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# Alterations of RNase H sensitivity of the 3′ splice site region during the *in vitro* splicing reaction

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## ABSTRACT

**We have developed a splicing assay system with an immobilized pre-mRNA to study the mechanism of the splicing reaction after spliceosome assembly. Using this system, we have found that the second step of the splicing reaction could be dissected into two stages. After the 5′ splice site reaction, at least two factors interact with the pre-formed spliceosome containing intermediate molecules in an ATP-independent manner to convert the spliceosome into a form competent for the 3′ splice site reaction. Then, the 3′ splice site reaction occurs on this spliceosome, if ATP is supplied to the reaction mixture. We have also investigated the dynamic state of the 3′ splice site region in the spliceosomes during the splicing reaction by probing with RNase H sensitivity. Prior to the 5′ splice site reaction, the 3′ splice site region was protected from RNase H attack. The region became sensitive immediately after the 5′ splice site reaction, and subsequently became resistant again as the spliceosome competent for the 3′ splice site reaction was formed. These results suggest that the interaction of the 3′ splice site region with some spliceosome components changes significantly during the splicing reaction.**

## INTRODUCTION

Nuclear pre-mRNA splicing occurs via two step reactions (for reviews see refs 1, 2). In the first step, pre-mRNA is cleaved at the 5′ splice site to produce a branched RNA molecule containing a second exon and an intron (lariat intermediate) and a linear first exon (5′ splice site reaction). The second step involves the cleavage at the 3′ splice site and the ligation of the two exons (3′ splice site reaction). Prior to the 5′ splice site reaction, pre-mRNA becomes associated with a number of components to form a complex, the spliceosome, in which the splicing reactions occur.

The spliceosomes were originally identified by density gradient sedimentation as 40–65S complexes formed under the conditions where the splicing reaction took place (3–5). A number of components including U1, U2, U4, U5, and U6 small nuclear ribonucleoprotein particles (snRNPs) are involved in these complexes (for reviews, see refs 6, 7). Affinity chromatography (8, 9) or native gel electrophoresis (10–15) of splicing reaction mixtures have revealed that these components are assembled in an ordered fashion to form the spliceosomes.

A few kinds of functional spliceosomes were isolated by density gradient sedimentation (16–18) or gel filtration chromatography (19, 20) from the reaction mixtures in which the reactions were blocked at specific stages of the splicing pathway. In mammalian system, the 5′ splice site reaction and the 3′ splice site reaction are blocked by adding EDTA to the reaction mixture (20) and by using a pre-heated nuclear extract (17, 19), respectively. In the yeast splicing system, when the extracts prepared from *prp*2 (16) and *prp*18 (18) mutants were used, the splicing reactions were blocked at the respective steps of the pathway. In all cases, the spliceosome fractions could be complemented by addition of various extracts which were inactive in the splicing reaction by themselves, suggesting that these spliceosomes represent the functional intermediate complexes in the splicing pathway. From these studies, it has been shown that both 5′ splice site and 3′ splice site reactions require additional protein factors and ATP. In mammalian system, however, these factors have not been characterized clearly, although factors required for the first and/or the second step reactions have been reported. In the case of the second step reaction, the requirement of at least two factors has been shown (21, 22). However, the functions of these protein factors have not been clarified. Nor has the role of ATP involved in the splicing reaction been understood.

To analyze the mechanism of the late events of the splicing pathway, we have developed a splicing assay system using an immobilized pre-mRNA (the solid-phase splicing assay). This system enabled us to recover splicing complexes including the spliceosomes rather easily and to characterize the complexes of the splicing reaction. Using this system, we could dissect the second step into two stages. Moreover, we have examined the interaction of spliceosome components with the 3′ splice site region using the RNase H-directed cleavage assay developed previously (23), and have found that the sensitivity of the 3′ splice site region to RNase H changes significantly during the splicing reaction.

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## MATERIALS AND METHODS

### Materials

Klenow fragment and RNase H were from Takara Shuzo Co. Amino-allyl dUTP was from Sigma. Sulfosuccinimidyl 6-(biotinamido) Hexanoate (NHS-LC-biotin) and streptavidin agarose were purchased from Pierce Chemicals. Oligodeoxynucleotides were synthesized as described previously (24).

### Immobilization of pre-mRNA

$^{32}$P-labeled δEX 14−15 pre-mRNA was synthesized *in vitro* as described previously (17). About 10 pmol of the pre-mRNA was hybridized to 150 pmol of an oligodeoxynucleotide of 41 nucleotides (nt) in length (5′-TTCATCCATTCATCCATT-CA*GGGTACCCGATCAGTAGACAG*), a 3′ portion of which (indicated by italics) was complementary to the 3′ end of the pre-mRNA, in 50 μl solution containing 14 mM Tris-HCl (pH 7.5), 40 mM NaCl and 0.2 mM EDTA, by heating at 100°C and slowly cooling to room temperature for 40 min. Then, the pre-mRNA was elongated at 37°C for 1 hr by addition of an equal volume of solution containing 14 mM $MgCl_2$, 0.4 mM each of dATP, dGTP and amino allyl dUTP, 2 mM DTT, 200 units of RNasin and 32 units of Klenow fragment. The elongated pre-mRNA was purified by electrophoresis on a 4% denaturing polyacrylamide gel, and then biotinylated with NHS-LC-biotin as described previously (25). About 100 fmol of the biotinylated pre-mRNA was bound to 5 μl of packed streptavidin agarose beads by gently vortexing at 4°C for 1 hr in buffer E (12 mM Hepes-NaOH (pH 7.9), 60 mM KCl, 1.5 mM $MgCl_2$, 0.12 mM EDTA, 12% glycerol) containing 0.1% NP-40, 0.1 mg/ml glycogen, 1 mg/ml BSA and 0.1 mg/ml yeast tRNA, and then the beads were washed with buffer E.

### Solid-phase splicing assay

HeLa cell nuclear extracts and the S100 fraction were prepared as described previously (26). Heat-treatment of the extracts was performed by incubation at 45°C for 16 min. MNase-treatment was carried out as described previously (27). Unless otherwise stated, the splicing reaction was performed in an incubator shaker in 20−50 μl solution containing 1.5 mM $MgCl_2$, 20 mM creatine phosphate, 0.5 mM ATP, 60% extracts or fractions in buffer D (26) and less than 5 μl RNA agarose. After each incubation, the RNA agarose was washed 5 times with buffer E, and transferred to new test tubes. After final incubation and subsequent washing, RNAs bound to the agarose beads were eluted by boiling in buffer G (0.2 M sodium acetate, pH 5.2, 10 mM EDTA, 1% SDS) for 3 min. The eluted RNAs were precipitated with ethanol and analyzed by electrophoresis on a 6% polyacrylamide/8 M urea gel.

### Fractionation of splicing factors

The S100 fraction (12 ml of 20 mg/ml protein) was dialyzed against buffer F (20 mM Hepes-NaOH (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 10% glycerol) containing 0.05 M KCl, and then loaded onto a DEAE-cellulose column (Whatman DE-52, 2.5×8 cm) which had been equilibrated with 0.05 M KCl/buffer F. After collecting the flow-through fraction (DEAE-I), the column was washed with 3 column volumes of 0.05 M KCl/buffer F. Subsequently, the column was eluted with 0.2 M KCl/buffer F and 2 ml fractions were collected. The absorbancy at 280 nm of each fraction was measured. The peak fractions were pooled, adjusted to 0.1 M KCl by dilution with buffer F, and applied to a Bio-Rex 70 column (Bio-Rad, 2.5×4 cm) which had been equilibrated with 0.1 M KCl/buffer F. After washing with 3 column volumes of 0.1 M KCl/buffer F, the column was eluted with 0.5 M KCl/buffer F. The pooled protein peak fractions were adjusted to 0.1 M KCl, and applied to a Mono Q column (Pharmacia, 1 ml) previously equilibrated with 0.1 M KCl/buffer F. After washing with 5 ml of 0.1 M KCl/buffer F, a linear gradient between 0.1 M KCl/buffer F and 0.5 M KCl/buffer F (total volume 15 ml) was applied and 0.5 ml fractions were collected. The HLF activity of each fraction was assayed by complementation assay with the heated nuclear extract. The active fractions were pooled (HLF). The pooled DEAE-I and HLF fractions were dialyzed against buffer D.

### RNase H-directed cleavage assay

Washed RNA agarose was incubated with 20 μl buffer E containing 2 units RNase H and 0.2 μg of an oligodeoxynucleotide of 16 nt in length complementary to the 3′ splice site region of the pre-mRNA (from −8 to +8 with respect to the 3′ splice site) for 20 min.

## RESULTS

### Immobilization of pre-mRNA on the agarose beads

The *in vitro* splicing assay system with an immobilized yeast pre-mRNA was previously constructed and used for the analysis of early events of the splicing reaction (25). In this system, the pre-mRNA was bound to a solid support through base-pairing with a small biotinylated RNA which was complementary to the 3′ end of the pre-mRNA. Although this assay system could also be potentially useful for the analysis of late events of the splicing reaction, there was a drawback in the procedure. During incubation with cell-free extracts, a substantial amount of the pre-mRNA was released from the solid support. This limited the applicability of this method to the analysis of late events of the splicing reaction. To circumvent this impasse, we developed another immobilizing system in which the pre-mRNA was directly biotinylated near its 3′ end (Figure 1A). In this system, pre-mRNA containing a single intron synthesized *in vitro* was elongated with Klenow fragment in the presence of amino-allyl

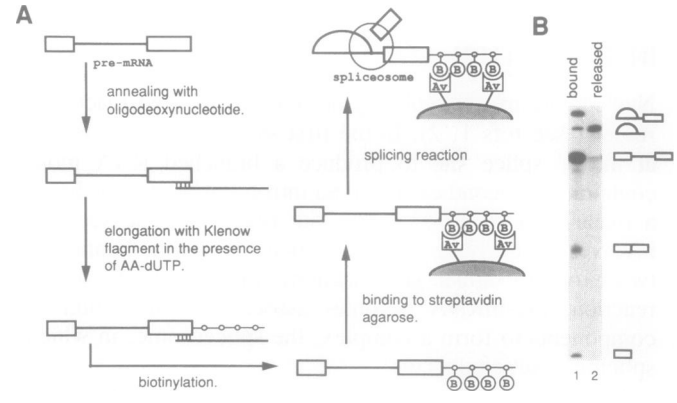


**Figure 1.** Solid-phase splicing assay. (A) Scheme for the immobilization of pre-mRNA. (B) The $^{32}$P-labeled immobilized pre-mRNA was incubated with a HeLa nuclear extract for 30 min. After incubation, RNAs were extracted from the agarose beads (lane 1) or from the first wash of the beads (lane 2), and electrophoresed on a 6% polyacrylamide/8 M urea gel. The gel was then autoradiographed. The structure of each RNA species is illustrated schematically on the right of the figure.

dUTP and an oligodeoxynucleotide complementary to the 3′ end sequence of the pre-mRNA. The resulting RNA-DNA chimeric molecule was biotinylated specifically at the sites of incorporated amino-allyl deoxyuridine residues and was bound to streptavidin agarose beads.

The immobilized pre-mRNA was efficiently spliced with a nuclear extract from HeLa cells (Figure 1B). After the splicing reaction, only the excised lariat intron which had no biotinylated residues was released from the agarose beads (lane 2), but other splicing intermediates and the spliced product remained bound to the solid support as well as the pre-mRNA (lane 1).

### Separation of the second step reaction into two stages

When the immobilized pre-mRNA was incubated with a heated nuclear extract, only the first step reaction occurred, leading to the accumulation of the lariat intermediate and the free 5′ exon (Figure 2, lane 1). This is consistent with the results obtained previously with the unimmobilized pre-mRNA (21). After extensive washing of the agarose beads containing ribonucleoprotein complexes (RNA agarose), a micrococcal nuclease (MNase) treated nuclear extract which is known to be inactive in the splicing reaction by itself was added without ATP. As consistent with the previous results (17, 19), the second step reaction did not occur under such conditions (Figure 2, lane 2). After this incubation, RNA agarose was extensively washed and subsequently incubated with buffer E containing ATP in the absence of any extracts and creatine phosphate. Upon this incubation, the second step reaction occurred efficiently and rapidly (lanes 3−7). The spliced product was detected after 2 min incubation (lane 3), and reached to the maximum level after 1 hr (lane 6).

It is unlikely that this reaction was due to residual non-specific proteins which remained bound to the beads even after the extensive washing, since it is difficult to imagine that this efficient reaction could occur with such scarcely remaining, if any, residual proteins. Moreover, the 3′ splice site reaction could not be observed if the second incubation was carried out at 4°C (Figure 2, lane 8). This finding seems to exclude the above argument, because such contaminating materials should remain bound to the beads even at 4°C and act in the subsequent incubation with ATP.

Accumulation of the 60−65S spliceosome was observed previously in the splicing reaction with the heated nuclear extract (17, 19). The intermediate molecules produced in the first step reaction were predominantly present in the spliceosome. These intermediate molecules were rapidly converted to the splicing products when the fractionated spliceosome was incubated with complementing extracts and ATP, suggesting that these intermediates reside in a functional spliceosome complex (17, 19). The spliceosome formed by incubation with the heated nuclear extract seems to be different from that formed by additional incubation with the MNase-treated extract, because the latter complex is capable of performing the 3′ splice site reaction if ATP alone is provided. The former spliceosome requires protein factors in addition to ATP to perform the 3′ splice site reaction (17, 19). The former complex is designated spliceosome IIa, while the latter is designated spliceosome IIb. The results shown in Figure 2 indicate that the second step

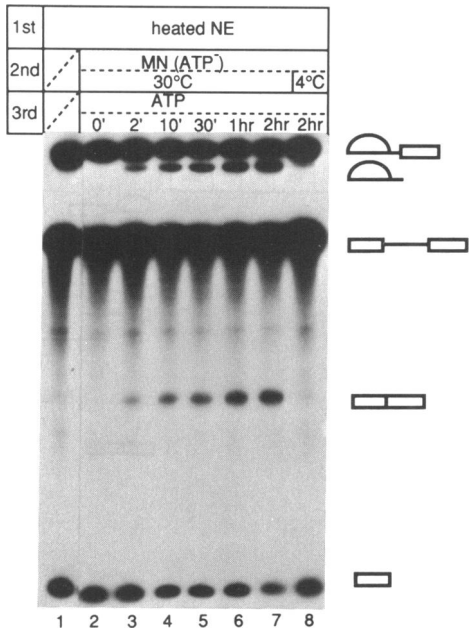


**Figure 2.** Two stages in the second step reaction. The immobilized pre-mRNA was first incubated with the heated nuclear extract for 90 min. The second incubation was performed with the MNase-treated nuclear extract in the absence of ATP and creatine phosphate for 10 min at 30°C (lanes 2−7) or at 4°C (lane 8). The third incubation was carried out in buffer E containing 1 mM ATP for the time indicated on the top of the each lane. Oblique dashed lines represent no further incubation. After incubations, RNAs were analyzed as in Figure 1. NE and MN in the figure indicate the nuclear extract and the MNase-treated extract, respectively.

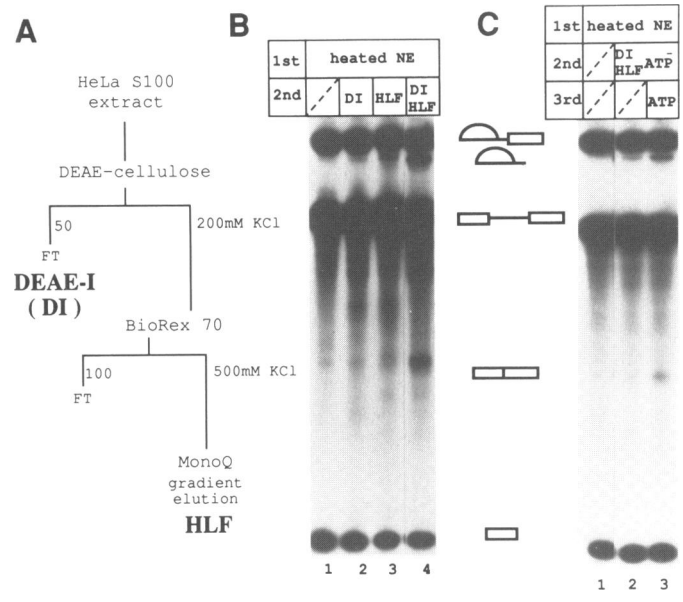


**Figure 3.** Requirement of HLF and DEAE-I for the formation of spliceosome IIb. (A) Scheme for the purification procedure of DEAE-I and HLF from the HeLa S100 fraction. The KCl concentrations of loading buffers or elution buffers are indicated. FT represents flow-through fractions. (B) The immobilized pre-mRNA was first incubated with the heated nuclear extract for 90 min. The second incubation was in 20 μl reaction mixtures containing 12 μl DEAE-I (lane 2), 12 μl HLF (lane 3), or 6 μl DEAE-I and 6 μl HLF (lane 4). After incubations, RNAs were analyzed as in Figure 1. (C) The second incubation was with equal volume of DEAE-I and HLF in the absence of ATP and creatine phosphate for 30 min. The third incubation was in buffer E containing 1 mM ATP for 1 hr. RNAs were analyzed as in Figure 1.

reaction involves the following two stages. First, protein factors interact with spliceosome IIa in an ATP-independent manner to convert it to spliceosome IIb. Second, the 3′ splice site reaction takes place in spliceosome IIb without need of further factors if ATP alone is supplied to the reaction mixture.

It is worth noting that after the 3′ splice site reaction occurred in spliceosome IIb by addition of ATP, a substantial amount of the excised lariat intron still remained on the agarose beads (Figure 2, lanes 3−7), whereas the analogous molecule produced in the splicing reaction with the nuclear extract was released from the beads (Figure 1B). This suggests that some factor(s) is required to release the lariat intron from the spliceosome after the 3′ splice site reaction occurs. The presence of such releasing factor has been shown recently in yeast (28).

## Requirement of two factors for the formation of spliceosome IIb

The heat labile factor (HLF) which is, in all likelihood, identical with SF3 reported previously by Krainer and Maniatis (21) is one of the activities required for complementing spliceosome IIa (19). This factor is present in the HeLa cytoplasmic S100 fraction as well as in the nuclear extract (21). To identify factors required for the formation of spliceosome IIb from spliceosome IIa, we partially purified HLF from the S100 fraction by three successive chromatographic procedures using the complementation assay with the heated nuclear extract (Figure 3A).

After the first step reaction occurred with the heated nuclear extract, the RNA agarose was washed extensively and then incubated with the partially purified HLF preparation in the presence of ATP. The second step reaction occurred slightly (Figure 3B, lane 3), but the reaction was greatly enhanced by the addition of a DEAE-cellulose flow-through fraction together with HLF (lane 4), indicating that the second step reaction requires some factor(s) in the DEAE-cellulose flow-through fraction (tentatively designated DEAE-I) in addition to HLF.

To examine whether these two factors are sufficient for the formation of spliceosome IIb, an experiment similar to the one described in Figure 2 was performed using HLF and DEAE-I instead of the MNase-treated crude nuclear extract in the second incubation (Figure 3C). During the final incubation with ATP, the 3′ splice site reaction occurred (Figure 3C, lane 3). These results show that HLF and DEAE-I are sufficient to convert spliceosome IIa to spliceosome IIb.

## Differential RNase H sensitivities of the 3′ splice site region

We have shown that the second step reaction occurs in two types of the spliceosomes: spliceosomes IIa and IIb. To understand the mechanism of the second step reaction, it is important to know how these two spliceosomes differ from each other. As an approach to this problem, we examined whether the conditions of the 3′ splice site are the same in the two spliceosomes. We employed the RNase H-directed cleavage assay which was developed previously (23).

After the first step reaction and the formation of spliceosome IIa were allowed to occur with the heated nuclear extract, the RNA agarose was extensively washed, and subsequently RNase H and an oligodeoxynucleotide complementary to the 3′ splice site were added. As shown in Figure 4, most of the lariat intermediate disappeared and instead, an RNA band which had the similar electrophoretic mobility with the lariat intron was generated (lane 2). However, this RNA band did not represent any products of the splicing reaction but the RNase H cleavage

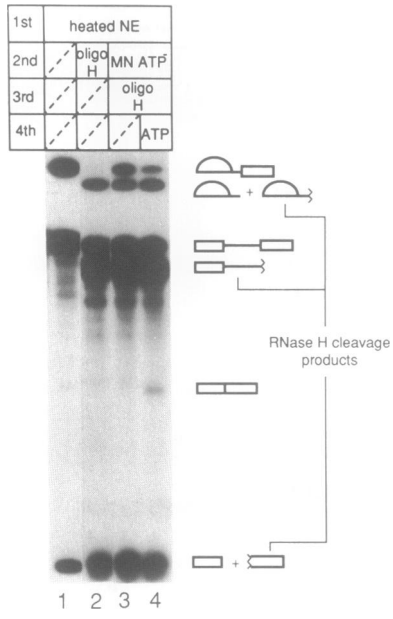


**Figure 4.** RNase H sensitivity of the 3′ splice site region in spliceosomes IIa and IIb. The immobilized pre-mRNA was first incubated with the heated nuclear extract for 90 min. The second incubation was with RNase H (H) and an oligodeoxynucleotide complementary to the 3′ splice site region (oligo) for 20 min (lane 2) or with the MNase-treated nuclear extract without ATP and creatine phosphate for 10 min (lanes 3 and 4). The third incubation was with RNase H and the oligodeoxynucleotide for 20 min. The fourth incubation was in buffer E containing 1 mM ATP for 1 hr. After incubations, RNAs were analyzed as in Figure 1.

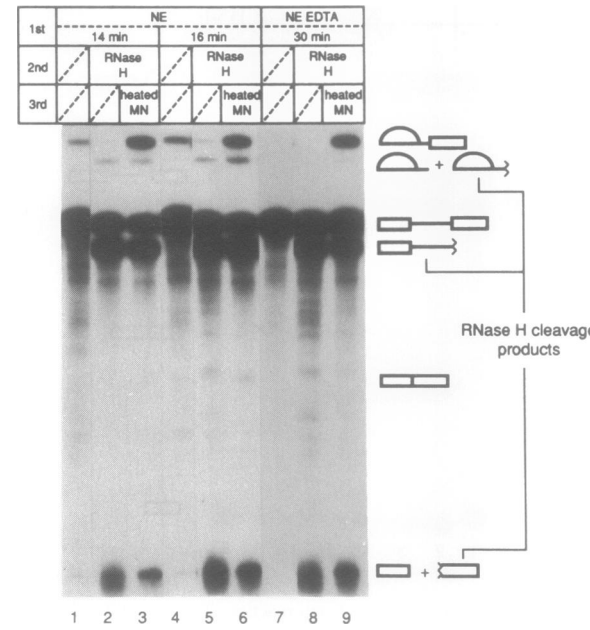


**Figure 5.** Alterations of RNase H sensitivity of the 3′ splice site region in the splicing reaction with a normal nuclear extract. The immobilized pre-mRNA was first incubated with the nuclear extract for 14 min (lanes 1−3), 16 min (lanes 4−6) or in the presence of 2.5 mM EDTA for 30 min (lanes 7−9). The second incubation was with RNase H and the oligodeoxynucleotide for 20 min. The third incubation was with the heated MNase-treated nuclear extract for 30 min.

product derived from the lariat intermediate, because the splicing factors (HLF and DEAE-I) and ATP were absent in the reaction mixture. We do not know, however, why the 5′ portion of the cleavage product was still retained on the agarose beads, despite the fact that they had no biotinylated residues. In any case, it is clear that the 3′ splice site region of the lariat intermediate in spliceosome IIa is sensitive to RNase H.

To examine the sensitivity of the 3′ splice site region in spliceosome IIb, the same assay was performed after incubation with the MNase-treated nuclear extract without ATP. During this incubation, spliceosome IIb should be formed as described in the previous section. As shown in Figure 4 (lane 3), about half of the lariat intermediate was resistant to the nuclease. If the RNA agarose was incubated with ATP after the RNase H treatment, the 3′ splice site reaction took place and the spliced product appeared with concomitant decrease of the lariat intermediate (lane 4). The occurrence of this reaction is possible only when the lariat intermediate survived from RNase H digestion is present in spliceosome IIb. These results led us to conclude that the 3′ splice site region in spliceosome IIb is resistant to RNase H.

### Sensitivity of the 3′ splice site region in the splicing reaction with normal nuclear extracts

The results described above suggest that RNase H is accessible to the 3′ splice site region in the early stage of the second step reaction but not in the late stage. To further confirm this observation, the RNase H-directed cleavage assay was applied to the splicing complexes assembled with the normal nuclear extract.

The immobilized pre-mRNA was first incubated with the normal nuclear extract for 14 min. During this incubation, the 5′ splice site reaction just began to occur, but the 3′ splice site reaction did not (Figure 5, lane 1). After this incubation, RNase H and the oligodeoxynucleotide were added. The lariat intermediate generated in the first incubation was extensively cleaved like the molecule in spliceosome IIa (lane 2). By contrast, when the first incubation was extended for 16 min instead of 14 min, we detected the lariat intermediate which was resistant to RNase H like the molecule in spliceosome IIb (lane 5). These results suggest that the RNase H sensitivity of the 3′ splice site region of the lariat intermediate changes during the second step reaction even with the normal nuclear extract.

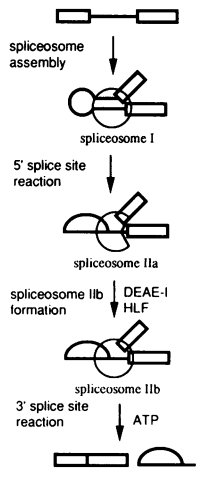


**Figure 6.** Schematic representation of the proposed four stages in mRNA splicing.

We also investigated the RNase H sensitivity of the 3′ splice site region before the 5′ splice site reaction. When the unimmobilized pre-mRNA was incubated for 14 min, the 65S spliceosomes containing unspliced pre-mRNA were observed by density gradient sedimentation analysis (data not shown). These spliceosomes which may be heterogeneous in the structure representing various stages of the complex formation are collectively designated spliceosome I. It is likely that spliceosome I was also formed when the immobilized pre-mRNA was incubated for 14 min. To analyze the sensitivity of the 3′ splice site region of spliceosome I, the RNase H-directed cleavage assay was performed after 14 min incubation of the immobilized pre-mRNA with the normal nuclear extract. As shown in Figure 5, about half of the pre-mRNA was resistant to RNase H digestion (lane 2). It is worth noting that the pre-mRNA was digested almost completely by RNase H if the first incubation with the nuclear extract was carried out in the absence of ATP (data not shown). These results suggest that the formation of early splicing complexes including spliceosome I renders the pre-mRNA resistant to RNase H. If the pre-mRNA becomes resistant to RNase H with the formation of the early splicing complexes, the 5′ splice site reaction can be complemented with a heated MNase-treated nuclear extract. Unless the entire sets of snRNPs are assembled in the early splicing complexes, the reaction may not occur with the heated MNase-treated nuclear extract in which snRNAs have been disrupted. As shown in Figure 5 (lane 3), the 5′ splice site reaction occurred efficiently with pre-mRNA which had survived from pre-treatment with RNase H, suggesting that some of the resistant pre-mRNA resided in spliceosome I. This is also the case when the first incubation with the nuclear extract was extended for 16 min (lanes 4–6). These results probably imply that the 3′ splice site region of unspliced pre-mRNA in spliceosome I is resistant to RNase H.

Essentially similar results were obtained when the first incubation was carried out in the presence of EDTA (Figure 5, lanes 7–9). It was previously reported that the 5′ splice site reaction was blocked but a functional spliceosome was formed under such conditions (20). As shown in Figure 5 (lane 9), after incubation in the presence of EDTA and subsequent RNase H treatment, the 5′ splice site reaction occurred by the addition of the heated MNase-treated extract, suggesting that the 3′ splice site region in the spliceosome that was formed in the presence of EDTA was resistant to RNase H.

## DISCUSSION

The immobilized pre-mRNA system that we developed in this study has been proved very useful for the analysis of late events of the *in vitro* splicing reaction. The salient feature of the system is that the immobilized pre-mRNA is extremely stable and not released from the solid support during prolonged incubation with HeLa nuclear extracts. This stability is apparently due to the DNA sequence of the pre-mRNA which contains amino-allyl deoxyuridine residues and is bound to streptavidin agarose beads. Using this immobilized system, it was possible to analyze the second step of the splicing reaction. In the present studies, we took a close examination on the interaction between spliceosome component(s) and the 3′ splice site region during the second step reaction.

The spliceosome complex containing the unspliced pre-mRNA was detected in the *in vitro* splicing system in which the EDTA treated nuclear extract was employed (20). The occurrence of

the spliceosome complexes containing either unspliced pre-mRNA or the intermediate molecules was observed in the *in vitro* system in which the heated nuclear extract was employed (17, 19). Of these spliceosomes, the one that contains only the intermediate molecules is designated spliceosome IIa. The intermediate molecules in spliceosome IIa were efficiently converted to the spliced product upon incubation with the complementing MNase-treated extract and ATP (19). The nuclease treated extract was substituted by at least two partially purified fractions, HLF and DEAE-I. Thus, spliceosome IIa appears to represent the complex which is through the first step reaction and ready for the second step. The 3′ splice site region of the lariat intermediate in spliceosome IIa is sensitive to RNase H digestion. We observed the similar nuclease sensitivity of the 3′ splice site region of the lariat intermediate in an early stage of the second step reaction with the normal nuclear extract. These results show that spliceosome IIa represents an authentic intermediate complex encountered in the splicing pathway.

Spliceosome IIa is converted to spliceosome IIb upon incubation with either the MNase-treated HeLa nuclear extract or both the HLF and DEAE-I fractions in the absence of ATP. The latter spliceosome must contain entire sets of factors necessary for the 3′ splice site reaction except for ATP, because the reaction takes place leading to the production of the spliced molecules by addition of only ATP. Unlike the case with spliceosome IIa, the 3′ splice site region of this complex is resistant to RNase H digestion. Although spliceosome IIb was first detected under the unnatural conditions in which ATP was not added to the reaction mixture, this complex appears to represent also an intermediate form of the normal splicing reaction, because we detected a lariat intermediate molecule whose 3′ splice site was resistant to RNase H in a late stage of the second step reaction with the normal HeLa nuclear extract. In a previous study on the RNase H sensitivity of the 3′ splice site region, however, such resistant lariat intermediate was not detected (23). It is likely that the intermediate molecules in spliceosome IIb were rapidly converted to the spliced product during the RNase digestion reaction, since the previous experiments were performed in the conventional splicing assay system in the presence of ATP.

Thus, on the basis of the sensitivity of the 3′ splice site region to RNase H, it appears that the splicing reaction proceeds via at least four stages (Figure 6). In spliceosome I which contains unspliced pre-mRNA, the 3′ splice site is resistant to the nuclease. This might be ascribed to some factors which have been reported to bind to this region such as IBP (29, 30), several hnRNP proteins (31), U2AF (32), and/or p62 (33). On the basis of the findings that the 3′ splice site region in spliceosome IIa is sensitive to RNase H but becomes resistant again as spliceosome IIb is formed, it is tempting to assume that HLF and/or DEAE-I interact with the 3′ splice site region during the formation of spliceosome IIb. Alternatively, this could also be due to long range effects induced by the binding of those factors or due to steric hindrance preventing the RNase H molecule to reach the 3′ splice site. It has been reported that different factors interact with the 3′ splice site region in the first and second steps of the splicing reaction (34). In yeast, a factor required for the second step reaction (*PRP*16) has recently been shown to bind to the spliceosome in an ATP-independent manner during the second step reaction, although the binding site remains to be determined (35). Clearly, more extensive characterization of the factors which associate with the 3′ splice site is needed to understand the mechanism of the second step splicing reaction.

## ACKNOWLEDGMENTS

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