
Requirement of ATP in the second step of the pre-mRNA splicing reaction

Hitoshi Sawa, Mutsuhito Ohno, Hiroshi Sakamoto and Yoshiro Shimura*

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan

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ABSTRACT

The requirement of ATP in the second step of mRNA precursor splicing was examined by dissecting the two steps of the in vitro splicing reaction using a heat-treated nuclear extract from HeLa cells. When a mRNA precursor containing two exons and a single intron from the δ -crystallin gene was initially incubated for 60 min with the heated extract, thereby allowing only the first step of the splicing reaction to occur, and subsequently with a normal extract for 10 min, the final spliced product was produced without any lag. The production of the spliced molecule during the second incubation with the normal extract represents conversion of the intermediates already formed with the heated extract into the spliced product. The conversion was stimulated by the addition of ATP during the second incubation and inhibited by a nonhydrolyzable ATP analogue. These results led us to conclude that ATP is required for the second step of the splicing reaction.

INTRODUCTION

The development of efficient in vitro splicing systems has led to considerable progress in understanding the mechanism of nuclear precursor mRNA (pre-mRNA) splicing (for review, see refs 1-4). It has been well established that the splicing reaction of pre-mRNA consists of two steps. In the first step, pre-mRNA is cleaved at the 5' splice site resulting in the production of two types of intermediate molecules, namely a linear first exon and a lariat RNA containing the second exon and intron. In the second step, cleavage at the 3' splice site and ligation of the exons occur, thereby releasing the intact intron in a lariat configuration.

The in vitro splicing reaction of pre-mRNA with mammalian and yeast extracts is known to require ATP (5-8). Other ribonucleotide triphosphates do not efficiently substitute for ATP, and ATP analogues containing nonhydrolyzable α - β or β - γ bonds do not support the splicing reaction (7-9). ATP is required for the first step of pre-mRNA splicing, (7,10,11). It has been shown that the association of splicing factors with pre-mRNA to form a splicing complex called the spliceosome does not occur without ATP (12,13). It appears that ATP is necessary for reaction(s) occurring at relatively early stages of spliceosome formation (14), although such a reaction(s) still remains to be clarified. It is not clearly understood whether ATP is required for the second step of the splicing reaction. We undertook a close examination of this problem, taking advantage of the previous finding (15) that the second step of the reaction is selectively inhibited if HeLa nuclear extracts are heated at 45°C for 10 min. Here we show that the second reaction step also requires ATP.

MATERIALS AND METHODS

Chemicals and Enzymes.

[α -³²P] GTP (400 Ci/mmol) was obtained from Amersham. Other nonradioactive nucleotides including cap analogues were purchased from Pharmacia. SP6 RNA polymerase and restriction enzymes were obtained from Takara Shuzo Co.

Plasmid Construction and Preparation of Pre-mRNA.

A DNA fragment containing two exons (exon 14 and 15) and an intron (intron N) of the δ -crystallin gene (16) was obtained from p δ EX14-15 (17,18) and inserted into the plasmid pSP65 that had been digested with EcoRI and SmaI. The new plasmid thus obtained (designated pSP14-15) was linearized with SmaI and transcribed in vitro with SP6 RNA polymerase as described previously (19). The pre-mRNA was purified by electrophoresis on a 5 % polyacrylamide gel containing 8 M urea.

In Vitro Splicing of Pre-mRNA

HeLa nuclear extracts were prepared as described previously (17). Unless otherwise stated, the splicing reaction was carried out in 10 μ l of the reaction mixture described previously (18) except that the concentration of ATP was lowered to 100 μ M. The moderate heat treatment of the extract was performed by incubation at 45°C for 10 min as reported previously (15). In all ATP-minus reactions, creatine phosphate was omitted to minimize the regeneration of ATP.

Analysis of Spliceosome Formation

The in vitro splicing reaction of δ EX14-15 pre-mRNA was carried out under the standard conditions except that the volume of the reaction mixture was increased to 100 μ l. The reaction mixture was chilled on ice and loaded on a 10-30 % glycerol gradient in 20 mM HEPES (pH 7.9), 50 mM KCl, 1.5 mM MgCl₂, 1 mM DTT and centrifuged at 50,000 rpm for 105 min in a Spinco SW55Ti rotor at 4°C. Fractions were collected from the bottom of the tube, and their Cerenkov count was measured.

RESULTS AND DISCUSSION

As reported previously (18), when δ EX14-15 pre-mRNA was incubated with a HeLa nuclear extract, the spliced product as well as intermediate molecules produced during the first step of the reaction were detectable after 30 min (Fig.1B, lane 2). None of these RNA molecules were produced in 10 min (Fig.1B, lane 1). Even after 30 min they were not detectable if ATP was not added to the reaction mixture (Fig.1B, lane 3). It has been demonstrated that if a HeLa nuclear extract is incubated at 45°C for 10 min, the first step of the in vitro splicing reaction of pre-mRNA occurs but the second step of the reaction is blocked (15). Thus, when δ EX14-15 pre-mRNA was incubated at 30°C for 60 min with a HeLa nuclear extract that had been heat-treated as above, the 5' exon (exon 14) and the intron-3' exon lariat RNA accumulated but no spliced

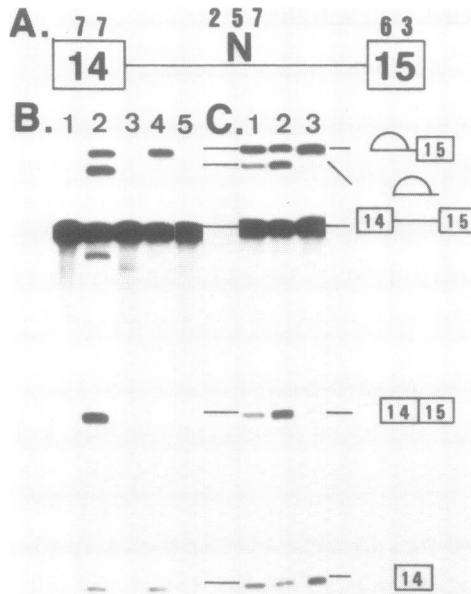


Fig.1. Requirement of ATP for the second step of pre-mRNA splicing. A. Schematic representation of δ EX14-15 pre-mRNA. The boxes represent exon sequences, and the line between them indicates an intron sequence. The numbers within the boxes and the letter above the line show specific exons and an intron, respectively, of the δ -crystallin gene (10). The 5' terminus of pre-mRNA is at the left side of the diagram. The lengths of the exons and the intron are indicated above them. B. *In vitro* splicing of δ EX14-15 pre-mRNA with a normal or a heat-treated HeLa nuclear extract. The reaction mixture was incubated with the normal extract at 30°C for 10 (lane 1) or 30 min (lane 2) in the presence of 100 μ M ATP or for 30 min in the absence of ATP (lane 3). With the heated nuclear extract, incubation was performed for 60 min in the presence (lane 4) or absence (lane 5) of 100 μ M ATP. C. *In vitro* splicing of δ EX14-15 pre-mRNA with a combination of the heated and normal HeLa nuclear extracts. The reaction mixture (10 μ l) was incubated at 30°C for 60 min with the heated nuclear extract in the presence of ATP, followed by further incubation for 10 min after addition of the reaction mixture (10 μ l) containing the normal nuclear extract with (lane 2) or without (lane 1) the addition of 100 μ M ATP, or of 100 μ M ATP plus 1 mM AMPPCP (lane 3). In all cases, pre-mRNA was absent in the second reaction mixtures.

product was detectable (Fig.1B, lane 4). It should be pointed out that this was also the case when the reaction mixture was incubated with the heated extract for 70 min or for 60 min

followed by additional incubation for 10 min with the further addition of 6 μ l of the heated extract and 100 μ M ATP (data not shown). As was consistent with the previous reports (5-8), the first step of the splicing reaction did not take place in the absence of ATP (Fig.1B, lane 5).

When the normal nuclear extract was added to the reaction mixture which had been incubated with the pre-heated extract at 30°C for 60 min, the spliced product was detectable after 10 min (Fig.1C, lane 1), indicating that the second step of the reaction occurred during the 10 min incubation. The production of the spliced product was greatly increased when ATP was added during this incubation (Fig.1C, lane 2). If a nonhydrolyzable ATP analogue, β - γ methylene ATP (AMPPCP), was added to the reaction mixture during the 10 min incubation in addition to ATP, the production of the spliced product was inhibited (Fig.1C, lane 3). These results suggest that ATP is required for the second step of the splicing reaction. The production of the spliced product without the addition of ATP during the second incubation (Fig.1C, lane 1) is likely due to the presence of residual ATP in the reaction mixture added during the initial incubation with the heated nuclear extract. The production of the spliced product during the additional incubation with the normal nuclear extract seems to be due to conversion of the intermediates produced during the incubation with the pre-heated extract and not to the de novo product from pre-mRNA during the additional incubation, because the spliced molecule was not produced when incubated with the normal extract for 10 min as described above.

To further confirm the notion that the splicing intermediates formed during incubation with the pre-heated extract were converted to the final spliced product, the following experiments were performed. The reaction mixtures incubated at 30°C for 30 min with a normal HeLa nuclear extract and for 60 min with the pre-heated extract were individually sedimented through a 10-30 % glycerol gradient to separate the spliceosome fractions. In the sedimentation profile obtained with the normal nuclear extract, we found at

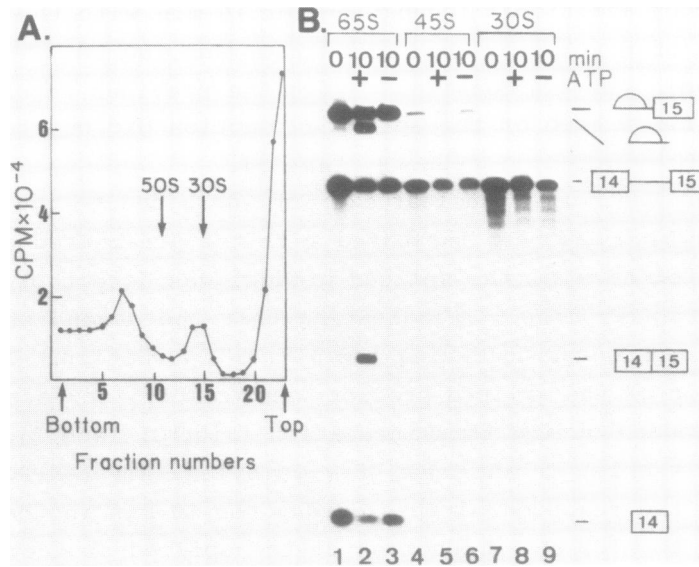


Fig.2. Requirement of ATP in conversion of intermediates in the spliceosome fractions into the spliced product. A. The splicing reaction was performed at 30°C with a heated HeLa nuclear extract for 60 min. After incubation 100 μ l of the reaction mixture (containing about 5×10^5 cpm pre-mRNA) was sedimented through a 10-30 % glycerol gradient as described in Materials and Methods. Nine-drop fractions (180 μ l) were collected from the bottom of the tube and their Cerenkov count was measured. The positions of the 50S and 30S ribosomes from *E. coli* are indicated. B. *In vitro* splicing reaction using the spliceosome fractions, obtained with the heated nuclear extract. Fractions 7, 11 and 15 in Panel A, representing the 65S, 45S and 30S complexes, respectively, were each divided into 3 equal portions and incubated for 10 min with a normal HeLa nuclear extract in the presence (lanes 2, 5 and 8) or absence (lanes 3, 6 and 9) of ATP. Lanes 1, 4 and 7 represent samples without incubation (0 time).

least three complexes sedimenting at about 65S, 45S and 20S (data not shown). Of these, the 65S complex represents the spliceosome, since the splicing intermediates are predominantly present in this complex (data not shown). The 45S and 20S complexes are thought to be a precursor form of the spliceosome and a non-specific complex, respectively (20, 21). In the case of the sedimentation profile obtained with the pre-heated extract, the 65S complex was formed but the 45S

complex was not apparent (Fig.2A). Instead a complex of approximately 30S was detected. It is worth noting, however, that if the reaction mixture was sedimented similarly after 30 min incubation, the 45S complex was clearly present and the amount of the 65S complex was much less than that observed in Fig.2A (data not shown). It is likely that the 45S complex was present in the reaction mixture with the pre-heated extract even after 60 min, though not as much as in the samples after 30 min incubation.

Fractions 7, 11 and 15 of Fig.2A corresponding to the 65S, 45S and 30S complexes, respectively, were individually incubated with a normal HeLa nuclear extract at 30°C for 10 min in the presence or absence of ATP. As shown in Fig.2B, the final spliced product was produced from the 65S complex if ATP was added to the reaction mixture. The spliced product was hardly produced, if at all, from the 45S complex and could not be detected from the 30S complex. These results indicate that the intermediate molecules present in the spliceosome fraction are converted into the final spliced product and that ATP is required for this conversion.

It has been reported that at least two nuclear factors (SF3 and SF4A) are required for the second step of pre-mRNA splicing and that one of the two factors (SF3) is inactivated by moderate heat treatment (15). The present study unambiguously shows that the second step of the splicing reaction, like the first step, requires ATP. It remains to be clarified, however, for what specific reaction(s) in the second step of pre-mRNA splicing, ATP is required. In view of the fact that the number of phosphodiester bonds is conserved during the second step as is the case with the first step, it is possible to assume that the second step of pre-mRNA splicing may proceed by an isoenergetic transesterification mechanism. Thus, the involvement of ATP in the second step would not necessarily be in the simple ligation reaction joining the two exons. It is tempting to assume that the requirement of ATP in the second step is associated with the function(s) of SF3 or/and SF4A. In any case, elucidation of

the functions of these factors will provide a vital clue to understanding the mechanism of the second step of pre-mRNA splicing.

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*To whom correspondence should be addressed

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