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HEAT TREATMENT OF NUCLEAR EXTRACT ALTERS SELECTION OF THE 3' SPLICE SITE IN PRE-mRNA SPLICING

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SUMMARY: We investigated *in vitro* splicing reaction using an artificially created mRNA precursor containing a single 5' splice site and tandemly duplicated 3' splice sites. We found that the 3' splice site proximal to the 5' splice site is predominantly used under the standard splicing conditions. However, when the preheated nuclear extract was employed, the intermediate in which the distal 3' splice site was selected accumulated exclusively. This shows that heat treatment of nuclear extract abolishes the activity involved in the selection of the 3' splice sites that are in cis-competition for the common 5' splice site. The results presented here suggest the presence of a factor(s) required for the selection of the proximal 3' splice site.

Splicing of mRNA precursor (pre-mRNA) is a process that requires extreme accuracy (for reviews see, 1, 2). This process occurs by sequential two-step cleavage-ligation reactions. In the first step, pre-mRNA is cleaved at the 5' splice site resulting in the production of two kinds of intermediate molecules, namely a linear 5' exon and a lariat RNA containing the 3' exon and intron (lariat intermediate). In the second step, the ligation of two exons proceeds, thereby releasing a lariat intron. For these steps to occur accurately, the 5' and 3' splice sites should be recognized correctly.

Previous studies have shown that the conserved sequences around the splice sites play an essential role in determining the splice sites. For the 5' splice site, the consensus sequence is AG/GUAAGU and for the 3' splice site, the consensus sequence is (Y)nNCAG/G (1). The sequence around the branchpoint in the intron also plays an important role (3, 4). The conserved sequences are recognized by multiple factors including U small nuclear ribonucleoprotein molecules (U snRNPs) (1). The recognition of the sequences is also thought to be influenced by other elements such as exon sequence adjacent to the splice site (5) and/or the secondary structure of pre-mRNA (6, 7).

A question in the mechanism of the splice site selection is why the closest 5' and 3' splice sites are usually selected for splicing, when multiple introns (therefore multiple splice sites) are present. Recent progress in biochemical characterization of splicing factors provides a clue to this question. It has been reported in *in vitro* splicing system that selection of the 5' splice sites that are in cis-competition for a common 3' splice site depends on the concentration of a splicing factor called

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SF2/ASF (8, 9). It has been shown that the high concentration of SF2/ASF promotes the selection of the 5' splice site that is closer to the common 3' splice site (8, 9).

In this paper, we have investigated the mechanism of 3' splice site selection using a model pre-mRNA containing a single 5' splice site and two tandemly duplicated 3' splice sites. We and others have previously shown that moderate heat treatment of nuclear extracts depletes the activity required for the second step reaction (10, 11). Here we report that heat treatment abolishes the use of the 3' splice site proximal to the common 5' splice site. Our results suggest that a factor(s) is required in the selection of the 3' splice site proximal to the 5' splice site.

MATERIALS AND METHODS

Chemicals and enzymes [α-^{32}P]GTP (400 Ci/mmol) was purchased from Amersham. Other non radioactive nucleotides and cap analog (m^7GpppG) were from Pharmacia. SP6 RNA polymerase and restriction enzymes were obtained from Takara Shuzo Co. Ltd. (Kyoto).
Oligonucleotide The synthetic oligonucleotide, 3SS-1 (5'-TTGTTGACCTGCAAAG-3'), used for RNaseH-directed digestion was synthesized by a DNA synthesizer A380 (Applied Biosystems Inc., Co.).
Plasmid construction To construct pSP215, a StyI-SmaI fragment containing a 3' portion of intron N and exon 15 of the chicken δ–crystallin gene (12) was excised from pSP14-15 (13) and inserted into the SmaI site of pSP14-15.
Pre-mRNA preparation pSP215 was linearized for transcription with SmaI. RNA precursor was synthesized *in vitro* with SP6 RNA polymerase as described previously (13), using this linearized plasmid as template. The transcript was purified by electrophoresis on a 5 % polyacrylamide gel containing 8 M urea.
Preparation of HeLa cell nuclear extract and DEAE fractions HeLa cell nuclear extracts were prepared as described previously (14). The DEAE fractions were prepared as follows: HeLa cell nuclear extracts (2 ml, 20 mg/ml protein) were dialyzed against buffer F (20 mM Hepes-KOH (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 0.2 mM APMSF, 10 % glycerol) containing 0.05 M KCl, and then loaded onto a DEAE-cellulose column (Whatman DE-52, 1.5 × 3 cm) which had been equilibrated with buffer F containing 0.05 M KCl. Bound materials were subsequently eluted with buffer F containing 0.2 M KCl to give fraction DI (3.5 mg/ml, 5 ml), and with buffer F containing 0.5 M KCl to give fraction DII (1.4 mg/ml, 5 ml). Solid ammonium sulfate (29.1 g / 100 ml, 50 % saturated at 0 °C) was added to the DII fraction, and the mixture was gently stirred on ice for 1 hour. This mixture was centrifuged for 20 min at 20,000 rpm in the type 70 rotor (Beckman), and the supernatant was used as ASS fraction. The precipitate was dissolved in 1 ml of buffer F containing 0.05 M KCl and designated ASP fraction. All these fractions were dialyzed against buffer F containing 0.05 M KCl.
***In vitro* splicing experiment** The splicing reaction with a HeLa cell nuclear extract was carried out in 10 µl of the reaction mixture as described previously (15). The heat treatment of nuclear extract was performed at 45 °C for 23 min. In complementation experiments, the splicing reactions were carried out at 30 °C for 1 hour with 3 µl of heat treated nuclear extract supplemented with 5 µl of DEAE fraction under otherwise the standard splicing conditions.
RNaseH-directed digestion and debranching reaction The lariat intermediate RNA was recovered from the gel as described previously (15). Then the RNA was incubated with buffer F containing 5 units RNaseH and 0.2 µg of oligonucleotide 3SS-1, complementary to the 3' splice site region of the SP14-15 pre-mRNA, at 30 °C for 10 min. Debranching reaction was carried out as described previously (15).

RESULTS AND DISCUSSION

Heat treatment of nuclear extract alters the pattern of the 3' splice site selection

3' splice site duplication experiments employing β-globin pre-mRNAs suggest that when multiple 3' splice sites are present, the use of the proximal 3' splice site is favored (5). In order to test

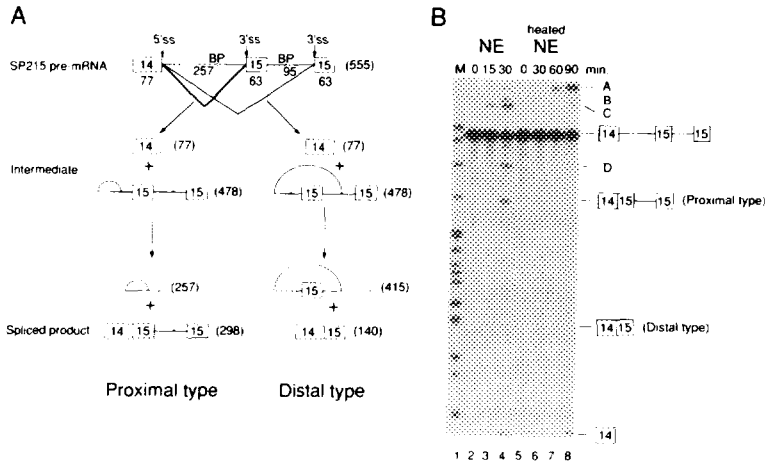

Fig.1. (A) Schematic representation of SP215 pre-mRNA and two possible splicing pathways. Exon sequences are shown by open boxes, and the lines between them show intron sequences. The numbers within the boxes indicate specific exons of the δ-crystallin gene (12). The length in nucleotides of the exons and introns is indicated below them. The total length in nucleotides of each RNA species is shown in parentheses on the right side of the figures. 5'ss, 5' splice site; 3'ss, 3' splice site; BP, branchpoint. (B) SP215 pre-mRNA was incubated at 30 °C with either a normal (NE, lanes 2-4) or a heat treated HeLa cell nuclear extract (heated NE, lanes 5-8). The reaction was terminated at the time indicated on the top of each lane. The RNA products were electrophoresed on a 4 % polyacrylamide gel containing 8 M urea and autoradiographed. The structures of each RNA species are shown on the right schematically. The products represented by A to D correspond to two types of the lariat intermediate (A, C) and two types of the lariat intron (B, D) (see text for details). M, [^{32}P]-labeled pBR322 DNA digested with HpaII used as a size marker.

whether such a phenomenon is observed with pre-mRNAs of other genes, we carried out similar 3' splice site duplication experiment using the chicken δ–crystallin gene. The construct, SP14-15 pre-mRNA, contains exons 14 and 15 and the intron between them of the δ–crystallin gene (13). The 3' half of this pre-mRNA was duplicated to produce SP215 pre-mRNA (Fig.1A). It is anticipated that the splicing occurs in two distinct pathways: either of the two 3' splice sites would be used to generate the products illustrated in Fig.1A. When we carried out *in vitro* splicing reaction of SP215 pre-mRNA with a HeLa cell nuclear extract, we observed several bands (Fig.1B lane 2-4). Among these bands, those depicted as A to D are presumably RNAs containing a lariat structure, as judged by their different mobilities when electrophoresed on different percentage acrylamide gels (data not shown). Other bands that should correspond to the linear RNA molecules, such as pre-mRNA, 5' exon (exon 14), proximal- and distal-type spliced products, were identified on the basis of their molecular size. By comparing the amounts of the spliced products of the proximal-type and of the distal-type, we examined the relative use of the duplicated splice sites. At 30 min incubation, the proximal product accumulated approximately 3-fold more at the molar basis than the distal-type (Fig.1B lane 4). Thus, the site proximal to the 5' splice site is used more efficiently than the distal site, even with a pre-mRNA derived from the δ–crystallin gene.

We and others have reported that *in vitro* splicing reaction can be blocked after the first step by using a heat treated nuclear extract (11, 13). To investigate whether the proximal 3' splice site is

predominantly used under these conditions, we performed the reaction using a nuclear extract that had been heat treated. We anticipated to detect two kinds of lariat intermediate, namely the lariat intermediates of the proximal-type and that of the distal-type (Fig.1A). However, we observed only a single band (band A) that is likely to be a lariat intermediate, as judged by its slow migration (Fig.1B lanes 7 and 8, band A). To confirm that this band represents a lariat intermediate, we excised this band from the gel and carried out the debranching reaction using a HeLa cell nuclear extract (15). By this reaction, the product was linearized and gave the size of the lariat intermediates (data not shown). We found that band C also has the length of the lariat intermediates (data not shown). Thus the bands A and C are thought to be the lariat intermediates either of the proximal- or the distal-type. We also found that the RNAs represented by B and D are the distal-type and the proximal-type lariat intron, respectively.

To determine which of the two 3' splice sites was used for the first step reaction with the product represented by band A, we employed the strategy as illustrated in Fig.2A. After recovering this lariat intermediate RNA from the gel (Fig.2B lane 2), this RNA was annealed to an oligonucleotide complementary to the 3' splice site, and digested with RNaseH. If this RNA is the lariat intermediate of the proximal-type, three cleavage products including lariat intron would be observed. On the other hand, if the RNA is the lariat intermediate of the distal-type, two products including Y-formed RNA would be produced (Fig.2A). When we carried out RNaseH-directed digestion, three bands were detected (Fig.2B lane 3). Of these bands, the band represented by the solid circle was found to be RNA which was incompletely digested by RNaseH (data not shown). Thus, two kinds of cleavage products were mainly generated by RNaseH treatment. The size of the lower band corresponded to that of exon 15. The mobility of the other band was different from the lariat intron of the proximal-type or the internal exon 15 with the adjacent intron.

Since it is likely that this product is the Y-form RNA, we recovered this cleavage product (Fig.2B lane 4) and carried out debranching reaction. If this product is Y-form RNA, debranching reaction would produce two kinds of linear RNA molecules whose sizes correspond to those of linear internal intron and the internal exon 15 with the adjacent intron, respectively (Fig.2A). As anticipated, two RNA molecules of appropriate sizes were generated (Fig.2B lane 5). The result shows that the lariat intermediate accumulated in a heat treated nuclear extract is the distal-type. In a similar experiment, we found that band C corresponds to the lariat intermediate of the proximal-type (data not shown). Thus, it is concluded that the heat treatment of a HeLa cell nuclear extract alters the pattern of the 3' splice site selection from the predominant use of the proximal site to the exclusive use of the distal site. We carried out similar experiments using a pre-mRNA containing tandemly duplicated 3' splice sites derived from the *Drosophila fushitarazu* (*ftz*) gene (16), and obtained essentially the same results (data not shown). This shows that the phenomenon is not specific to δ-crystallin pre-mRNA.

Fractionation of HeLa cell nuclear extract reveals the presence of the activity required for the selection of the proximal 3' splice site

The results described above suggest that two activities sensitive to the heat treatment are present in HeLa cell nuclear extract: namely, the activity that is required for the second step reaction (10, 11) and the activity that is required for the use of the proximal site of duplicated 3' splice sites. To test

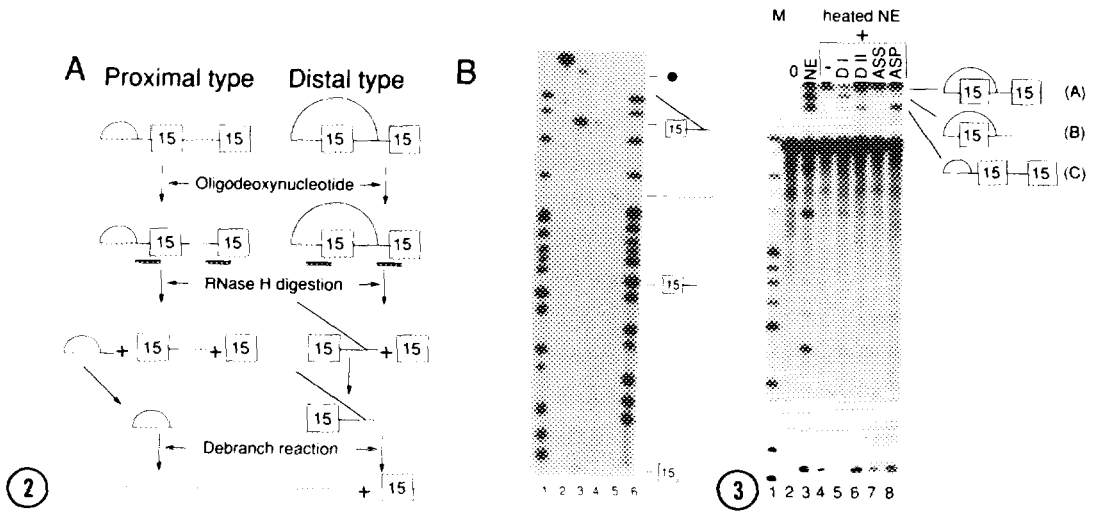

Fig.2. (A) Scheme for analysis of the lariat intermediate is shown. The RNA species considered to be produced in each pattern are illustrated. The solid thick line indicates an oligonucleotide complementary to the 3' splice site. (B) The RNA represented by A in Fig. 1B was recovered from the gel and was electrophoresed on a 4 % polyacrylamide gel containing 8 M urea (lane 2). An oligodeoxynucleotide complementary to the 3' splice site was hybridized to this RNA, and RNaseH digestion was carried out at 30 °C for 10 min (lane 3). The RNaseH cleaved product that is considered to be a Y-form RNA was recovered from the gel (lane 4) and was incubated with a HeLa cell nuclear extract under the splicing conditions (lane 5). The structures of each RNA species deduced by these procedures are indicated on the right side schematically. The band in lane 3 that is shown by the solid circle is a product that was incompletely digested by RNaseH. [α-^{32}P] labeled pBR322 DNA digested with HpaII was used as a size marker (lanes 1 and 6).

Fig.3. Complementation experiment with the DEAE-column fractions of HeLa cell nuclear extract. Splicing of SP215 pre-mRNA was carried out with a normal HeLa cell nuclear extract (lane 3). Splicing was carried out with a heat treated nuclear extract supplemented with buffer F containing 0.05 M KCl (lane 4), DI, DII, ASS or ASP fraction (lanes 5-8) as described in MATERIALS AND METHODS. The recovered RNAs were analyzed by electrophoresis on a 4 % polyacrylamide gel containing 8 M urea. The positions of the distal-type lariat intermediate (band A), the distal-type lariat intron (band B) and the proximal-type lariat intermediate (band C) are indicated at right. M, [^{32}P]-labeled pBR322 DNA digested with HpaII used as a size marker.

whether these activities are separable, we fractionated HeLa cell nuclear extracts by DEAE column chromatography into two fractions (DI and DII fraction), and added these fractions to the heat treated nuclear extracts. If a column fraction added to the heat treated nuclear extract contains the activity required for the second step reaction, the second step reaction occurs, resulting in the final spliced product and the lariat intron. In contrast, if a fraction contains the activity required for the use of the proximal 3' splice site, the lariat intermediate of the proximal-type is generated. The presence of these activities can be detected by monitoring the band B or C that corresponds to the lariat intron of the distal-type or the lariat intermediate of the proximal-type, respectively.

As shown in the previous section, the heat treatment of nuclear extract resulted in the accumulation of the lariat intermediate of the distal-type (Fig.3 lane 4). When DI fraction was added to a heat treated nuclear extract, the band corresponding to the lariat intron of the distal-type (band B) was detected (Fig.3 lane 5). However, the lariat intermediate of the proximal-type (band C) was hardly detected (Fig.3 lane 5). On the other hand, when DII fraction was added to a heat treated nuclear extract, the lariat intermediate of the proximal-type (band C) was mainly detected (Fig.3 lane 6), but the lariat intron of the distal-type (band B) was rarely detected. When DII

fraction was concentrated by ammonium sulfate precipitation (ASP fraction), the lariat intermediate of the proximal-type was detected intensely, whereas the lariat intron of the distal-type was hardly detected (Fig.3 lane 8). Therefore, the activity required for the proximal 3' splice site selection that is mainly in DII fraction seems to be distinct from the activity required for the second step reaction that is in DI fraction. We also found that addition of DII fraction to normal nuclear extract alters the selection of the 3' splice sites of a construct containing duplicated 3' splice sites (data not shown).

The experiments presented here indicate the presence of a factor(s) that is involved in the selection of the 3' splice sites. We designate this proximal site selection activity PSF (Proximal 3' splice Site selection Factor). The distal 3' splice site was used for the first step reaction, even when this activity was depleted by the heat treatment (Fig.1B). Moreover, when we added DI fraction to the heat treated nuclear extract, the splicing reaction proceeded to the final step without using the proximal 3' splice site (Fig.3). It is therefore conceivable that PSF serves as a modulator in the splice site selection.

As to the 5' splice site selection, it has been shown that the selection depends on the relative concentration of two protein factors *in vitro* (17). SF2/ASF (7, 8) promotes the selection of the 5' splice site proximal to the 3' splice site, whereas another protein hnRNP A1 promotes the use of the 5' splice site distal to the 3' splice site (17). On the basis of these findings, we suggest that the mechanism of the 3' splice site selection may also depend on the balance of several factors. It should be determined whether a single or more than one factor (s) is involved in the 3' splice site selection.

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