anneals to 60 and 36% of the *E* strand of SV40 fragments C and D, respectively (Fig. 6g and h). This is equivalent to the fraction of SV40 sequences present in Ad2⁺ND₁ DNA. The concentrations of RNA sequences complementary to these two fragments differ significantly: the *E* strand of SV40 C fragment is saturated at a late, cytoplasmic Ad2⁺ND₁ RNA concentration of 0.1 mg/ml, but more than 0.5 mg of RNA per ml is required to saturate the *E* strand of SV40 fragment D. A similar effect was observed in experiments using late, nuclear Ad2⁺ND₁ RNA, in which 58 and 35% of the *E* strand sequences of SV40 C and D fragments, respectively, were saturated (data not shown). Finally, late nuclear RNA extracted from Ad2⁺ND₁-infected cells appears to contain small amounts of RNA complementary to part of the *L* strand sequences of both SV40 fragment C and fragment D DNA (data not shown). This result is analogous to that found with late Ad2 nuclear RNA and is probably a consequence of the fact that late in adenovirus infection the nuclei contain RNA sequences complementary to all of both strands of the viral genome (25, 30; Flint and Sharp, Brookhaven Symp., in press).

DISCUSSION

The strands of the four fragments of SV40 DNA produced by cleavage with *Eco*RI and *Hpa*1 have been separated by electrophoresis in 1.4% agarose, and used to assay for virus-specific RNA present in cells infected with the Ad2-SV40 hybrid viruses, Ad2⁺ND₁ and Ad2⁺ND₃. The method of analysis, hybridization of a small amount of single-stranded, ³²P-labeled DNA in the presence of a large excess of unlabeled RNA, is subject to several uncertainties. One of these is that a 50-base pair segment (5% of the SV40 C fragment, for example) is probably the shortest length of duplex DNA that will bind to hydroxylapatite columns (36); this could be the limit of accuracy in the determination of the fraction of a DNA strand that is complementary to RNA. However, this inability of small DNA fragments either to anneal to complementary RNA or to bind to hydroxylapatite probably tends to compensate for overestimation of hydrid produced by binding of a large segment of ³²P-labeled DNA to a shorter segment of RNA. As Table 1 shows, only 90% of the ³²P-labeled SV40 fragment strands will chromatograph as hybrid when annealed to vast excesses of SV40 complementary RNA. Although we have previously observed saturation values of close to 100% (30), these probably vary with both the DNA preparation and batch of hydroxylapatite used. Given these uncertainties, it is reassuring that late nuclear Ad2⁺ND₁ RNA is found to be comple-

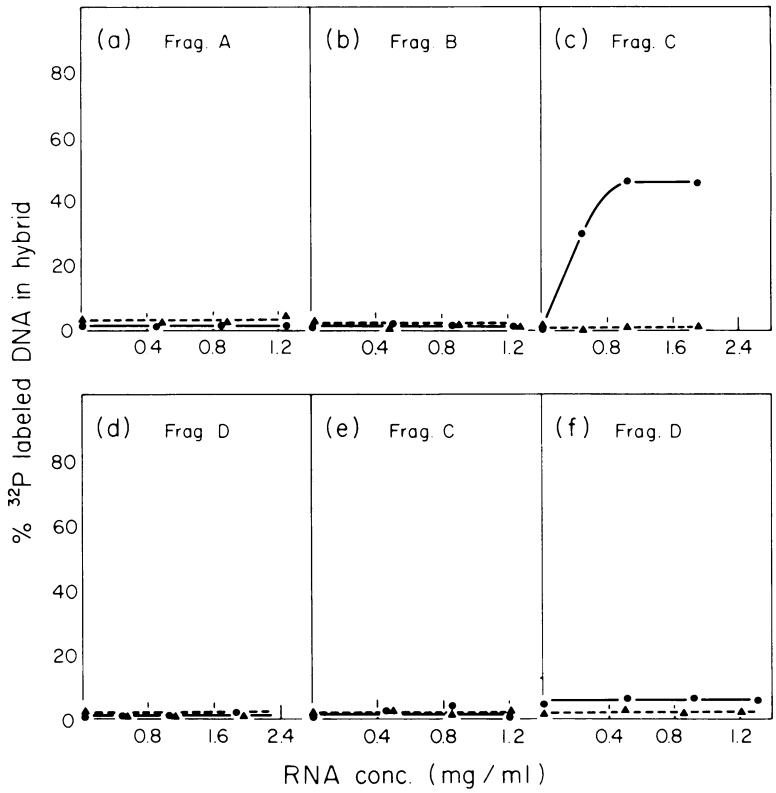

FIG. 5. *Hybridization of ^{32}P-labeled strands of the four SV40 fragment to early Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$ RNA. Two hundred counts/min of ^{32}P-labeled strand of each SV40 fragment was annealed to early hybrid virus cytoplasmic RNA as described in the legend to Fig. 3. The percentage of DNA entering hybrid was determined by assay on hydroxylapatite columns. ^{32}P-labeled strands of the four SV40 fragments were separated by agarose gel electrophoresis as described in the text. Panels a, b, c, and d show the annealing of early Ad2$^+$ND$_1$ RNA to the ^{32}P-labeled E and L strand of the SV40 fragments A, B, C, and D, respectively. Panels e and f show the hybridization of SV40 fragments C and D, respectively, to Ad2$^+$ND$_3$ early RNA. In all cases, the E strand is denoted by (●) and the L strand by (▲).*

mentary to only 60% and 35% of the SV40 C and D fragment, respectively. Ad2$^+$ND$_1$ DNA contains 58% and 35% of the DNA sequences found in SV40 C and D fragments, respectively (see Fig. 1).

Our interpretation of the hybridization results obtained with early Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$ cytoplasmic RNA is summarized in Fig. 7. Three conclusions can be drawn from these data. (i) The early Ad2 mRNA complementary to the *l* strand of both *Eco*RI fragments D and E is not present in cells infected by either hybrid virus. This observation supports our previous suggestion that the Ad2 early mRNA complementary to *Eco*RI-Ad2 fragments D and E constitutes one chain of RNA located at the D-E junction (Flint et al., J. Mol. Biol., in press). Hence, cells infected with either hybrid virus must lack at least one early gene product (presumably nonessential) expressed during Ad2 infection. (ii) The Ad2 sequences at the 5′ end of the novel early RNA species present in cells infected by Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$ are identical and are not present as stable cytoplasmic RNA during the early stage of Ad2 infection. The implications of this observation are discussed below. (iii) The 3′ ends of Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$ early cytoplasmic RNA complementary to SV40 DNA are probably the same and map in the vicinity of 0.18 on the SV40 genome.

Previous estimates, using hybridization of single-stranded DNA in the presence of excess RNA, have placed the 3′ end of early SV40 mRNA between positions 0.24 and 0.21 on the SV40 genome (9, 28). More recently, Khoury et al. (10) have mapped the early 3′-terminus at position 0.18, whereas Dhar et al. (3) have described oligonucleotides in fingerprints of in vivo labeled early SV40 mRNA which map at position 0.17. When the *E* strand of SV40 C fragment was prepared by gel electrophoresis

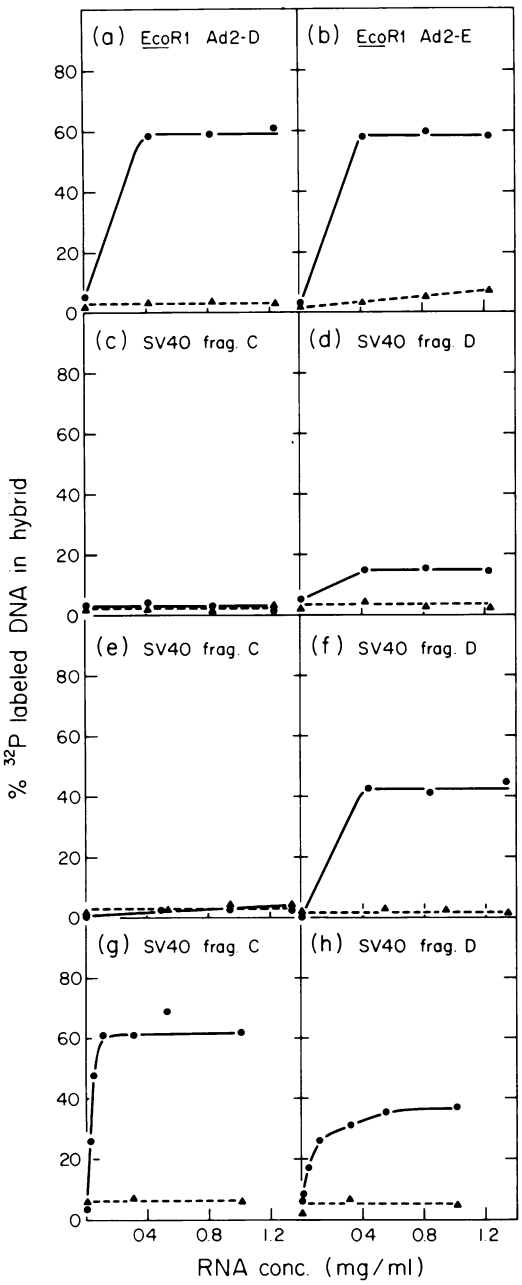

FIG. 6. *Hybridization of late cytoplasmic Ad2⁺ND₁ and Ad2⁺ND₃ RNA to ³²P-labeled strands of EcoRI-Ad2 fragments D and E and SV40 fragment C and D. ³²P-labeled strands and cellular RNA were mixed and incubated as described in the legend to Fig. 3. Late cytoplasmic RNA was extracted from infected cells 18 h after infection and cellular RNA was treated to remove any viral DNA as described. Panels (a) and (b) show the annealing of ³²P-labeled l (●) and h (▲) strands of EcoRI fragments D and E, respectively, to late Ad2⁺ND₃ RNA. Panels (c) and (d) show the annealing of ³²P-labeled SV40 fragments C and D strands, respectively, to late Ad2⁺ND₃ cytoplasmic RNA. Panels (e) and (f) show the annealing of Ad2⁺ND₃ late, nuclear RNA to separated strands of SV40 fragments C and D, respectively. Panels (g) and (h) show the fraction of ³²P-labeled strands of SV40 fragments C and D that enter hybrid during incubation with cytoplasmic late Ad2⁺ND₁ RNA. In all panels (c)-(h), the E strand of a SV40 fragment is denoted by (●) and the L strand by (▲).*

and annealed to a large excess of early SV40 cytoplasmic RNA, about 85% of the ³²P-labeled DNA entered hybrid, whereas 90% of the probe entered hybrid during incubation with a vast excess of asymmetric SV40 complementary RNA (Table 1). This would be consistent with 0.18 being the 3′ terminus of early SV mRNA. Thus, Ad2⁺ND₁, Ad2⁺ND₃, and SV40 early cytoplasmic mRNA probably share the same 3′ termini, which map at about 0.18 on the SV40 genome. A base sequence at this position in the SV40 DNA must specify either a signal to terminate transcription or a site of cleavage during RNA processing.

Sequences complementary to the *E* strand of SV40 fragment C are more abundant that those complementary to the *E* strand of SV40 fragment D in late Ad2⁺ND₁ cytoplasmic RNA. This suggests that an Ad2-SV40 hybrid mRNA with its 3′ terminus at 0.18 on the SV40 genome and a second less abundant RNA species complementary to the SV40 D fragment are present in the cytoplasm of Ad2⁺ND₁-infected cells. Khoury et al. (11) also observed that all the SV40 sequences in Ad2⁺ND₁ are expressed at late times during infection and that some of these SV40-specific sequences were less abundant than others. Although cytoplasmic RNA was used in these experiments, the less abundant RNA sequences could arise through leakage during cell fractionation from the nucleus of untranslatable RNA, as large amounts of additional viral RNA sequences which are not normally transported to the cytoplasm accumulate in the nucleus during the late phase of the lytic cycle (25, 30; Flint and Sharp, Brookhaven Symp., in press).

The Ad2-SV40 hybrid virus, Ad2⁺ND₁, lytically infects human and monkey cells with an equal efficiency, whereas the parental virus Ad2 and sibling Ad2⁺ND₃ form plaques at only 1/1,000 the efficiency on simian cells. U antigen, an early SV40 antigen found in transformed cells, may be responsible for the efficient growth of Ad2⁺ND₁ on monkey cells. The immunological studies of Lewis and Rowe (17) indicate that U antigen is expressed during the early phase of an Ad2⁺ND₁ infection, but the amount of U

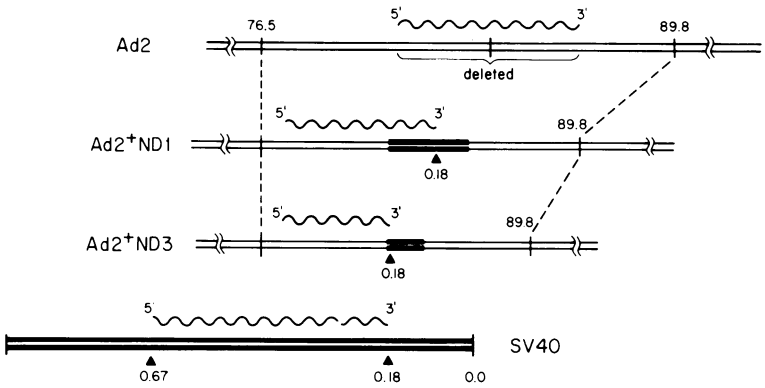

FIG. 7. *Proposed map of early cytoplasmic RNA sequences in Ad2-, Ad2$^+$ND$_1$-, and Ad2$^+$ND$_3$-infected cells. Justification of the Ad2 and SV40 DNA content of the hybrid viruses is given in Fig. 1 and the Introduction. The thick lines represent SV40 DNA sequences and the thin lines, Ad2 DNA. The positions of 0.18 and 0.67 on the SV40 genome are denoted by arrows (▲). The 5' and 3' polarity of the RNA sequences were assigned from the polarity of the complementary Ad2 and SV40 strands (12, 29–31).*

antigen increases markedly once viral DNA replication begins. In agreement, we find that the concentration of Ad2$^+$ND$_1$ late cytoplasmic RNA necessary to half saturate the *E* strand of SV40 fragment C is 1/20th of that of early cellular RNA required (compare Fig. 6g and 5c). If early Ad2$^+$ND$_1$ cytoplasmic RNA codes for U antigen, then the data presented here suggests that the SV40-coded component of this antigen must be smaller than 18,000 daltons (from 0.28 to 0.17 on the SV40 genome) and the total protein encoded by the hybrid Ad2/SV40 mRNA smaller than 50,000 daltons.

One thousand bases of Ad2 DNA at the 3' end of the SV40 DNA segment are expressed as cytoplasmic RNA in early AD2$^+$ND$_1$- and Ad2$^+$ND$_3$-infected cells and are not found early during Ad2 infection. (As saturation values in Fig. 4 extend over only a threefold increase in RNA concentration, any viral RNA sequences present at a fivefold or less abundancy would not be detected.) Hence, a promoter site for transcription must lie to the left of these 1,000 bases of DNA in the hybrid genome. The expression of the additional 1,000 bases of early mRNA may be the result of: (i) the deletion of a segment of Ad2 DNA from both hybrid genomes which contains a sequence that is normally transcribed in Ad2-infected cells and is responsible for degradation of this RNA chain, about 1,000 bases in length; (ii) a mutation in the base sequence of Ad2 DNA at a position about 1,000 bases pairs to the left of the SV40 segment in the hybrid virus DNAs, creating either a new RNA polymerase promoter site or an RNA base sequence that protects the chain in the 3' direction from degradation during processing; or (iii) the interaction of a base sequence in the region of the hybrid mRNA chain coded for by SV40 (i.e., at the 3' end of the RNA chain) with an Ad2-coded RNA sequence at the 5' end of the hybrid virus mRNA to stabilize the intervening RNA chain during processing. The hypothetical interaction between the 3' and 5' ends of the RNA chain could occur either by direct base sequence interaction or by means of a recognition protein binding to the 3' end of the RNA chain.

We cannot distinguish between these models at the present time, partly because the mechanism by which viral mRNAs are synthesized remains obscure, but study of other nondefective Ad2/SV40 hybrid viruses may allow one, or all, of these models to be dismissed. Whereas the DNA of the Ad2 virus isolated from African green monkey cells infected with kidney Ad2^{2+} (pool 2), the seed stock used for isolation of both Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$ (15, 17), is transcribed into early mRNA in a fashion identical to the wild-type Ad2 stock used in experiments, the closely related Ad5 serotype does express the additional 1,000 of the *l* strand of *Eco*RI fragment D as mRNA early during infection (Flint, unpublished observations). This, we feel, argues strongly in favour of model (ii). The inability of certain nonconditional mutants of Ad2$^+$ND$_1$ (4) and of Ad2$^+$ND$_5$ (16) to replicate efficiently in simian cells may therefore be the result of mutation at this site.

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