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# *In vitro* splicing of purified precursor RNAs specified by early region 2 of the adenovirus 2 genome

(RNA processing/MOPC-315 cell extract/RNA·DNA hybridization–selection)

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**ABSTRACT** **Early region 2 of the adenovirus 2 genome (map position 61–75) specifies two poly(A)$^+$ nuclear RNAs (28S and 23S) that appear to be precursors of the 20S cytoplasmic mRNA [Goldenberg, C. J. & Raskas, H. J. (1979) *Cell* 16, 131–138]. Splicing of these nuclear RNAs *in vitro* has been obtained with a whole cell extract prepared from MOPC-315 mouse myeloma cells. The *in vitro* reaction excises sequences from two introns and attaches 5′ sequences to the mRNA body. The splicing reaction was demonstrated by two procedures: (*i*) hybridization of pulse-labeled RNA fractionated by size and (*ii*) annealing of RNAs with radioactive DNA probes followed by nuclease digestion. The first procedure provided evidence that sequences from the large 2300-nucleotide intron (74.6–68.8) were excised and 5′ transcripts were spliced to the mRNA body. Utilizing both S1 and Exo VII nucleases, the second procedure demonstrated excision of sequences from the smaller 720-nucleotide intron (68.5–66.3), the splicing of sequences from the second leader (68.8) to the mRNA body, and the formation of an mRNA body of 1700 nucleotides, the size found *in vivo*. These findings provide evidence that an *in vitro* system that splices viral RNAs to yield products comparable to those found *in vivo* is now available.**

Functional RNA molecules that contain covalently linked transcripts of noncontiguous DNA segments have now been observed in many systems, including a variety of viral and eukaryotic mRNAs and also tRNAs (for reviews see refs. 1–3). Structural studies of several *in vivo* precursor molecules have provided compelling evidence that a splicing mechanism must function *in vivo* to excise intervening RNA sequences (for review see ref. 4). The biochemical steps in splicing of mRNAs and the regulation of this process have not been elucidated until now, primarily because of the absence of an *in vitro* system for analysis.

As a model system for studying processing and maturation of mRNA precursors, we have focused on one of the four regions expressed at early times after adenovirus 2 infection, early region 2 (E2). The polypeptide product of E2 is a 72,000-dalton DNA-binding protein (7–9). E2 cytoplasmic mRNA consists of 50 nucleotides from positions 75–74.6 spliced to 170 nucleotides from 68.8–68.5 and 1700 nucleotides from 66.3–61.6 (see Fig. 1) (10–12). Studies of pulse labeled nuclear RNA revealed three major poly(A)$^+$ species (Fig. 1) (13, 14). The largest (28S) precursor is approximately 4700 nucleotides in length and contains all the intervening sequences. A 23S species appears to be a processing intermediate lacking the sequences between 74.6 and 68.8. The nuclear 20S RNA has a structure indistinguishable from the cytoplasmic mRNA and presumably is the direct precursor of the functional mRNA. To facilitate detailed biochemical studies of RNA processing *in vitro*, we initiated experiments with purified RNAs and whole cell extracts. In this report we describe an extract that splices purified precursor E2 RNAs that have been obtained from cultures early in adenovirus infection.

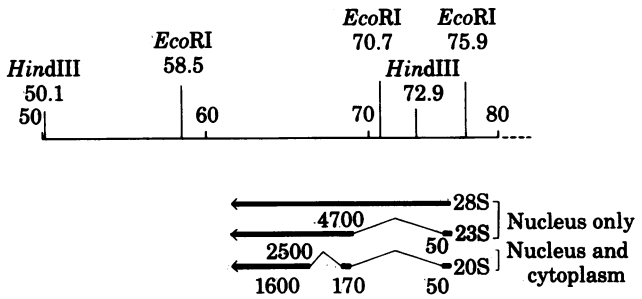


FIG. 1. Pulse-labeled poly(A)$^+$ RNAs transcribed from E2 at early times in infection. Solid bars represent the structures of nuclear and cytoplasmic E2 RNAs (10, 11, 12, 14). The arrows indicate direction of transcription. Caret-shaped symbols indicate sequences covalently joined by splicing. Numbers below the solid bars represent the colinear transcript length in nucleotides. Relevant cleavage sites of restriction endonucleases *Eco*RI and *Hin*dIII have been reported previously (14). The map positions indicate the relative distance of the cleavage site from the left end of the genome.

## MATERIAL AND METHODS

**Cell Culture, Virus Infection, and RNA Preparation.** Maintenance of KB (human) cell suspension cultures, procedures for adenovirus 2 infections, labeling conditions with [$^3$H]uridine, and isolation of nuclear and cytoplasmic RNA were performed as described (13, 14).

**RNA·DNA Hybridizations.** Conditions for digestion with restriction enzymes, purification of DNA fragments, fractionation of RNAs by electrophoresis in polyacrylamide/formamide gels, and RNA·DNA hybridization conditions have been described (13, 14). The S1 and Exo VII nuclease digestion of RNA·DNA hybrids and the analysis of the nuclease-resistant DNA on alkaline 1.4% agarose gels were performed as described by Berk and Sharp (12).

**Preparation of Extracts with Splicing Activity.** Whole cell extracts containing RNA-splicing activities were prepared essentially as described by Manley *et al.* (15). Suspension cultures of MOPC-315 (mouse) cells were grown to a density of approximately $1.5 \times 10^6$ cells per ml and whole cell extracts were prepared as described in the text. After the cell lysate was precipitated with 0.4 M ammonium sulfate, the supernatant was removed and reprecipitated with 55% (wt/vol) ammonium sulfate. The precipitate was collected by centrifugation and stored at −70°C. Extracts have retained full activity for at least 3

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Abbreviation: E2, early region 2.

months. Portions of precipitated extracts were thawed and resuspended in incubation buffer [20 mM Hepes, pH 7.8/100 mM KCl/10 mM $Mg(OAc)_2$, 2 mM dithiothreitol, 10% (vol/vol) glycerol]. This suspension was applied directly to a Bio-Gel P-2 column (Bio-Rad; 4 × 0.7 cm) previously equilibrated with incubation buffer. The material eluting in the void volume of this column (1 ml) was used to direct processing of precursor RNAs *in vitro*. Reactions were in 100 μl of incubation buffer containing 0.5 mM each GTP, CTP, UTP, and ATP, 10 mM creatine phosphate, creatine kinase at 100 μg/ml, and 300 μg of total cytoplasmic RNA purified from uninfected KB cells. Incubations were at 30°C for 15 min. Reactions were terminated by the addition of 2% sodium dodecyl sulfate, 7 M urea, 0.35 M NaCl, 1 mM EDTA, and 10 mM Tris·HCl at pH 8, and the RNAs were purified as described above.

## RESULTS

**Whole Cell Extracts Convert E2 28S RNA to the Size of 20S Cytoplasmic Molecules.** To assay *in vitro* splicing activity we have utilized two procedures. Most experiments utilized a radioactive 28S precursor RNA from region E2 prepared as outlined in the left of Fig. 2: Poly(A)$^+$ nuclear RNA was purified from infected cultures that had been pulse labeled for 30 min with [$^3$H]uridine. E2-specific RNAs were then purified by hybridization with a single-stranded probe specific for region E2

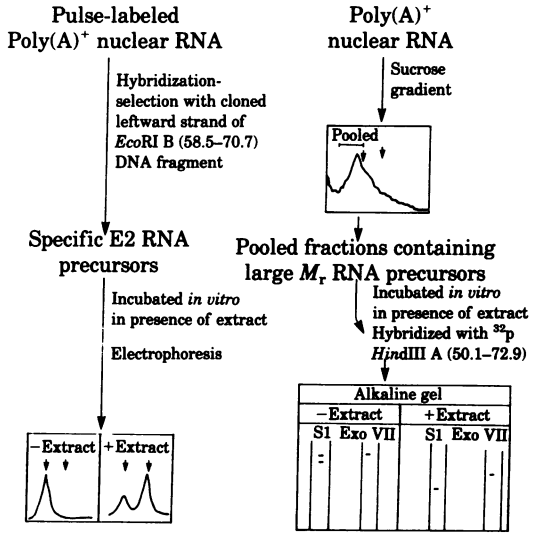


FIG. 2. Two methods used to detect *in vitro* splicing of E2 RNAs. One method relies on analysis of the radioactivity in pulse-labeled RNA; the second method utilizes hybridization with radioactive DNA probes and nuclease digestion to determine RNA structure. The first method is depicted on the left. Infected cultures are labeled with [$^3$H]uridine. Poly(A)$^+$ nuclear RNAs are purified and specific E2 RNAs are prepared by hybridization-selection (14). The DNA probe for the hybridization is a single-stranded M13 phage that contains the leftward strand of adenovirus 2 *Eco*RI B (58.5–70.7) (unpublished data). The selected RNA is then incubated *in vitro* as described in *Materials and Methods*. After incubation, the RNA is purified, fractionated by oligo(dT)-cellulose chromatography, and subjected to electrophoresis on formamide/polyacrylamide gels (14). The size distribution of the fractionated RNA and the RNA sequences in each size class provide evidence related to splicing. In the second procedure (right), high molecular weight poly(A)$^+$ RNA precursors are purified by fractionation on a 15–30% sucrose gradient. Ribosomal RNA markers ($^{14}$C) are added to the sample prior to centrifugation. Fractions containing RNA 28 S in size and larger are pooled and incubated *in vitro* with the processing extract. The processed RNAs are then purified and hybridized to $^{32}$P-labeled *Hin*dIII A fragments (50.1–72.9). The RNA·DNA hybrids are digested with S1 or Exo VII nuclease, and the resultant products are analyzed on alkaline agarose gels.

(*Eco*RI B fragment, coordinates 58.5–70.7). A typical profile of E2 precursor RNA prepared by this procedure is shown in Fig. 3*A*. The [$^3$H]RNA preparation is at least 80% 28 S in size. The poly(A)$^+$ E2 precursor RNAs could then be incubated *in vitro* in the presence of various extracts. Two criteria could be used to determine if splicing occurred: changes in size distribution and accompanying changes in sequence content and structure.

We chose to assay splicing activity in soluble whole-cell extracts prepared essentially as described by Manley *et al.* (15). Extracts were prepared from MOPC-315 cells, a transplantable plasmacytoma originally induced with mineral oil in a BALB/c-2 mouse (16). Cells were harvested and subjected to hypotonic shock, Dounce homogenization, and a subsequent hypertonic shock. The resulting crude extract was made 0.4 M in ammonium sulfate, and the supernatant from this precipitation was adjusted to 55% ammonium sulfate. After precipitation the extract was solubilized and applied to a Bio-Gel P-2 desalting column. Material eluting in the void volume was assayed for processing activity. The total protein in whole cell extracts obtained from 1 × $10^9$ MOPC-315 cells was about 40–50 mg.

The results of *in vitro* incubations of 28S E2 RNA with the MOPC-315 extract are shown in Fig. 3. As a control, precursor RNA was incubated for 15 min at 30°C in incubation buffer without extract. After incubation the RNA was selected by oligo(dT)-cellulose chromatography and fractionated by electrophoresis in a formamide/polyacrylamide gel. The major 28S species remained intact as judged by its size distribution (Fig. 3*A*); on the basis of the gel analysis the 28S preparation contained trace amounts of RNA migrating as 20S molecules, an amount estimated at 90 cpm. When an equal portion of RNA precursors was incubated in the presence of a whole cell MOPC-315 extract, a new peak of 375 cpm of 20S RNA was generated (Fig. 3*B*). Approximately 80% of the 28S RNA, on a molar basis, was converted to a 20S species. In three independent experiments not shown here, 420, 285, and 390 cpm of 20S [$^3$H]RNA were produced from 28S preparations containing 130, 50, and 75 cpm migrating as 20S molecules. The 20S species produced *in vitro* has the same migration rate as the cytoplasmic E2 mRNA (see ref. 14). The *in vitro* size change occurred rapidly, being completed in 15 min. The resulting 20S RNAs were stable

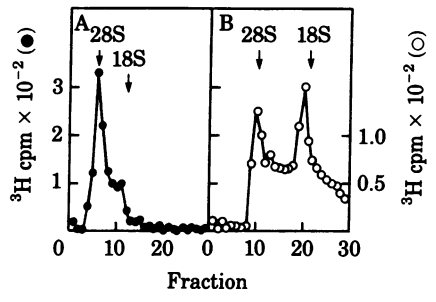


FIG. 3. *In vitro* processing of E2 RNA precursors in presence of MOPC-315 extract. Suspension cultures of KB cells were labeled with [$^3$H]uridine for 30 min (200 μCi/ml, 5 × $10^6$ cells per ml; 1 Ci = 3.7 × $10^{10}$ becquerels) beginning 5.5 hr after infection. Infections were performed in the presence of cytosine arabinonucleoside to block DNA replication (see ref. 14). Poly(A)$^+$ nuclear RNA was purified and annealed with the E2 DNA probe as described in the legend to Fig. 2. Specific E2 RNAs from 1 × $10^9$ KB cells (approximately 3 × $10^4$ cpm) were incubated *in vitro* with the MOPC-315 cell extract (0.6 mg/ml) as described in Fig. 2. Processed RNAs were selected by oligo(dT)-cellulose and subjected to electrophoresis in formamide gels. Gel fractions were solubilized in ammonium hydroxide and radioactivity was measured directly (14). (*A*) RNAs incubated in the absence of extract. (*B*) RNAs incubated in the presence of extract. For each sample 1.2 × $10^4$ cpm was loaded on the gel; the positions of 28S and 18S markers are represented by arrows.

after 1-hr incubation (data not shown), indicating that no extensive degradation occurs after processing is completed.

**Concentration Dependence of Processing Activities Contained in MOPC-315 Extracts.** As a first step in characterizing the putative processing activity in MOPC-315 extracts, we determined the optimal extract concentration for maximal size conversion from 28S to smaller poly(A)$^+$ molecules (Fig. 4). Increasing concentrations of extract were added to reaction mixtures containing equal amounts of E2 RNA precursors. In the absence of added extract the RNA remained intact. Some change in size distribution was observed at low extract concentrations (0.2 mg/ml) (data not shown), and the greatest effect was attained when the protein concentration of the extract was about 0.6 mg/ml. When the protein concentration in the reaction mixture was increased from 0.7 to 1.3 mg/ml, the conversion of E2 RNA precursors dramatically decreased, suggesting the presence of inhibitors in the extract. Therefore optimal processing can be obtained only within a narrow range of protein concentrations when a crude whole cell extract is the source of the processing activity.

Because the processing reaction appeared to be inhibited at protein concentrations greater than 1 mg/ml (see Fig. 4), we performed a further fractionation in an attempt to separate the processing activity from a possible inhibitor. After being desalted by a Bio-Gel P-2 column, the extract, in incubation buffer, was applied directly onto an anion-exchange column, DEAE-cellulose. Only a third of the RNA was converted to smaller RNAs by the fraction that did not bind to DEAE-cellulose in the presence of incubation buffer (data not shown; the profile was similar to the 1.3 mg/ml profile in Fig. 4). With the protein fraction that was eluted with incubation buffer containing 0.3 M ammonium sulfate, approximately two-thirds of the RNA was converted. When the two column fractions were added together the incubation resulted in a profile very similar to that obtained with the fraction that did not bind to DEAE-cellulose; only a third of the RNA was converted to the 20 S size. This result strongly suggests that the inhibition of RNA processing at high concentrations of MOPC-315 extract is caused by components that do not bind to DEAE-cellulose.

**The 20S E2 RNAs Produced by *in Vitro* Processing Are Spliced to 5′ Sequences from the Leader Region and Lack Sequences from the 74.6–68.8 Intron.** In the experiments described above, incubation with MOPC-315 extracts resulted in a size change for the nuclear E2 RNAs: a decrease in 28S molecules and an appearance of 20S RNA having the same migration rate as the cytoplasmic mRNA. This 20S molecule produced *in vitro* is likely to contain the original poly(A) stretch on the 3′ end of the molecule, because the processed molecules still bind to oligo(dT)-cellulose. Thus the decrease in size from a molecule of 4700 nucleotides to one of less than 2000 probably was accomplished by excision of internal sequences, by removal of 5′-terminal nucleotides, or by both processes.

The mature 20S RNA has two blocks of intervening sequences (see Fig. 1). To determine the fate of the sequences in the large intron (74.6–68.8) during *in vitro* incubation, the following experiment was performed: Nuclear E2-specific RNA was purified (Fig. 5*A*) and then incubated with MOPC-315 extract, yielding reduced amounts of 28S RNA and a new peak of 20S molecules (Fig. 5*B*). The RNAs processed *in vitro* were then rehybridized to two fragments, one (72.9–75.9) specific for the 5′ leader sequence and a second (70.7–72.9) specific for sequences within the large intron. The 28S RNA that remained after *in vitro* incubation hybridized efficiently to both probes (Fig. 5*C* and *D*), as expected for a molecule transcribed continuously for 4700 nucleotides. In contrast, the 20S RNA produced *in vitro* hybridized only to the 72.9–75.9 fragment, the fragment containing the 5′ nucleotide leader sequences of the cytoplasmic mRNA. The amount of 20S RNA hybridizing to the 72.9–75.9 DNA was 31% of the amount of 28S RNA hybridizing

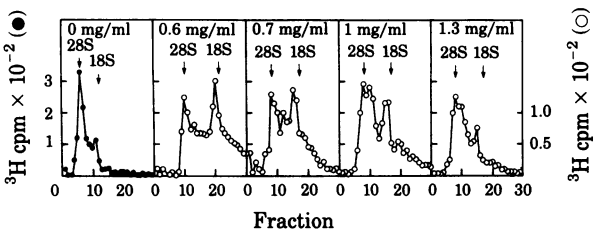


FIG. 4. Effect of different concentrations of MOPC-315 whole cell extracts on the processing of E2 RNA precursors. Poly(A)$^+$ nuclear E2 RNAs were purified by hybridization selection as described in Figs. 2 and 3. The RNA was incubated in the presence of the indicated concentrations of MOPC-315 whole cell extracts, as described in Fig. 2. For each reaction 3 × 10$^4$ cpm of specific precursors was used. After the reactions, RNAs were purified by oligo(dT)-cellulose chromatography and analyzed by electrophoresis in formamide gels. Gel fractions were solubilized in ammonium hydroxide and radioactivity was measured directly (14). Approximately 1 × 10$^4$ cpm was loaded on each gel.

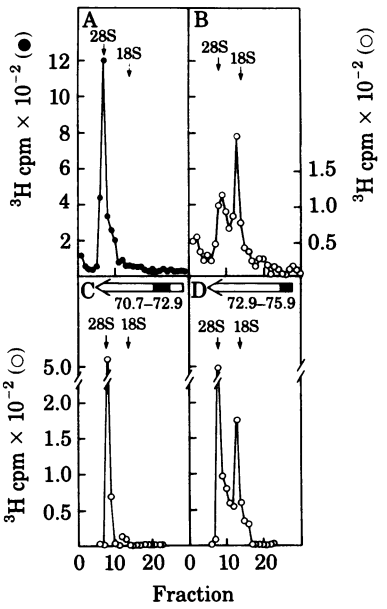


FIG. 5. Splicing of leader sequences from map position 75 to the body of the 20S E2 RNA produced by *in vitro* processing. Specific E2 [$^3$H]RNA precursors were purified from 3 × 10$^9$ infected KB cells (4.5 × 10$^5$ cpm) as described for Figs. 2 and 3. The E2 RNAs were incubated *in vitro* in the presence of whole cell MOPC-315 extract (0.6 mg/ml) as described in Fig. 2. This was followed by purification of the poly(A)$^+$ RNAs on oligo(dT)-cellulose and electrophoresis in formamide gels. After electrophoresis the RNAs in individual gel fractions were annealed with various viral DNA fragments. (*A*) Size distribution of purified E2 RNAs before *in vitro* incubation. Approximately 1.8 × 10$^4$ cpm was applied to the gel. Gel slices were solubilized in ammonium hydroxide and their radioactivities were measured. (*B*) Size distribution of E2 RNAs after *in vitro* incubation. Approximately 70 × 10$^3$ cpm was applied to the gel. After electrophoresis the RNA was eluted from each gel slice in 300 μl of 0.9 M NaCl/0.09 M sodium citrate at 66°C for 48 hr; the radioactivities of 6-μl fractions (1/50th of the total eluted volume) were measured directly. (*C* and *D*) Rehybridizations of the E2 processed RNAs shown in *B*. Eluted RNAs (in 300 μl) were annealed simultaneously with 5 μg equivalents (the amount of the DNA fragment that would be derived from 5 μg of whole genome DNA) of the indicated DNA fragments. After hybridizations the filters were treated with RNase (13, 14). The arrows inserted at the top of *C* and *D* indicate the structure of the 28S precursor RNA shown in *A*. The solid bars indicate the positions of the DNA probes used for the hybridization, 70.7–72.9 for *C* and 72.9–75.9 for *D*.

to the same fragment (compare *A* and *D* of Fig. 5). This is the expected result if the only 72.9–75.9 transcripts in the 20S RNA are the leader sequences present on the 20S RNA spliced *in vivo*. The 20S RNA did not hybridize to the 70.7–72.9 probe, which specifies only intron transcripts. This is the same result obtained when cytoplasmic E2 20S RNA is analyzed (14) and allows several conclusions: (*i*) at least some of the 74.6–68.8 intron sequences are missing in the 20S RNA produced *in vitro*; (*ii*) the 20S RNA contains sequences from the DNA fragment that encodes the 50-nucleotide leader tract present on the functional mRNA; (*iii*) these 5′ sequences must be covalently linked to transcripts that form the body of the mRNA *in vivo*.

**The 20S E2 RNAs Produced by *in Vitro* Processing Lack Sequences from the 68.8–66.3 Intron.** To analyze the 20S RNAs produced *in vitro* for the second splice junction, 68.8–66.3, the structure of steady-state RNA populations was determined by hybridization with [$^{32}$P]DNA followed by nuclease digestion (see right of Fig 2). This assay for processing activity required a preparation of nuclear RNA precursor that was essentially free of 20S cytoplasmic RNA. To obtain the necessary substrate, poly(A)$^+$ nuclear RNA was fractionated on a 15–30% sucrose gradient. RNAs 28 S and larger in size were pooled. To determine the size of the pooled RNAs, aliquots were fractionated in glyoxal gels, transferred to activated cellulose paper, and hybridized to 3′-end-labeled single-stranded DNA fragments from E2 (Fig. 6). The specific activity of the probe was $10^8$ cpm/$\mu$g, and $1 \times 10^6$ cpm was used for each hybridization. The total poly(A)$^+$ nuclear preparation was predominantly 20S RNA, with detectable amounts of slower migrating species (Fig. 6, lane 3). The pooled high molecular weight RNA contained a major 28S band. Most important, the pooled high molecular weight RNA showed no evidence of 20S E2 RNA, the cytoplasmic species.

To characterize further the high molecular weight RNAs obtained by sucrose gradient fractionation, the preparation was annealed with [$^{32}$P]DNA and subsequently digested with either S1 or Exo VII nuclease (12). The S1 digestion degrades all single-stranded regions in RNA·DNA hybrids. Exo VII digests free single-stranded ends of DNA molecules but not the internal single-stranded regions produced by DNA hybridization with RNA molecules having deleted intron sequences. After nuclease digestion the hybrids were treated with alkali and analyzed on agarose gels. The [$^{32}$P]DNA used for the annealing reactions was *Hin*dIII A, coordinates 50.1–72.9. The results expected if splicing occurs *in vitro* can be predicted from Figure 1. The intact 28S RNA should yield an S1- and Exo VII-nuclease resistant band of 3900 nucleotides stretching from position 72.9 to the 3′ termini of the poly(A)$^+$ 28S molecules. The expected result was obtained with both nucleases (Fig. 7, lanes 2 and 3). To conserve the precursor sample, lower amounts of RNA were used for the sample treated with Exo VII; accordingly DNA·DNA hybridization was observed in addition to the expected 3900 nucleotide band. The absence of 20S cytoplasmic RNA from the preparations, as shown in the blot hybridization analyses (Fig. 6), was confirmed by the Exo VII digestion (Fig. 7, lane 3); the preparations did not yield the 2500-nucleotide DNA that would be expected from *Hin*dIII A hybridization with E2 cytoplasmic RNA (see Fig. 1). The S1 nuclease digestion products of the 28S RNA preparations did include a small percent of a 1700-nucleotide DNA. This 1700-nucleotide DNA is possibly derived from an RNA that has been visualized in the electron micro-

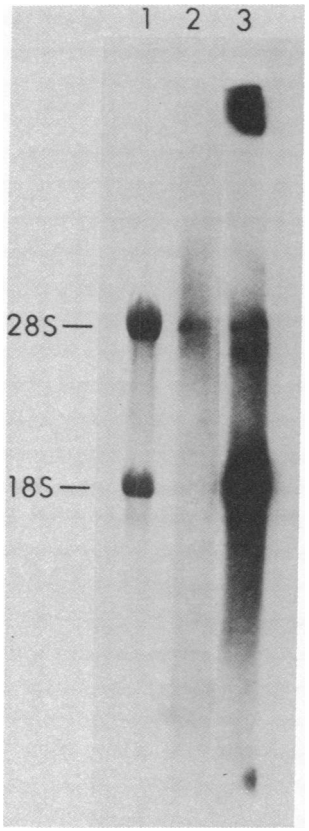


FIG. 6. Blot hybridization of E2 poly(A)$^+$ nuclear RNAs after 15–30% sucrose gradient fractionation. Fractions containing RNA 28 S in size and larger were pooled, subjected to electrophoresis on glyoxal/1.1% agarose gels, and transferred to diazobenzyloxymethylcellulose paper. Single-stranded M13 phage that contains the leftward strand of adenovirus 2 *Eco*RI B (58.5–70.7) DNA was digested with *Hae* III and 3′-end-labeled with $^{32}$P. Hybridizations were performed as described by Alwine *et al.* (17). Approximately $1 \times 10^6$ cpm was used per hybridization ($1 \times 10^8$ cpm/$\mu$g). After hybridization the strips were exposed to x-ray film with an intensifying screen for 72 hr at −70°C. Lane 1, ribosomal ($^{14}$C) markers ($1 \times 10^4$ cpm) were subjected to electrophoresis, transferred to diazobenzyloxymethylcellulose paper, and exposed to x-ray films. Lane 2, high molecular weight poly(A)$^+$ nuclear RNAs pooled from a sucrose gradient (RNA from approximately $5 \times 10^6$ cells). Lane 3, total poly(A)$^+$ nuclear RNA (RNA from approximately $5 \times 10^6$ cells).

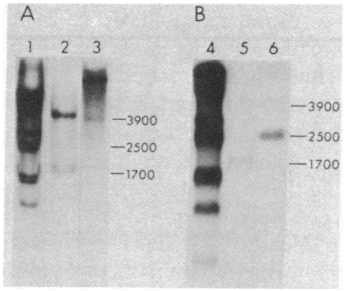


FIG. 7. Evidence from nuclease digestion of RNA·DNA hybrids that sequences from position 75 are spliced to the body of the E2 RNA during *in vitro* processing. High molecular weight poly(A)$^+$ nuclear RNAs were purified by sucrose gradient centrifugation and incubated *in vitro* in the presence of MOPC-315 cell extract as described in the legend to Fig. 2. After incubation the processed RNAs were selected by oligo(dT)-cellulose. RNA samples were annealed with $^{32}$P-labeled *Hin*dIII A (50.1–72.9) DNA fragment, and digested with S1 or Exo VII nuclease, and the nuclease-resistant DNA was analyzed on alkaline agarose gels (12). (*A*) Nuclease analysis of high molecular weight RNAs prior to *in vitro* incubations. Lane 1, adenovirus 2 [$^{32}$P]DNA digested with *Sma* I. Lane 2, S1 nuclease digestion products (RNA from $2 \times 10^8$ cells). Lane 3, Exo VII digestion products (RNA from $3 \times 10^7$ cells). (*B*) Nuclease analysis of poly(A)$^+$ RNAs after *in vitro* incubation in presence of extract. Lane 4, adenovirus 2 [$^{32}$P]DNA digested with *Sma* I. Lane 5, S1 nuclease digestion products (RNA from $3 \times 10^8$ cells). Lane 6, Exo VII digestion products (RNA from $3 \times 10^8$ cells).

scope (18). This RNA contains 2350 nucleotides from positions 75–68.5 spliced to 1700 nucleotides from 66.3–61.6—i.e., a molecule in which only the smaller intron has been excised (see Fig. 1). Alternatively, this DNA might be derived from a rightward strand transcript and therefore be unrelated to E2 expression; such transcripts from the r-strand were detected early in infection (14). On the basis of the absence of the 20S species from the pooled high molecular weight RNA (Figs. 6 and 7), we proceeded to utilize these preparations in *in vitro* processing studies.

The nonradioactive high molecular weight RNAs were then incubated in the presence of the whole cell MOPC-315 extract. After the reaction, RNAs were annealed with $^{32}$P-labeled *Hin*dIII A fragment. After S1 nuclease digestion of the hybrids, the major detectable band was 1700 nucleotides in length (Fig. 6, lane 5), the size expected from 20S E2 mRNA. The Exo VII-resistant DNA was primarily a single band of 2500 nucleotides (Fig. 6, lane 6). This combination of S1 and Exo VII nuclease products can be obtained only if the major RNA product has the second splice junction present in the cytoplasmic 20S mRNA. Identical S1 and Exo VII nuclease patterns were obtained in control experiments with cytoplasmic RNA prepared from infected cultures (data not shown). Accordingly this experiment leads to the following conclusions: (*i*) the *in vitro* reaction excises sequences from the 68.5–66.3 intron; (*ii*) leader sequences from position 68.8 are spliced to the body of the functional mRNA; (*iii*) the 1700-nucleotide RNA body produced by the splicing reaction is identical to that of the functional mRNA (10–12) within 100 nucleotides, the uncertainty of the assay.

## DISCUSSION

A crude extract having the ability to splice purified mRNA precursors *in vitro* has now been developed. The description of this extract is only the initial step in characterizing a system that should help to unravel the biochemical steps in mRNA processing. We have previously investigated the feasibility of studying adenovirus RNA processing *in vitro* (19–21). Isolated nuclei were incubated *in vitro*, and the size distribution of viral transcripts was determined. We demonstrated the quantitative conversion of 28S E2 RNA to 20S RNA that contained spliced leader sequences. A similar system was described by Blanchard *et al.* (22). In spite of these successes, the complexity of the assay—i.e., the requirements for freshly labeled nuclei and for a cytosol extract, and the limited ability to fractionate the components—made further studies difficult. The MOPC-315 extract described here should overcome these earlier limitations.

It is interesting that our initial report of splicing activity was obtained with a concentrated crude cell extract. In that respect this finding parallels the first successes with several other *in vitro* systems—i.e., the initiation of eukaryotic protein synthesis (23) and transcription with RNA polymerases II and III (15, 24–26). Our first attempts at RNA processing with whole cell extracts utilized cultured KB cells, a source that yielded protein concentrations about 1/5th to 1/4th of the MOPC-315 extract. With KB extracts we obtained processing activity, but we were not able to preserve this activity during storage, either in liquid nitrogen or after reprecipitation with ammonium sulfate and storage at −70°C (data not shown).

Although the system we have characterized clearly possesses splicing activity for adenovirus E2 RNA, it is important to define the extent of our conclusions from these data: We have demonstrated the removal of sequences from both E2 introns, the splicing of sequences from both leaders to the body of the RNA, and the formation of an RNA with a 1700-nucleotide body, comparable to that found *in vivo*. From these experiments we cannot conclude that the splicing has the same precision as occurs *in vivo*. Whether the identical sequence excision and leader sequence attachment occurs *in vitro* as compared to *in vivo* is unknown at present. Moreover, we do not know the precise biochemical requirements for this processing activity, if this processing activity is generally functional, if it can act on other viral and cellular precursor RNAs, or if it is similar to the activity that can splice yeast tRNA precursors (27–29). Furthermore, the use of a mouse cell extract to splice a viral RNA that is normally produced in infected human cells implies that the crude extract does not have species specificity.

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