In vitro-synthesized adenovirus 2 messenger RNA precursors are accurately spliced by nuclear extracts

(Salmonella phage 6—early region 2 pre-RNA/mRNA splicing/MOPC-315 nuclear extract/RNA sequencing)

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ABSTRACT Precursor mRNAs were synthesized in vitro from a plasmid in which the early region 2 gene of adenovirus 2 is fused to an efficient bacteriophage promoter (Salmonella phage 6). The RNAs were purified and used as substrates for in vitro splicing in the presence of nuclear extracts prepared from MOPC-315 mouse myeloma cells. The in vitro splicing was accurate at the nucleotide level. The reaction occurs rapidly and without any detectable lag. The concentration of the pre-mRNA precursor during incubation appears to be an important factor for high efficiency (60%-80%) of in vitro RNA splicing. Fractionation of the splicing components as well as modifications of the DNA template to study the nucleotide-sequence requirement for in vitro splicing can now be accomplished with this system.

The mRNA precursors of many eukaryotic genes contain introns, which are removed by splicing. Introns have also been found in genes encoding transfer RNAs and ribosomal RNAs. The splicing machinery is thought to be different for each class of RNA (1-4). Although several models for mRNA splicing have been proposed, the biochemical mechanisms of intron removal and the regulation of this process are not known (5, 6). The invariant consensus 5' and 3' splice junction sequences identified as the result of the nucleotide sequence analysis of numerous eukaryotic genes are so far the only recognized RNA signals for mRNA splicing (4, 7). Several reports indicate that large portions of the introns can be removed without affecting the accuracy of splicing (8-12). Mutations in the nucleotide sequence of the splice junction consensus sequences decreased the splicing efficiency or generated alternative splice sites (13-15). The mechanisms as well as the enzymology of mRNA splicing should be addressed in in vitro systems, which would allow the characterization and purification of the individual components. It was reported previously that splicing of purified adenovirus precursor mRNAs occurs accurately and efficiently in nuclear extracts prepared from MOPC-315 mouse myeloma cells (16, 17). These experiments, however, were limited by the inability to purify precursor RNAs from infected cells in large quantities. In this report, I have used in vitro-synthesized pre-mRNAs as substrates for splicing. Truncated RNAs encoded by adenovirus 2 (Ad2) early region 2 (E2) were synthesized in vitro by using a plasmid in which a highly efficient bacteriophage promoter, Salmonella phage 6 (SP6), is fused to the E2 gene. In vitro transcription of this plasmid with SP6 RNA polymerase generated large quantities of single RNA species that could be labeled to a high specific activity. Here, I describe experiments that demonstrate accurate and efficient (60%-80%) in vitro splicing of the E2 in vitro-synthesized RNAs. The nucleotide sequence across the splice junctions in the processed RNAs unambiguously showed that in vitro splicing has the same precision as in vivo. Several kinetic parameters as well as optimum conditions and requirements for the splicing reaction were also defined.

MATERIALS AND METHODS

Cloning of the Ad2 E2 DNA fragments in the plasmid DNA containing the SP6 bacteriophage promoter was as indicated in the figure legends. In vitro transcriptions for 1 hr at 37°C containing 1 uCi of DNA template and 16 units of SP6 RNA polymerase (New England Nuclear) in the presence of 10 &muCi of [a-32P]GTP (600 Ci/mmol; 1 Ci = 37 GBq) were as described (19). Usually, 3 &muCi of RNA was obtained from 1 &muCi of the linearized DNA template. The purified RNAs (36 pmol of the complete 3.3-kilobase (kb) pre-RNA equivalent to 0.3 &muCi in a 15-μl reaction mixture (volume) were incubated in the presence of a nuclear extract prepared from MOPC-315 mouse myeloma cells as described (17). After the reaction was complete, the RNAs were extracted as described (17).

RESULTS

As a model system for studying in vitro splicing of mRNA precursors, we focused on one of the early regions expressed at early times after Ad2 infection (20). E2 is transcribed mainly from two different promoters, the early (from position 75; the Ad2 map from the left-hand end is divided from 0 to 100) and the late (from position 72). We reported that E2 mRNA precursors purified from Ad2-infected cells are spliced accurately and efficiently in vitro by nuclear extracts prepared from MOPC-315 mouse myeloma cells (17). However, the detailed biochemical study of the splicing reaction as well as the purification and identification of the different splicing components requires an easily obtainable source of large quantities of mRNA precursors. To obtain large amounts of E2 RNA precursors, I used a recombinant plasmid containing DNA sequences encoding the promoter from the bacteriophage SP6 fused to the E2 gene (Fig. 1). The advantage of this transcription system is that SP6 RNA polymerase initiates transcription exclusively at the SP6 promoter and is able to transcribe long transcripts with great efficiency (19, 21). The expected size of runoff RNAs transcribed from the plasmid DNA SH2 containing the E2 gene fused to the SP6 promoter are shown in Figs. 1A and 2A (lanes 5-7) and 1B (lanes 1 and 5). To determine whether the in vitro-synthesized E2 RNAs can serve as substrates for in vitro splicing, the DNA plasmid was linearized with Sal I and Bgl II restriction enzymes (Fig. 1). Transcription of this DNA with SP6 RNA polymerase produced a discrete 3.3-kb RNA (Fig. 2A and B, lanes 5). This RNA, which is not capped or polyadenylated, contains the signals for splicing two introns (Fig. 1).

Abbreviations: Ad2, adenovirus 2; E2, early region 2; SP6, Salmonella phage 6; kb, kilobase(s).
The solid bars represent the runoff RNAs and the numbers above the bars indicate the nucleotide length of the expected RNAs. The arrows indicate the direction of transcription. The structure of the expected spliced RNAs to be detected if pre-RNAs (3.3 kb), transcribed from the linearized Sal I/Bgl II pSH2 DNA, were used as substrates for in vitro splicing as shown. The donor splice site of the first 5' exon in the 2.2- and 1.5-kb RNAs is 70 nucleotides from the cap site (position 72 in the text) of the late E2 mRNA.

Time Course of the in Vitro Processing Reaction. $^{32}$P-labeled 3.3-kb RNA was synthesized and incubated for different times in the presence of a nuclear extract purified from mouse myeloma MOPC-315 cells (Fig. 2A and B). After incubation, new RNA species were detected that comigrated on agarose gels as expected for RNAs in which one or both introns have been removed. The in vitro size change occurred rapidly without any detectable lag. After 2 min (Fig. 2A, lane 1), ~30% of the total detectable RNA was converted to the 2.7-, 2.2-, and 1.5-kb RNA, and the reaction was terminated between 15 (Fig. 2A, lane 3) and 30 min of incubation (Fig. 2A, lane 4; Fig. 2B, lane 2). The resulting in vitro RNA species were stable even after 60 min of incubation (Fig. 2B, lanes 3 and 4). The data of Fig. 2C show a percentage of conversion yield rather than a reaction rate, because undiluted nuclear extract (protein concentration of 4.86 mg per ml of reaction) was used in this experiment (see Fig. 5 and below).

To calculate the percentage of conversion, the bands derived from the processed RNAs (2.7, 2.2, and 1.5 kb) and the unspliced 3.3-kb RNA were excised from the gel, and their radioactivity was measured and normalized to molar amounts. The sum of cpm in the spliced RNAs was then divided by the sum of cpm in all of the bands to obtain the percentage of conversion. Although this value does not represent an absolute percentage of conversion (i.e., percentage of spliced RNAs versus input RNA in the incubations), this quantitation method was used because only 60%-70% of the input RNA was recovered in the bands present in the agarose gels.

Accurate Splicing of in Vitro E2 Transcripts. The accuracy of the in vitro splicing reaction at the nucleotide level was examined. The linearized DNA plasmid SH2 (3 μg) was transcribed with SP6 RNA polymerase in the presence of unlabeled nucleotides. The unlabeled 3.3-kb precursor RNA was purified and incubated in the presence of the nuclear extracts. The in vitro-processed RNAs were glyoxylated and fractionated on agarose gels. The nucleotide sequence of the splice junctions in the in vitro-processed 1.5-kb RNA was compared to the 3.3-kb precursor RNA. The E2 intron nucleotide sequence was expected to be detected in the 3.3-kb RNA precursor (Fig. 3D). Fig. 3 A and B shows the result of this experiment. The nucleotide sequence in the in vitro-processed 1.5-kb RNA demonstrated faithful and accurate in vitro splicing for the splice junction between the 5' acceptor of the main body and the 3' donor of the second leader sequence. The accuracy of in vitro splicing for the splice junction between the first and second leader sequences in the 1.5-kb RNA was not determined directly. However, primer extension analysis revealed the expected size for the major cDNA product, indicating accurate in vitro splicing between the first and second leaders (data not shown).

To determine the structure of the in vitro-generated 2.7- and 2.2 kb RNAs (Fig. 2), the 3.3-kb $^{32}$P-labeled precursor RNA was incubated in the presence of the nuclear extract. After incubation, the RNAs were purified and hybridized to a single-stranded DNA from a recombinant M13 phage that contains the leftward strand of the Ad2 DNA (coordinates 58.5-70.7; 1900 nucleotides upstream of the 77-nucleotide second leader; see Fig. 1). Hybrids were digested with RNase T1 and filtered through nitrocellulose filters under conditions that only single-stranded DNA will bind (23). The RNAs were then eluted from the filter and analyzed on glyoxal gels. As shown in Fig. 4 (lanes 3–5), the in vitro processed RNAs generated a major RNase T1-resistant band derived from the 1.5-kb spliced RNA of 1.1 kb. The RNase T1-resistant RNA bands derived from the RNA precursor (2.6 kb) and the other in vitro-spliced RNAs (1.9 and 1.6 kb) are fainter than the protected 1.1-kb RNA (Fig. 4, lanes 3–5; see faint bands migrating above the 1.1-kb RNA). The relative intensities of those bands agree with the results shown by direct sizing (compare Fig. 2B, lane 2, and Fig. 4, lanes 3–5). These results suggest that the 2.7- and 2.2-kb RNAs detected after incubations are RNAs in which either the 3' or the 5' intron (see Fig. 1) has been removed during in vitro splicing.

Reaction Parameters. To characterize the splicing reaction, I examined the optimal concentration of the nuclear extract during in vitro incubations. Increasing dilutions of the extract were added to the reaction mixture. The higher percentage of conversion was attained with undiluted extract (4.86 mg of protein per ml of reaction mixture). However,
Fig. 2. Time course of the *in vitro* processing for SP6-E2 RNAs. The plasmid pSH₂ was linearized with Sal I and Bgl II restriction enzymes and *in vitro* transcriptions were carried out as described (19). The pre-RNAs (36 pmol of the total 3.4-kb RNA in 15-μl reaction mixture volume and 30,000 cpm) were incubated for the indicated times in the presence of a nuclear extract (final concentration in reaction, 4.86 mg/ml) prepared from MOPC-315 cells (17). Equal aliquots (33% of the splicing reaction; 10,000 cpm) were subjected to electrophoresis in a 1.4% agarose gel. The bands were excised, solubilized with NCS, and counted. Molecular weight RNA standards transcribed from the SH₂ plasmid DNA linearized with a battery of restriction enzymes, at different points from the SP6 promoter, were run in parallel. (A) *In vitro*-transcribed RNAs (10,000 cpm) from the SH₂ plasmid linearized with Sal I and *Kpn I* (lane 7), *Xho I* (lane 6), and *Bgl II* (lane 5). The *in vitro*-transcribed 3.3-kb RNA from the SH₂ digested with *Sal I* and *Bgl II* after incubation with the nuclear extract for 2 min (lane 1), 5 min (lane 2), 15 min (lane 3), and 30 min (lane 4). (B) *In vitro*-transcribed RNAs from the SH₂ plasmid linearized with *Sal I* and *Xho I* (lane 1) or *Bgl II*, 2000 cpm (lane 5). The *in vitro*-transcribed 3.3-kb RNA after incubation with the nuclear extract for 30 min (lane 2), 45 min (lane 3), and 60 min (lane 4). (C) The percentage of conversion was calculated as described in the text.

Fig. 3. Accuracy of the *in vitro* splicing reaction. The plasmid SH₂ DNA (2 μg) was linearized with *Sal I* and *Bgl II* and transcribed *in vitro* with SP6 RNA polymerase in the presence of unlabeled nucleotides. Approximately 720 pmol of the total 3.4-kb RNA (6 μg of RNA) in a 300-μl reaction mixture volume was incubated for 30 min at 30°C in the presence of a nuclear extract prepared from MOPC-315 mouse myeloma cells. After incubation, the RNAs were sedimented through a 5.7 M CsCl cushion, glyoxylated, and fractionated on 1.4% agarose gels. 32P-labeled RNAs were run in parallel as markers. The RNAs were excised from the gel, eluted as described (22), and hybridized to a 147-nucleotide *Xho I/Bal I* DNA primer 32P-la
ded at the 5' end *Xho I* site (C; see ref. 17). The DNA-RNA hybrids were extended with reverse transcriptase in the presence of a mixture of unlabeled deoxy- and 2',3'-dideoxynucleotides as described (17). (A) Nucleotide sequence of the splice junctions in the *in vitro*-processed 1.5-kb RNA. (B) Nucleotide sequence of the exon—intron boundary in the unspliced SP6—E2 3.3-kb pre-RNA. The same amount of unlabeled in *vitro*-transcribed 3.3-kb RNA as for the splicing reaction was used. (C) Representation of the nucleotide sequence analy-
sis. (D) Nucleotide sequence in the RNA precursor and the RNA product. A, C, G, and T denote the specific dideoxy NTP inhibitor used in the reaction.
RNA precursors in a 15-μl reaction mixture volume was used, the percentage of conversion was low (data not shown).

DISCUSSION

I have shown that in vitro-synthesized Ad2 E2 pre-mRNAs were accurately and efficiently spliced in vitro by nuclear extracts prepared from MOPC-315 mouse myeloma cells. The pre-mRNAs were synthesized in vitro by using a plasmid in which a highly efficient SP6 bacteriophage promoter was fused to the Ad2 E2 gene. The nucleotide sequence analysis across the splice junctions in the in vitro-processed RNAs unambiguously showed that in vitro splicing has the same precision as in vivo splicing.

The system described in the present study uses RNA substrates that can be prepared in large quantities and labeled to a high specific activity, allowing relatively simple assays. This in vitro splicing system has a high efficiency of conversion (60%-80%) and, therefore, has great advantages over the reported coupled or uncoupled systems (23-26).

The in vitro-synthesized RNA used in the present study as a splicing substrate contains a 3' exon 982 nucleotides downstream from the acceptor splice site (Fig. 1). Sequences toward the 3' end of the genuine E2 mRNA precursor are therefore dispensable for in vitro splicing. These results indicate that polyadenylation is not a required step for in vitro splicing, in accordance with the observation of others in in vivo systems (19, 23-26) and also in vivo (27). The in vitro-synthesized SP6–E2 pre-RNAs are not capped; however, the role of capping in splicing is uncertain, because we do not know at present if the transcripts are capped during incubations with the extracts.

In vitro-synthesized RNAs that have only a 5' donor splice site were not cleaved in this in vitro splicing system (unpublished results). This result suggests that the endonucleolytic cleavage requires nucleotide sequences of the donor and acceptor splice sites. The excised intron sequences were not detected in this system; however, I cannot rule out a rapid degradation of those RNAs during the incubations. Further purification of the nuclear extract might enable the detection of those RNAs.

In vitro splicing occurs rapidly and without any detectable lag. I have detected RNA splicing even after 2 min of incubation. The lag reported by workers in other laboratories (23–
Fig. 6. Effect of temperature of incubations on in vitro splicing. SP6–E2 3.4-kb pre-RNAs labeled with $^{32}$P were incubated (as in Figs. 2 and 5) at different temperatures and for different times in the presence of a 1:20 dilution (in buffer described in Fig. 5) of the nuclear extract (final protein concentration in reaction, 0.25 mg/ml). Lane 1, SP6–E2 pre-RNA without extract; lanes 2 and 3, pre-RNAs incubated at 0°C for 10 or 20 min, respectively; lanes 4 and 5, pre-RNAs incubated at 10°C for 10 or 20 min, respectively; lanes 6 and 7, pre-RNAs incubated at 20°C for 10 or 20 min, respectively; lanes 8 and 9, pre-RNAs incubated at 30°C for 10 or 20 min, respectively; lane 10, SP6–E2 pre-RNA incubated in the presence of a 1:10 dilution of the nuclear extract for 30 min.

26) could be due to the different reaction conditions, particularly the pre-RNA substrate concentrations used. The time courses described in this study were carried out with 36 pmol of pre-mRNA in a 15–μl reaction mixture volume. In contrast, 1/4000 that amount of precursor RNA was used by Hernandez and Keller (26). It is also important to point out that the percentage of conversion was very low when <0.05 pmol of E2 pre-mRNAs in a 15-μl reaction mixture volume was used. The same result was obtained when in vitro-synthesized E2 RNAs (0.002 pmol in 15–μl reaction mixture volume) in HeLa cell extracts from the major late promoter of Ad2 were used as substrates (data not shown). One explanation for the described results is that the in vitro formation of the heterogeneous ribonucleoprotein-like structures, which would serve as the actual substrates for the splicing enzymes, may depend on the concentration of pre-mRNA added to the reaction mixtures. Alternatively, it could be that different RNA substrates have different reaction parameters for splicing. Although conditions in which splicing proceeds with linear kinetics (Figs. 5 and 6) were attained in this study, further kinetic analyses were hampered by the fact that only 60–70% of the input RNA was recovered in the bands present in the agarose gels. Therefore, absolute rates of conversion are difficult to calculate in this system. Further purification of the activity may overcome this problem. The splicing activity has an optimal ionic strength between 25 and 50 mM ammonium sulfate, and it does not appear to require divalent cations (data not shown). This characteristic distinguishes mRNA splicing from that of tRNA splicing in which the ligase requires divalent cations (28) and the autocatalytic splicing of Tetrahymena RNA, which also requires divalent cations (18). The splicing activity requires exogenous nucleotides (ATP and/or GTP), and the optimum pH in Tris·HCl buffer was between 6 and 7.4 (data not shown). We have recently attained a 10- to 20-fold purification of the splicing activity by blue-dextran chromatography (unpublished results). This result indicates that further purification of the splicing activity can now be achieved using the system described in this study.

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