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Accurate and efficient *in vitro* splicing of purified precursor RNAs specified by early region 2 of the adenovirus 2 genome

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ABSTRACT

Polyadenylated and deproteinized nuclear RNA precursors encoded by early region 2 of the adenovirus 2 genome are spliced in vitro by nuclear extracts 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 nucleotide sequence across the splice junctions in the E_2 RNAs processed in vitro was investigated by performing primer extensions in the presence of dideoxynucleotides and direct sequencing on polyacrylamide gels. We conclude that the in vitro splicing reaction is accurate and has the same precision as that of in vivo E_2 cytoplasmic mRNA prepared from Ad2 infected cells. The efficiency of in vitro splicing by the nuclear extracts is very high. Approximately 80% of E_2 RNA precursor, on a molar basis, are spliced in vitro to a mature RNA. These findings provide evidence that a nuclear extract prepared from MOPC-315 mouse myeloma cells is capable of accurate and efficient splicing of E_2 RNA precursors.

INTRODUCTION

Since the existence of spliced adenovirus 2 (Ad2) messenger RNAs was first demonstrated, a large number of viral and eukaryotic protein-coding genes and also tRNAs and rRNAs have been shown to be split (for review see 1-6). Structural studies of several in vivo RNA precursor molecules have provided evidence that split genes are transcribed into colinear precursors from which the intervening sequences (sequences separating the exons) are removed by a cleavage-ligation reaction (splicing) (for review see 4,5). Elucidation of the biochemical steps in splicing of mRNAs is undoubtedly of fundamental importance for the understanding of mRNA synthesis and regulation of gene expression. The study of enzymes and components involved in the splicing reaction requires in vitro cell-free systems which are able to splice accurately and efficiently mRNA precursors. In vitro transcription and splicing of viral (7) and human β globin RNA (8) in the whole cell extracts from HeLa cells was reported. However, the efficiency of the splicing reaction in both systems are extremely low.

Recently (9), we have presented evidence that splicing of purified

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adenovirus precursor mRNA occurs very efficiently in a whole cell extract prepared from MOPC-315 mouse myeloma cells. The mRNA precursor that we have used for the in vitro splicing reaction is encoded by one of the four regions expressed at early times after Ad2 infection (10,11), early region 2 (E_2). The polypeptide product of E_2 is a 72,000 dalton DNA-binding protein (12-14). E_2 mRNA is copied from the strand transcribed in the leftward direction (15,16). This mRNA consists of two exons (each approximately 70 nucleotides) transcribed from positions 75 and 68.5, respectively, spliced to 1700 nucleotide mRNA body encoded from positions 66.3-61.6 of the Ad2 genome (17-19). Studies of pulse-labeled polyadenylated nuclear RNA revealed three major species (20,21). The largest (28S) precursor contains all the intervening sequences. A 23S species appears to be a processing intermediate lacking the large 5' intervening sequences. The nuclear 20S RNA has a structure identical to the cytoplasmic mRNA and presumably is the direct precursor of the functional mRNA. Studies measuring steady state levels of E_2 nuclear RNA also detected an RNA molecule (26S) in which the smaller 3' intervening sequence has been excised (22).

The experiments we have performed with purified and deproteinized E_2 mRNA precursors and MOPC-315 mouse myeloma whole cell extracts demonstrate excision of intervening sequences and ligation of leader transcripts to the mRNA body (9). However, from these experiments we cannot conclude that the splicing has the same precision as occurs in vivo. In this report, we describe a nuclear extract that splices with a very high efficiency purified deproteinized and polyadenylated E_2 mRNA precursors that have been obtained from cultures early in adenovirus infection. The nucleotide sequence of the in vitro processed E_2 RNAs across the splice junctions is identical to that of the in vivo E_2 cytoplasmic mRNA. Therefore, the nuclear extract prepared from MOPC-315 mouse myeloma cells is capable of efficient and accurate in vitro splicing of E_2 mRNA precursors.

MATERIALS AND METHODS

Cell culture, virus infection, and RNA preparation. Maintenance of KB (human) and MOPC-315 mouse myeloma cell suspension cultures, procedures for Ad2 infections, and isolation of nuclear and cytoplasmic RNA were performed as described (21). Infections were performed in the presence of 25 µg/ml cycloheximide and harvested at 6 hr after infection. High molecular weight poly(A)$^+$ RNA precursors to be used as substrates for in vitro incubations were highly purified by fractionation on three successive 15-30% sucrose

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gradient sedimentations as described previously (9,23).

Preparation of nuclear extracts with splicing activity. Nuclear extracts containing splicing activity were prepared as described previously (9) with the following modifications. Suspension cultures of MOPC-315 mouse myeloma cells were grown to a density of approximately 1.5 x 10^6 cells per ml. Cells were harvested and resuspended in isotonic buffer (10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM DTT, 75 mM $(NH_4)_2SO_4$) containing 0.025% Nonidet P-40. After 3-5 minutes on ice, the nuclei were collected by centrifugation at 10,000 g. The pelleted nuclei were resuspended in hypotonic buffer (10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM DTT) containing 1% Nonidet P-40. After incubation for 30 min on ice, the nuclei were homogenized by four strokes in a dounce, and adjusted dropwise to 0.25 M sucrose and 0.4 M ammonium sulfate. After this addition, the lysate was gently stirred for 30 minutes at 4°C. The extract was then centrifuged at 50,000 rpm for 1 hour in a Beckman 60 Ti rotor. The supernatant was removed and reprecipitated by addition of solid ammonium sulfate (0.51 gm per ml of suspension). The precipitate was collected by centrifugation and stored at -70°C. The total protein in nuclear extracts obtained from 1.5 x 10^9 MOPC-315 cells was 30-40 mg. To test the splicing activity of the post-nuclear fraction, the supernatant of the 10,000 g centrifugation was adjusted to 1% Nonidet P-40, 0.25 M sucrose, and precipitated by addition of solid ammonium sulfate as described above. The precipitated extracts were thawed and resuspended in incubation buffer (60 mM Tris-HCl, pH 7.5, 150 mM $(NH_4)_2SO_4$, 3 mM DTT, 20% sucrose). This suspension was applied directly to a Bio-Gel P_2 desalting column as described before (9). The material eluted in the void volume of this column was used to direct processing of RNAs *in vitro*. Reactions were in a total volume of 150 μl containing 50 μl of extract (600 μg of protein, equivalent to 3 x 10^7 MOPC-315 cells), 0.5 mM each GTP, CTP, UTP, and ATP, 10 mM creatine phosphate, 100 μg/ml creatine kinase, 300 μg of total cytoplasmic RNA purified from KB cells, and 10 mM $MgCl_2$. Incubations were at 30°C for 50 minutes. Reactions were terminated and RNAs purified as described (9).

Preparation of a primer DNA fragment. The Ad2 DNA fragment 59.5-70.7 cloned in pBR322 plasmid was linearized by digesting sequentially with restriction endonucleases *Eco*RI and *Xho*I (see Figure 1). The cleaved DNA was subsequently treated with calf intestinal phosphatase, 5'-end-labeled with T_4 polynucleotide kinase and γ-^{32}P ATP (24), and subcleaved with *Bal*I. The ^{32}P-labeled 147 base pair primer fragment, extending from the *Xho*I to

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the BalI site and labeled at the XhoI site (see Figure 1), was isolated by electrophoresis in an 8% nondenaturing acrylamide gel. The DNA fragment was recovered from the gel slice as described (24).

Hybridizations of DNA primer with RNAs. Hybridizations of the ^{32}P 5'-end-labeled 147 DNA primer (0.5 μg) with polyadenylated cytoplasmic mRNA purified from 9 x 10^8 KB cells infected at early times with Ad2 or *in vitro* E_2 processed RNAs (E_2 precursor RNAs purified from 6 x 10^9 KB infected cells) were performed as described (25). After hybridization for 18 hr, nucleic acids were diluted 10-fold with 0.5 M KCl, 10 mM Tris-HCl, pH 7.5, and applied to an oligo dT-cellulose column (26). Molecules composed of poly(A) terminal RNA and bound ^{32}P DNA were eluted with water and precipitated with ethanol.

Sequencing by primer extension. Nucleotide sequencing by performing chain extensions with dideoxynucleotides (ddNTP's) was performed as described (27,28). Hybrids were dissolved in the reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, 0.1 M KCl, 10 mM $MgCl_2$, and 1 mM DTT), divided into aliquots supplemented with one of the four ddNTP's (P-L Biochemicals) and deoxynucleotides (dNTP's) at 57 μM each, except for dATP at 28.5 μM. The following ratios were used: dATP/ddATP, 0.49; dCTP/ddCTP, 2; dGTP/ddGTP, 1; dTTP/ddTTP, 1.35. Reverse transcriptase reactions were carried out in a final volume of 70 μl for 15 minutes at 40°C using 22 units of AMV reverse transcriptase (Life Science). Subsequently, 20 μl of a solution containing 250 mM of all four deoxynucleotides in the reaction buffer was added, and the incubation was continued for an additional 10 minutes. The reactions were terminated by adding 10 μl of 1 M sodium hydroxide and then heated at 100°C for 5 min. The reactions were neutralized, phenol extracted, and precipitated with ethanol. The products were resuspended in formamide dye sample buffer for sequencing gels, and analyzed by electrophoresis on 85 cm x 17.5 cm x 0.04 cm sequence gels containing 6% acrylamide and 7 M urea. Electrophoresis was carried out for 18 hr at 2000 V (29,30).

RESULTS

Nuclear Extracts Convert E_2 Nuclear RNA Precursors to the Size of the Mature mRNA

To assay for *in vitro* processing activity, we have utilized E_2 nuclear RNA precursors. The E_2 polyadenylated nuclear RNA contains three large size classes (28S, 26S, and 23S) in addition to molecules the size of cytoplasmic 20S mRNA (Figure 1). The larger forms contain intervening

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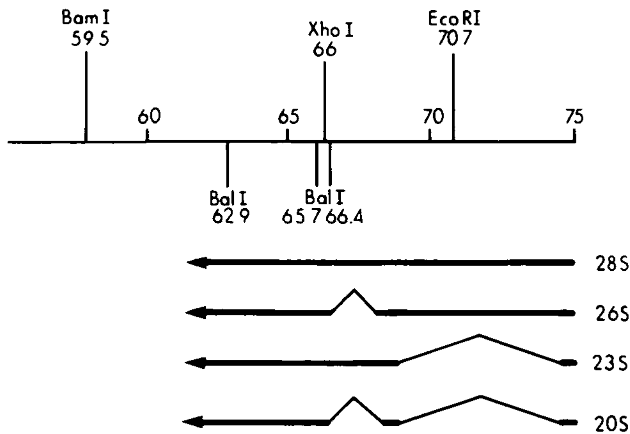

Figure 1. Nuclear poly(A)$^+$ RNA transcribed from E_2 at early times in infection. Solid bars represent the structure of nuclear E_2 RNAs (18,19, 21,22). The arrows indicate the direction of transcription. Caret-shaped symbols indicate sequences covalently joined by splicing. The cytoplasmic mRNA has the structure of the 20S species (9). Relevant cleavage sites of restriction endonucleases EcoRI, BamI, XhoI, and BalI have been reported previously and confirmed at the nucleotide sequence level (25,41). The map positions indicate the relative distance of the cleavage site from the left end of the genome.

sequences that are absent from the nuclear 20S RNA. The nuclear 20S has a structure indistinguishable from the cytoplasmic 20S mRNA and presumably is the direct precursor of the functional mRNA. To analyze the 20S RNAs produced in vitro, we characterized the RNAs by fractionation in agarose gel followed by Northern blots and hybridization to a ^{32}P-labeled 59.5-70.7 DNA fragment cloned in PBR322 which encodes E_2 exons and intervening sequences (Figure 1). This assay for processing activity required a preparation of nuclear RNA precursor that was essentially free of 20S nuclear RNA. To obtain the necessary substrate, polyadenylated nuclear RNA was fractionated on a 15-30% sucrose gradient. RNAs 28S and larger in size were pooled, precipitated, and run again twice on 15-30% sucrose gradients. The pooled high molecular weight RNA contained 20S E_2 RNA molecules after the first fractionation on sucrose gradients (Figure 2A, lane 5). However, after the second and third fractionation on sucrose gradients, the high molecular weight nuclear RNA precursors showed no evidence of 20S E_2 RNA (Figure 2A, lanes 2 and 3). Aliquots equivalent to 6 x 10^7 infected cells of this highly purified and deproteinized high molecular weight nuclear RNA precursor were incubated for 50 min at 30°C in incubation buffer without extract (see

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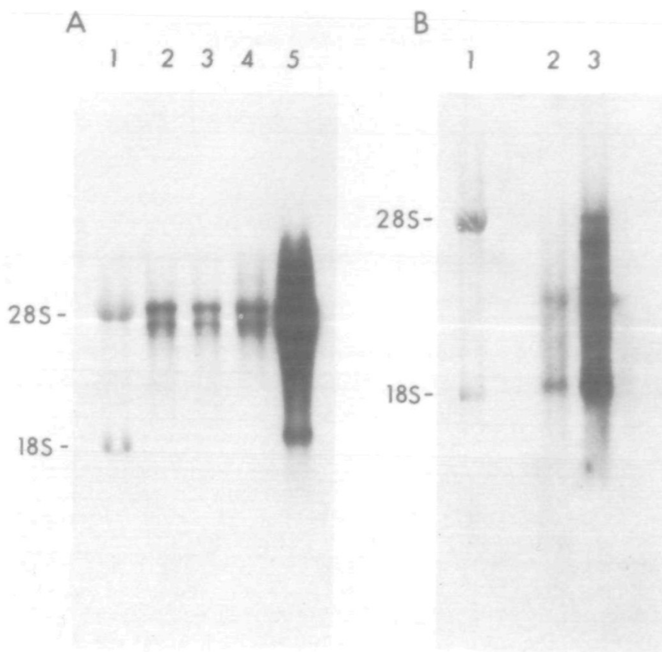

Figure 2. Assay for *in vitro* processing of E_2 poly(A)$^+$ nuclear RNA precursors. High molecular weight nuclear poly(A)$^+$ RNA precursors to be used as substrates for *in vitro* incubations were fractionated on three successive 15-30% sucrose gradient sedimentations (9,23). Fractions 28S in size and larger were pooled, subjected to electrophoresis on glyoxal/1.1% agarose gels, and transferred to nitrocellulose paper. The nick translated 59.5-70.7 Ad2 DNA fragment cloned in PBR322 (3 x 10^8 cpm/ug) was annealed to the immobilized RNA (42). After hybridization, the strips were exposed to X-ray film with an intensifying screen for 48 hrs at -70°C.

Panel A: Blot hybridization of E_2 poly(A)$^+$ nuclear RNAs after successive fractionations on 15-30% sucrose gradient sedimentation. Lane 1: ribosomal ^{14}C markers (1 x 10^4 cpm). Lane 5: high molecular weight poly(A)$^+$ nuclear RNA pooled from the first sucrose gradient (RNA purified from 6 x 10^7 infected cells). Lanes 2 and 3: nuclear RNA pooled from the second and third sucrose gradients (RNA purified from 6 x 10^7 infected cells). Lane 4: high molecular weight nuclear RNA from the third gradient incubated *in vitro* in incubation buffer at 30°C for 50 min in the absence of extract as described in the experimental procedure (RNA purified from 6 x 10^7 cells).

Panel B: Blot hybridization of E_2 poly(A)$^+$ nuclear RNA precursors after incubation with a nuclear extract prepared from MOPC-315 mouse myeloma cells. Lane 1: ribosomal ^{14}C markers (1 x 10^4 cpm). Lanes 2 and 3: high molecular weight poly(A)$^+$ nuclear RNAs purified by three successive sucrose gradient sedimentations after *in vitro* incubation with the nuclear extract as described in the experimental procedure (lane 2: RNA purified from 6 x 10^7 infected cells, and lane 3: RNA purified from 6 x 10^8 infected cells).

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Materials and Methods). After incubation the RNA was selected by oligo(dT)-cellulose chromotography and fractionated on glyoxal agarose gel. Figure 2A (lane 4) showed that the major 28S and 26S RNA species remained intact as judged by their size distribution. When the E_2 precursor RNA was incubated in the presence of nuclear extracts prepared from MOPC-315 mouse myeloma cells and aliquots equivalent to 6 x 10^7 and 6 x 10^8 infected cells assayed for RNA processing, two new bands of 23S and 20S RNA were generated (Figure 2B, lanes 2 and 3). As reported with the whole cell MOPC-315 extract (9), approximately 80% of the E_2 precursor RNA, on a molar basis, was converted to a 20S species (Figure 2, densitometry scan not shown).

The preparation of the extracts isolated from MOPC-315 mouse myeloma used in the present study differs from that previously reported (9). This extract was prepared from the nuclear fraction of the MOPC-315 cell and was isolated in the presence of Nonidet P-40 detergent. In order to demonstrate that all the RNA processing components were present in the nuclear fraction, the 10,000 g supernatant of the extract utilized for the experiment shown in Figure 2B was assayed for processing activity (see Materials and Methods). After incubation with the post-nuclear fraction, the E_2 RNA precursors remained the same (data not shown), indicating that no major leakage of the processing activities occurred during fractionation.

Nucleotide Sequence of the Splice Junctions in the In Vitro Spliced E_2 RNAs

In order to investigate the accuracy of the *in vitro* splicing reaction at the nucleotide level, polyadenylated nuclear RNA was isolated from 6 x 10^9 KB cells at early times during Ad2 infection, and fractionated on sucrose gradients as described in the legend of Figure 2. Since approximately 80% of the E_2 precursor RNAs was converted *in vitro* to a 20S species (Figure 2B), the remainder of the E_2 precursor RNAs may originate bands in a sequencing gel corresponding to the E_2 intervening sequences. To obtain *in vitro* spliced RNAs 20S in size and free of precursor RNAs, the *in vitro* RNA products after incubation with the nuclear extract were fractionated on a 15-30% sucrose gradient. RNAs 20S in size were pooled and the nucleotide sequence of the splice junctions was determined by performing RNA-DNA primer extensions with reverse transcriptase in the presence of 2'-3' dideoxynucleoside triphosphates as specific chain terminators. For this study we have chosen a primer DNA fragment of 147 nucleotides, which hybridizes 13 nucleotides downstream from the splice junction between the 5'-end main E_2 mRNA body and the second RNA leader (Figures 1 and 4). The ^{32}P-labeled primer *XhoI*-*BalI* fragment 5'-end labeled at the *XhoI* site (see Figures 1 and

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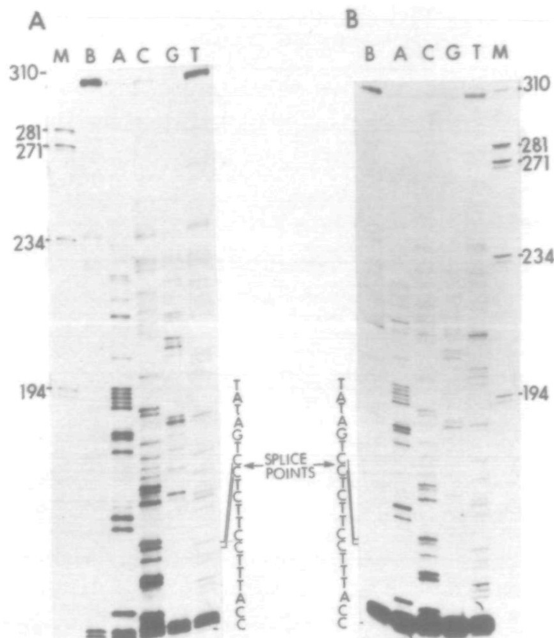

Figure 3. Sequencing of the splice junctions of E_2 RNA precursors processed in vitro. High molecular weight nuclear RNAs were purified from 6 x 10^9 KB cells as described in the legend of Figure 2 and incubated in the presence of a nuclear extract prepared from MOPC-315 cells. Aliquots were tested for in vitro processing (Figure 2B). The in vitro processed RNAs were then fractionated on a 15-30% sucrose gradient sedimentation, and fractions containing RNA 20S in size were pooled and precipitated. The nucleotide sequence of the splice junctions in the in vitro processed RNAs was determined by performing chain extensions with reverse transcriptase in the presence of 2'-3'-dideoxinucleoside triphosphates. The 147 nucleodite XhoI-BalI DNA primer (Figures 1 and 4) labeled at the 5'-end XhoI site was hybridized and extended as described in the Materials and Methods.

Panel A: Nucleotide sequence of the splice junctions in the in vivo cytoplasmic E_2 mRNA. Exposure to X-ray film with an intensifying screen was for 15 hrs at -70°C.

Panel B: Nucleotide sequence of the splice junctions in the in vitro processed 20S E_2 RNA. Exposure to X-ray film with an intensifying screen was for 7 days at -70°C. A, C, G, and T denote the specific ddNTP inhibitor used in the reaction. B denotes a reaction in the absence of ddNTP's inhibitors, M markers ØX174 DNA 5'-end labeled.

4) was isolated and hybridized to the in vitro processed E_2 20S RNAs. The hybrids were selected by oligo(dT)-cellulose chromatography and precipitated with ethanol. Subsequently, reverse transcription was carried out in the presence of dideoxynucleotides as described in the Materials and Methods. The nucleotide sequence of the splice junctions of the in vitro processed RNA

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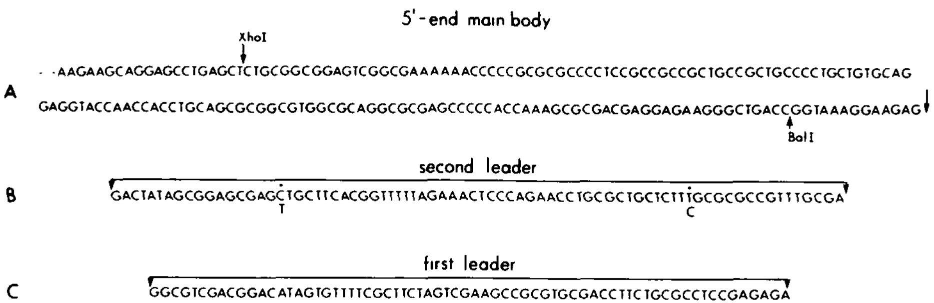

Figure 4. Nucleotide sequence of the splice junctions of the E_2 mRNA. From experiments like the one shown in Figure 3, the nucleotide sequence of the E_2 mRNA second leader and 13 nucleotides from the BalI site to the splice point in the 5'-end main body of the E_2 mRNA were derived. The nucleotide sequence of the 5'-end main body, the second leader and the first leader are indicated in the corresponding RNA sequences (A, B, and C). The positions of the splice points are indicated by vertical arrows. The sites for restriction endonucleases XhoI and BalI are indicated in Panel A. The nucleotide differences present in the Ad5 second leader are indicated underneath the Ad2 sequence. The nucleotide sequence of the Ad2 5'-end main body (41) as well as the first leader and the second leader of Ad5 of the E_2 mRNA have been reported previously (25).

was directly compared to the E_2 cytoplasmic mRNA isolated from KB cells early during Ad2 infection in the presence of cycloheximide. Figure 3 shows the nucleotide sequence of the splice junctions derived from the cytoplasmic E_2 mRNA (Figure 3A) and the *in vitro* processed E_2 RNAs (Figure 3B). Both nucleotide sequences were identical demonstrating faithful and accurate *in vitro* splicing for the splice junction between the 5' acceptor E_2 mRNA body and the 3' donor of the second RNA leader sequence (see Figures 1 and 4). The accuracy of the *in vitro* splicing for the junction between the second and first leader sequences was analyzed using the primer extension technique in the absence of dideoxynucleotides. Figure 3A and B (lane B) shows the expected cDNA product of 290 nucleotide long, generated by both the *in vitro* spliced E_2 RNAs and the cytoplasmic E_2 mRNA. The nucleotide sequence of the splice between the first and second leaders was not determined directly, but the appropriate size for the major cDNA product indicates accurate *in vitro* splicing between the first and second E_2 RNA leaders. The nucleotide sequence of the 5'-end main body and the second and first E_2 mRNA leaders is interpreted in detail in Figure 4. The nucleotide sequence for the second E_2 mRNA leader has been reported in Ad5 (23). By comparing the sequences of the E_2 mRNAs in Ad2 and Ad5, we found in Ad2 two nucleotide alterations

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in the 77 nucleotides of the second E_2 mRNA leader. A C-residue has been changed to a T, and a T-residue has been changed to a C in the Ad2 E_2 mRNA (Figure 4).

DISCUSSION

We have shown that adenovirus E_2 precursor RNA was accurately and efficiently spliced *in vitro* by nuclear extracts prepared from MOPC-315 mouse myeloma cells. The nucleotide sequence analysis of the *in vitro* E_2 RNA products by the dideoxy chain termination method demonstrated unambiguous evidence that cleavage and ligation, of both leader sequences to the RNA body, have the same precision as occurs *in vivo*. From these experiments, we conclude that all the components of the splicing apparatus necessary to recognize and splice E_2 RNAs are present in the nuclear extracts prepared from MOPC-315 cells. For future studies and purification, the nuclear extract may be a valuable source of splicing activity.

The efficiency of splicing obtained with the nuclear extracts, 80% on a molar basis, was the same as reported previously with whole cell extracts (9). One of the factors which may have contributed to obtaining the high efficiency of *in vitro* splicing was the fact that we have used polyadenylated mRNA precursor isolated from cultures early in Ad2 infection. This RNA appears to be the precursor to the 72K DNA binding protein mRNA (20,21). RNAs transcribed *in vitro* by concentrated whole HeLa cell extracts were reported to be spliced *in situ* with only a very low efficiency, between 2 and 5% (7,8). Other investigators were not able to detect any splicing in very similar *in vitro* systems (31). The discrepancies in splicing efficiencies between the *in vitro* system reported here and the coupled *in vitro* transcription-splicing systems could be due to several factors. The *in vitro* transcribed RNAs could lack sequences or nucleoside modifications for attaining similar structures to those found *in vivo* and therefore may not be ideal substrates for the splicing apparatus. For example, specific transcription termination and polyadenylation do not occur in the available eukaryotic *in vitro* transcription systems (32,33). Alternatively, inactivation of an essential component for splicing or of proteins that organize the precursor RNA into mRNP's *in vivo* may occur during preparation of the whole HeLa cell extracts. We are currently testing the ability of the nuclear MOPC-315 extracts to splice run-off RNAs which have been synthesized in *in vitro* transcription systems. Preliminary results suggest a low efficiency of *in vitro* splicing (unpublished data). Further experiments on this question

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are underway. Any manipulations to analyze the in vitro splicing reaction requires relatively rapid and reasonable assays. We have previously utilized assays which required the purification of large amounts of nuclear RNA from infected cells. By using Northern blots, we were able to increase the sensitivity for detection of the in vitro spliced RNAs. This assay will facilitate future characterization of the splicing activities and/or co-factors such as ribonucleoprotein particles containing small nuclear RNAs (snRNP's) that have been implicated in splicing (34,35). Development of in vitro splicing systems are crucial for the understanding of the biochemical steps in RNA processing. For example, major features of the reaction mechanism have been described for the splicing of yeast tRNA precursors (36-38) and the ribosomal RNA precursors of tetrahymena pigmentosa (39,40). Hopefully, further experiments utilizing the in vitro splicing system described here will help to unravel the biochemical steps in mRNA splicing.

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