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In vitro RNA–RNA splicing in adenovirus 2 mRNA formation

(mRNA processing)

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ABSTRACT **"Splicing" of the precursor to an adenovirus mRNA was accomplished in isolated cell-free extracts. Nuclei were prepared from hypotonically swollen cells that had been labeled with [^{3}H]uridine for 10 min prior to nuclear isolation. Addition of a "cytoplasmic" fraction was required for the splicing to occur. The nuclear precursor, a poly(A)-terminated RNA molecule approximately 5 kilobases long, contained sequences complementary to the *58.5–75.9* region of the adenovirus 2 genome, including those sequences spliced out of the mature mRNA molecule. The *in vitro* spliced product was a poly(A)-terminated RNA molecule identical in size to the cytoplasmic 72,000 M_r protein mRNA (2 kilobases long) in which the sequences encoded in the *70.7–75.9* region of the viral genome were spliced to those encoded at *58.7–65.6*, with the sequences encoded at *66.1–70.7* deleted.**

Both the mRNAs that are produced from viral DNA in the cell nucleus (1–7) and many cellular mRNAs (8–11) contain sequences from noncontiguous regions of DNA. The only detectable nuclear primary RNA transcripts from adenovirus 2 (Ad-2) DNA (12, 13), as well as the genes for hemoglobin (14–16) and immunoglobulin light chains (17), also contain mRNA-specific sequences in a noncontiguous form. Therefore, it has been suggested that RNA–RNA "splicing" is necessary during mRNA biogenesis (1, 3). Recently, a precursor to yeast tRNA was described that contains ≈15 nucleotides intervening between two ≈40-nucleotide regions that constitute the mature tRNA (18–20). Cell-free extracts are capable of removing these intervening sequences and splicing the remaining segments together (20). In this report we describe the *in vitro* conversion of an Ad-2 nuclear mRNA precursor to a molecule with the size and sequence composition of the mature mRNA. We conclude that RNA–RNA splicing of parts of a nuclear precursor RNA molecule is probably a general mechanism in mRNA manufacture in higher cells.

MATERIALS AND METHODS

The growth of HeLa cells, procedures for Ad-2 infection, and virus and virus DNA purification have all been described (21, 22), as have techniques for extraction of RNA from isolated nuclei, sucrose sedimentation analysis (22), and gel analysis of the Ad-2-specific RNA (23).

Preparation of DNA fragments by restriction endonuclease digestion has been described (24). Enzymes used for this work were purchased from New England BioLabs.

The preparation of isolated nuclei basically followed the technique described earlier for *in vitro* RNA studies (22). Details of the nuclear preparation and incubation are in the figure legends.

Hybridization at 65°C in 0.3 M NaCl/0.01 M EDTA/0.2 % sodium dodecyl sulfate/0.01 M {[tris(hydroxymethyl)methyl]-amino}ethanesulfonic acid (Tes) and elution of hybridized RNA without nuclease or measurement of RNase-resistant hybrids have been described (25).

RESULTS

Early in Ad-2 infection at least five individual transcription units, scattered on both strands of the Ad-2 DNA, are active in producing mRNA (12, 26–28). The average size of these transcription units is approximately 2–8 kilobases (kb), considerably smaller than the major late Ad-2 transcription unit (12, 13). However, like the late Ad-2 mRNAs, virtually all of the early Ad-2 mRNAs are composed of noncontiguously encoded sequences, some of which are at least 5 kb apart in the Ad-2 genome (29, 30). Previously, Craig and Raskas (31) demonstrated that poly(A)-terminated nuclear RNA larger than mRNA was complementary to the DNA of two of these transcriptional units. We chose to investigate further one of these, that which produces the mRNA for the 72,000 M_r DNA binding protein (26, 29, 30, 32). Fig. 1 shows a map of the sequences that are present in the mRNA for this protein, from ≈60 to 75*, reading leftward in the viral genome. This mRNA will be referred to as 72,000 M_r, mRNA. We had earlier identified a transcriptional start site for this RNA between *70.7* and *75.9*, with transcription proceeding leftward into the *58.5–70.7* region (12). More recent experiments suggest that transcription proceeds all the way to approximately 55 or a total distance of approximately 7 kb from the beginning to the end of this transcriptional unit (J.-M. Blanchard, J. R. Nevins, and J. E. Darnell, unpublished results). The 3′ end of the 72,000 M_r mRNA has been positioned by electron microscopy and gel analysis of RNA–DNA hybrids at about *62* on the physical map (29, 30). Therefore, the nuclear poly(A)-terminated molecule (approximately 28S or 5 kb) identified by Craig and Raskas (31) could extend from about *75* to *62*. We reasoned that this ≈5-kb RNA molecule (listed as 4900 nucleotides in Fig. 2) might be the only (or the predominant) labeled poly(A)-terminated [poly(A)$^+$] RNA complementary to the *75–62* region of the viral genome if a short enough labeling time were used. After a 10-min [^{3}H]uridine label time, more than 95% of the total cell poly(A)$^+$-labeled RNA complementary to *58.5–70.7* was indeed in the nucleus in the form of ≈5-kb RNA molecules (Fig. 2). After a 90-min labeling time, more than 90% of the RNA complementary to *58.5–70.7* was in the cytoplasm and had a size, approximately 20 S or 2 kb, equal to that reported for the mRNA of the 72,000 M_r protein. The fraction of radioactivity in the nuclear ≈5-kb RNA that hybridized to *70.7–75.9* was about 40% that which hybridized to *58.5–70.7*, consistent with a molecule whose 5′ terminus was approximately at 75 and 3′ terminus at *62* (Fig. 3).

The presence of a single major poly(A)-terminated nuclear

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Abbreviations: Ad-2, adenovirus 2; kb, kilobase.

* The adenovirus genome is divided into 100 units (350 base pairs per unit), with 0 to the left end of the rightward transcribed strand (25). Two coordinates, italic (e.g., *58.5–70.7*), are used to describe any region and one coordinate indicates a specific site in the DNA.

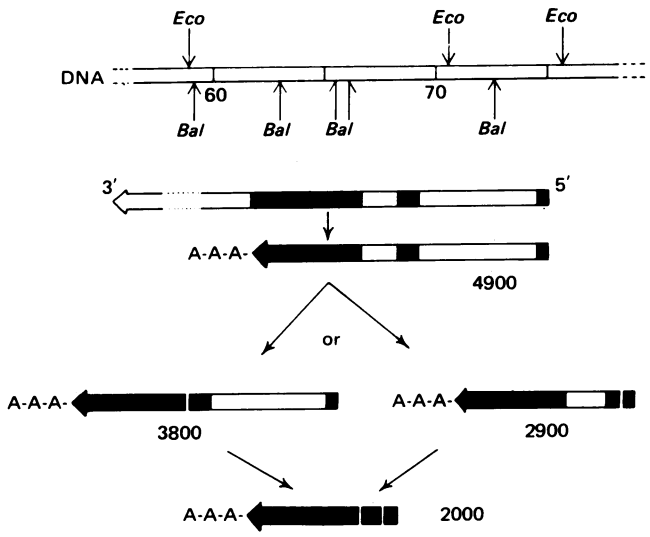

FIG. 1. The transcriptional unit for the mRNA of the Ad-2 72,000 M_r protein. The details diagrammed here come from refs. 12, 29, and 30. The start site for RNA synthesis in this leftward-reading transcript (12) as well as the map site for the 5′ end of the 72,000 M_r mRNA (29, 30) is near *75*. Synthesis proceeds past the site where poly(A) is added (≈*62*) at least to ≈*55* (unpublished data). The sequences contained within the spliced mRNA are shown in black. The total length of the mRNA, including poly(A) (indicated by A-A-A-), is about 2000 nucleotides. Two possible intermediates (2900 and 3800 nucleotides long) in the splicing of the ≈4900 nucleotide poly(A)$^+$ molecule are shown. The sites for restriction endonucleases *Eco*RI and *Bal* I are shown, and the coordinates come from the data distributed at the 1976 Adenovirus Workshop at Cold Spring Harbor.

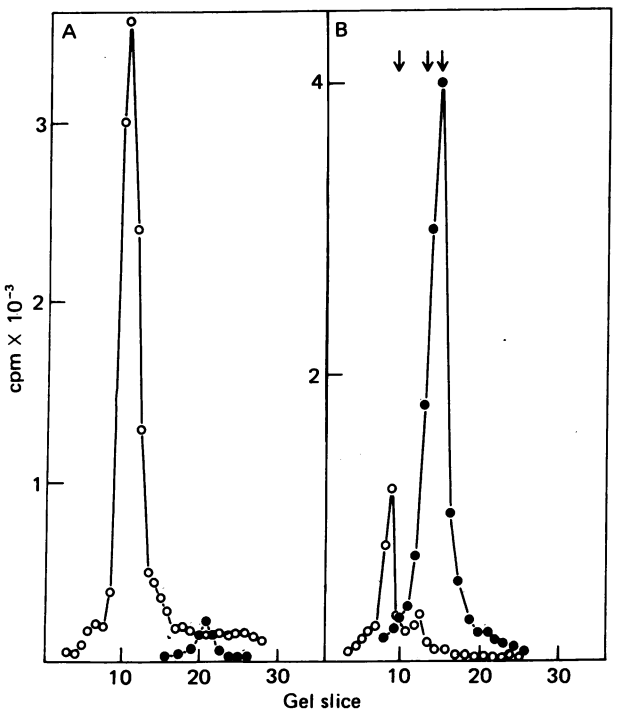

FIG. 2. Gel electrophoresis of RNA complementary to *58.5–70.7*. Ad-2-infected cells (≈2 × 10^8 cells) were labeled with [^{3}H]uridine (20 mCi/μmol) (1 Ci = 3.7 × 10^{10} becquerels) for either (*A*) 10 min (400 μCi/ml; 10^7 cells per ml) or (*B*) 90 min (20 μCi/ml; 10^6 cells per ml) concluding at 4 hr of infection, and cytoplasmic and nuclear poly(A)$^+$ RNA were selected by poly(U)-Sepharose chromatography (33). The RNA was denatured and subjected to polyacrylamide gel electrophoresis (23). RNA was eluted from gel slices and a portion was hybridized at 65°C to filter-bound DNA (25) from *58.5–70.7* on the Ad-2 genome. RNase-resistant hybrids were scored. ○, Nuclear poly(A)$^+$ RNA; ●, cytoplasmic poly(A)$^+$ RNA. Migration is from left to right; arrows indicate positions of 28S, ≈22S, and ≈20S RNA complementary to *58.5–70.7*.

RNA after a 10-min labeling period suggested that nuclei labeled *in vivo* could furnish a "substrate" for an RNA-processing reaction *in vitro* that yielded one predominant mRNA.

At the outset we feared that random, nonspecific degradation of the nuclear RNA during incubation *in vitro* would preclude the observation of specific cleavage and/or splicing of the nuclear precursor RNA. However, (Fig. 3), when the nuclei were incubated *in vitro* at 37°C for 30 min in the presence of a mixture of Mg^{2+}, ribose triphosphates, buffers, and KCl, no degradation and no processing of the 5-kb, poly(A)$^+$ nuclear RNA were observed (Figs. 3 *A* and *B*). Addition of various cell extracts during nuclear incubations *in vitro* could therefore be tested for their ability to accomplish a "processing" event.

In the presence of cytoplasmic extract from either infected or uninfected cells, a portion (30–50%) of the ≈5-kb RNA complementary to *58.5–70.7* was reduced in size. Results from three such *in vitro* incubations carried out for 25–30 min are shown in Fig. 4. The poly(A)$^+$ ≈5-kb RNA, which contains sequences complementary to both *58.5–70.7* and *70.7–75.9*, yielded a mixture of two poly(A)$^+$ RNAs, one approximately 22 S (3 kb) and the other approximately 20 S (2 kb). The 20 S poly(A)$^+$ RNA comigrated during polyacrylamide gel electrophoresis with authentic ^{32}P-labeled cytoplasmic 72,000 M_r mRNA (Fig. 4 *B* and *C*). Both the 22S and the 20S RNA molecules processed *in vitro* appeared to have lost the majority of sequences complementary to *70.7–75.7* DNA region (Fig. 4*A*). Preliminary observations on nuclei incubated for various times from 5 min to 30 min suggest that the 22S poly(A)$^+$ species may be formed before the 20S species.

If the labeled 20S poly(A)$^+$ RNA that appeared during the incubation *in vitro* were truly equivalent to the spliced 72,000 M_r mRNA, it should have retained approximately 100 to 200 nucleotides complementary to *70.7–75.9* (the *Eco*RI F DNA fragment; see map in Fig. 1 and refs. 29 and 30). Furthermore, if the 20S molecule processed *in vitro* were spliced as is the mRNA, the *70.7–75.9* sequences should be closely linked (within ≈1500 nucleotides) to sequences transcribed from *58.7–62.9* and *62.9–65.6*, but should be missing most of the RNA complementary to *66.1–70.7*. These regions of the Ad-2 DNA can be obtained by digestion of the *Eco*RI B fragment (*58.5–70.7*) with the endonuclease *Bal* I (Fig. 1). In contrast to the 20S RNA, in the 28S poly(A)$^+$ molecule all of the originally transcribed RNA sequences complementary to the *70.7–75.9* region should be present and be closely linked to sequences complementary to *66.1–70.7*. To test whether, in fact, these predictions were true, a "two-step" hybridization experiment was performed. Poly(A)$^+$ RNA was prepared from nuclei incubated in the presence or absence of cytoplasmic extract and subjected to electrophoresis in polyacrylamide gels (as shown in Fig. 4). The RNAs in the 20S and the 28S regions of the gel were eluted (almost no RNA was obtained from the 20S region of the gel from the control sample incubated without cytoplasmic extract), and each was treated with alkali to disaggregate the RNA and reduce the size to 1200 ± 400 nucleotides. (The size of the RNA was tested by polyacrylamide gel electrophoresis; data not shown.) The first step of the hybridization was carried out by hybridizing the alkali-generated RNA fragments of the original poly(A)$^+$ 20S or 28S RNA with nitrocellulose filters containing *70.7–75.9* DNA. The hybridized RNA was eluted without any RNAse treatment so that RNA sequences adjacent to those that hybridized to the DNA would remain covalently linked. The eluted RNA was then digested

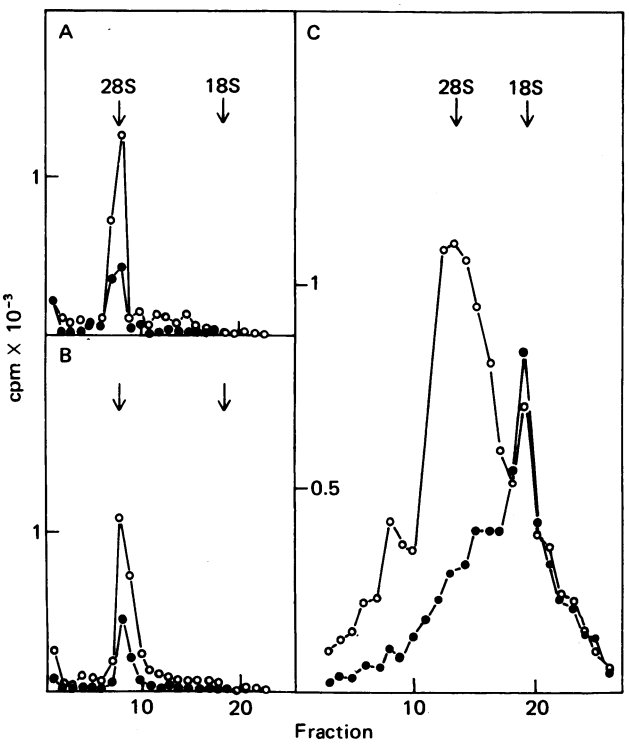

FIG. 3. Effect of *in vitro* incubation on poly(A)$^+$ Ad-2-specific RNA. Ad-2-infected cells (4×10^8 cells) labeled for 10 min as described in Fig. 2 were washed twice in phosphate-buffered saline (0.14 M NaCl/3 mM KCl/1.5 mM KH_2PO_4/4 mM NaH_2PO_4) at 4°C, resuspended at 3×10^7 cells per ml, and swollen in a hypotonic buffer (10 mM Tris·HCl, pH 7.4/10 mM NaCl/5 mM $MgCl_2$) for 10 min (34), and homogenized in a tight-fitting Dounce homogenizer (10 strokes); nuclei were sedimented at 1000× *g* for 5 min after addition of KCl to a concentration of 0.1 M. One sample of the nuclei was phenol extracted immediately (*A*). The other sample was resuspended in 1 ml containing 5 mM dithiothreitol, 5 mM $MgCl_2$, 100 mM KCl, 10 mM Tris·HCl, pH 7.8, 0.75 mM ATP, 0.25 mM GTP, CTP and UTP, and 25% (vol/vol) glycerol (22) and incubated for 30 min at 37°C before the RNA was extracted (*B*). Poly(A)$^+$ RNA was selected (see Fig. 2) and the RNA was subjected to gel electrophoresis (*A* and *B*). A sample of the poly(A)$^-$ RNA left after selection was sedimented through a 15–30% sucrose gradient (*C*) (22). Samples from the gels or gradient were hybridized to filters bearing the *58.5–70.7* (○) or *70.7–75.9* (●) region of the Ad-2 genome. Approximately 2 μg equivalent of DNA (that amount of a given region present in 2 μg of total Ad-2 DNA) was present on each filter. RNase-resistant material was assayed as in Fig. 2.

with DNAse to remove contaminating DNA and rehybridized to sections of the Ad-2 genome between *58.5* and *75.9*. After the second round of hybridization the RNA·DNA hybrids were treated with RNase so that only those portions of the RNA that were base-paired were scored. In this way the 20S and the 28S RNA could be tested for the presence of sequences from various parts of the Ad-2 genome that were linked (within ≈1200 nucleotides, the size to which the RNA was alkali fragmented) to RNA sequences complementary to the *70.7–75.9* region. During the second round of hybridization, the RNA fragments from the original 20S RNA produced *in vitro* formed RNase-resistant hybrids with DNA from *58.7–62.9* and *62.9–65.6*, but very little RNA hybridized to DNA from *66.1–70.7*. The same hybridization pattern was demonstrated by the cytoplasmic mRNA for the 72,000 M_r protein (Table 1). After the second round of hybridization the RNA fragments from the original 28S RNA formed RNase-resistant hybrids with all the DNA fragments from *58.5–75.9*, including the region from *66.1–70.7* (Table 1 and Fig. 1). Thus it appears that the incubation *in vitro* in the presence of cytoplasmic extract produced a new ≈20S RNA molecule from the 28S RNA that contained the same nucleotide sequences as the authentic mRNA.

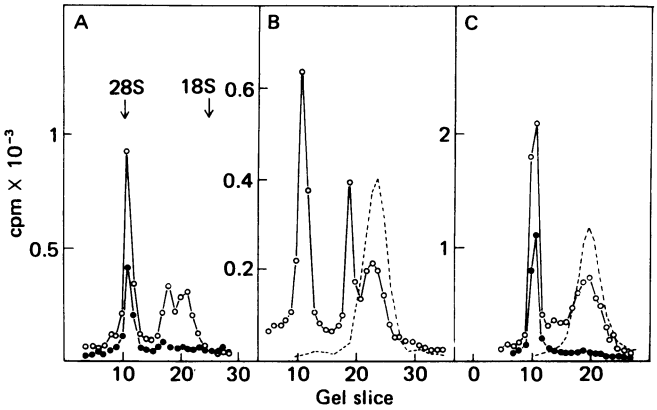

FIG. 4. *In vitro* processing of 28S poly(A)$^+$ nuclear RNA to yield Ad-2 72,000 M_r mRNA. Ad-2-infected cells labeled as in Figs. 2 and 3 were collected and nuclei were prepared as for Fig. 3 in three separate experiments, *A*, *B*, and *C*. The nuclei were incubated for 30 min in the presence of 1 ml of postnuclear supernatant prepared by homogenization with a tight-fitting Dounce homogenizer of unlabeled cells washed once with 10 mM Tris·HCl, pH 7.4/10 mM NaCl/5 mM $MgCl_2$ and then swollen in the same buffer at 10^8 cells per ml. The cytoplasmic extract was freed of nuclei by a 5-min spin at 1000 × *g*. The cytoplasm from infected cells (4 hr after infection) was used in experiment *A* and uninfected cells for experiments *B* and *C*. After the end of the incubation the RNA was extracted and analyzed as for Figs. 2 and 3. ○—○, RNA hybridized to *58.5–70.7*; ●—●, RNA hybridized to *70.7–75.9*; - - -, marker cytoplasmic [^{32}P]RNA from infected cells labeled with ^{32}P 2–5 hr after infection and hybridized also to *58.5–70.7* (*B*) and to *58.5–70.7* and *70.7–75.9* (*C*).

All of the results described so far have concerned poly(A)$^+$ RNA molecules. Because the cytoplasmic mRNA for the 72,000 M_r protein is mainly if not exclusively a poly(A)$^+$ species (26, 27, 29–31) and because the majority of the labeled poly(A)$^+$

Table. 1. Analysis of the sequences present in the 28S precursor and the *in vitro* spliced 20S 72,000 M_r mRNA

Exp.	RNA	cpm hybridized to map coordinates: *58.7–62.9*	*62.9–65.6*	*66.1–70.7*	*70.7–75.9*
1	28S	122	123	301	430
	20S	182	322	6	133
2	28S	89	153	281	2630
	20S	68	394	30	120
	mRNA	243	1347	47	267

Labeled nuclear poly(A)$^+$ RNA was eluted from 20S and 28S regions of gels similar to those shown in Fig. 4 and precipitated with ethanol. The dissolved RNA was made 0.1 M in NaOH at 4°C (35) for 10 min, neutralized, and hybridized to a filter containing 50 μg equivalents of *70.7–75.9* DNA. After incubation at 45°C for 48 hr in 30% (vol/vol) formamide in 10 mM Tes, pH 7.4/0.5 M NaCl/10 mM EDTA/0.4% (wt/vol) sodium dodecyl sulfate plus yeast RNA at 200 μg/ml, the filters were washed once with the hybridization buffer and then extensively with 0.3 M NaCl/0.03 M sodium citrate, and RNA was eluted as described (25). The samples were precipitated with ethanol, digested for 20 min at 37°C with pancreatic DNAse I (25), and rehybridized to fragments indicated in the table. Fragment coordinates and enzyme digestions necessary for preparations are shown in Fig. 1. The results are expressed in RNase-resistant hybridized cpm. The numbers for the mRNA were obtained by hybridization of the poly(A)$^+$ 20S cytoplasmic RNA labeled for 3 hr between 1 and 4 hr after infection. In other experiments, ^{32}P-labeled cytoplasmic RNA hybridized 94% to *58.5–70* and 6% to *70.7–75.9*. Note that the numbers in the last line (mRNA) correspond to a one-step hybridization at 66°C as described in *Materials and Methods*.

RNA in nuclei after a 10-min label is in the ≈5-kb RNA species (Figs. 2 and 3), it seems most likely that this RNA is the precursor to the poly(A)$^+$ 20S molecule produced *in vitro*. Moreover, it has been shown that both late Ad-2 mRNA (37) and hemoglobin (15) mRNA have poly(A) added before splicing is completed. In an effort to rule out the possibility that the mRNA of the 72,000 M_r protein was formed *in vitro* from already spliced poly(A)$^-$ RNA simply by poly(A) addition during incubation, the poly(A)$^-$ nuclear RNA was examined (Fig. 3*C*). Because of the large amounts of total RNA involved this sample had to be separated in a sucrose gradient rather than by gel electrophoresis. About 50% of the total Ad-2 RNA specific for the *58.5–70.7* region was in the poly(A)$^-$ fraction, but the great majority was contained in molecules about 5 kb in size. Very little of the total labeled Ad-2 RNA therefore was in the 20S size range in a poly(A)$^-$ form, while as much as 25–35% of the total labeled RNA complementary to *58.5–70.7* was converted *in vitro* to 20S poly(A)$^+$ molecules (Fig. 4). Moreover the 20S region of the poly(A)$^-$ RNA was found to hybridize to DNA from *58.5–62.9*, *62.9–65.6*, and *66.1–70.7* unlike the spliced 20S mRNA, so that virtually no potential spliced poly(A)$^-$ molecules preexisted before incubation. It is of course not possible to be certain that some large 28S poly(A)$^-$ molecules both become poly(A)$^+$ and spliced during the reaction, but it seems most likely that the splicing reaction observed involved the poly(A)$^+$ 28S molecules.

An interesting result emerged from the analysis of the poly(A)$^-$ RNA (Fig. 3*C*). A species of RNA about 1.5 to 2 kb in length and complementary to *70.7–75.9* was observed. This RNA is a candidate for the "spliced out" region that lies between ≈*68* and ≈*75* on the Ad-2 genome.

DISCUSSION

The discovery that Ad-2 mRNAs contained sequences not contiguously encoded in the DNA (1–3, 29, 30) coupled with previous experiments that defined Ad-2 transcription units (12, 13, 22, 28) made it highly likely that RNA–RNA splicing occurred. However, it remained to be demonstrated that such a reaction could occur in cell-free extracts. The experiments reported here demonstrate that such a reaction can occur for an Ad-2 mRNA molecule. The success of the experiment depended first on the availability of nuclei containing only one major labeled product from the 72,000 M_r protein transcription unit. Second, we assume that this ≈5-kb nuclear RNA is present in whatever complex is necessary to carry out the various enzymatic cleavages and subsequent splicing events to form the mRNA for the 72,000 M_r protein. At any rate, simple addition of labeled virus-specific RNA to nuclear extracts has not yet produced any evidence of specific processing. In the present experiments the correct enzymatic cleavages are demonstrated by the reduction in size of nuclear RNA molecules containing sequences complementary to *58.5–70.7* (Fig. 3) so that comigration with mRNA was observed. Splicing is indicated by the retention of sequences in the *in vitro*-generated 20S RNA that were complementary to *70.7–75.9*, *58.5–62.9*, and *62.9–65.5*, but not to *66.1–70.7*, while the 28S RNA contained sequences complementary to all these regions of the viral genome.

The cell location and nature of the enzyme(s) responsible for this splicing reaction remain uncertain. The isolated nuclei alone will not carry out the reaction but require the addition of "cytoplasmic" extract. Because, however, this extract was prepared by swelling cells in hypotonic buffer (22, 34) and stripping the cytoplasm during homogenization, it is possible, in fact probably likely, that some enzyme or cofactor "leaks" from the nuclei during this preparation. For example, late in Ad-2 infection, spliced mRNA products can be transiently observed in the nucleus after a short label, suggesting the nucleus as the site of RNA–RNA splicing (23). Furthermore, many enzymes [e.g., DNA polymerases (36)] and small RNA precursor molecules (38, 39) that are present in nuclei isolated by nonaqueous techniques are found in the "cytoplasm" when nuclei are prepared by hypotonic swelling. The loss of the processing (splicing) function from nuclei subjected to hypotonic swelling, however, may be a fortunate occurrence that will enable purification of the activity to proceed expeditiously.

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