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Three distinct activities possibly involved in mRNA splicing are found in a nuclear fraction lacking U1 and U2 RNA

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ABSTRACT

A nuclear extract from HeLa cells was fractionated by DEAE-Sepharose chromatography, and the fractions were assayed for the binding activity for a small RNA transcript carrying a splice junction or branch point sequence. The binding activity for the RNA carrying a 5' splice junction was localized in the small nuclear ribonucleoprotein (snRNP) fraction together with binding activity for the RNA carrying a 3' splice junction or branch point sequence. However, stronger binding activities for the 3' splice junction RNA and for the branch point RNA were discovered in the flow-through fraction where no small nuclear RNA were detected. When the flow-through fraction was added to purified U1 ribonucleoprotein, the binding activity for the 5' splice junction RNA was markedly enhanced. We propose that the factors responsible for the three types of activities found in the flow-through fraction play a role in the splicing of mRNA precursor.

INTRODUCTION

Until recently, U1 ribonucleoprotein (U1-RNP) has been the only well-characterized factor that was shown to be involved in the splicing of an mRNA precursor (1-3). Mount et al. (4) showed that U1-RNP selectively bound to a 5' splice junction in a transcript from mouse β-globin gene. We showed that a partially purified U1-RNP fraction or U1-rich snRNP fraction bound selectively to both 5' and 3' splice junction sequences , whereas highly purified U1-RNP bound only to a 5' splice junction sequence (5). Recently, Black et al. (6) have reported that U2-RNP is also required for in vitro splicing and that it becomes associated during the splicing reaction with a branch point region in an mRNA precursor.

We have now found major binding activities for a small in vitro transcript carrying either a 3' splice junction sequence or branch point sequence. These activities are found in a nuclear fraction in which neither U1 nor U2-RNA were detected. We have

also found a factor (or factors) that selectively enhances the binding activity of U1-RNP for the 5' splice junction RNA. We propose that the factors responsible for such activities in the flow-through fraction play an important role in mRNA splicing as a part of the splicing complex.

MATERIALS AND METHODS

Preparation of RNA carrying a splice junction or branch point sequence

Chemical syntheses of DNAs OS-11, 12, 14 and 15 were carried out as described previously (5). Other synthetic DNAs were synthesized by an automated DNA synthesizer (Applied Biosystem model 380A or Nippon Zeon Genet H-4). Cloning of a double stranded synthetic DNA between the HindIII and AccI sites of pSP64 was done using a method similar to that described previously (5). The standard reaction mixture for transcription (25 µl) contained 2.5 µg DNA template, 40 mM Tris.HCl (pH 7.5), 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, 25 units RNasin (Biotec), 100 µCi of α-32P-UTP (410 Ci/mmol), 0.5 mM each ATP, GTP and CTP, and 7 units of SP6 RNA polymerase (Boehringer). It was incubated at 40°C for 1.5 hr. The RNA products were extracted with a phenol/chloroform mixture, precipitated with ethanol and subjected to electrophoresis in 20% polyacrylamide/7 M urea gel. After brief autoradiography, the desired RNA was eluted from the gel and precipitated with ethanol together with 20 µg of yeast tRNA. 2-8x10^7cpm (3-10pmol) of RNA was obtained routinely.

Preparation and fractionation of a nuclear extract and purification of U1-RNP

The nuclear extract was prepared from 2-4x10^9 HeLa cells and fractionated on a DEAE-Sepharose column as described by Kinlaw et al. (7), except that 0.1 mM phenylmethylsulfonyl fluoride and 5 µg/ml each of pepstatin, leupeptin and chymostatin (Protein Research Foundation, Osaka) were added to the medium throughout. Protein concentration was determined by the dye-binding method using a reagent kit (Bio-Rad). Small nuclear RNAs were identified by electrophoresis of a phenol extract of 200 µl or 300 µl of each fraction in 10% polyacrylamide/7 M urea gel, followed by staining with ethidium bromide. Further purification of U1-RNP

was carried out by chromatography on heparin-agarose and Blue-Sepharose as described previously (7).

RNA-binding assay

The binding activity for an RNA transcript was assayed using a modification of the filter-binding method described previously (5). The sample to be assayed was incubated in 200 µl reaction mixture containing 10 fmol ^{32}P-labeled RNA (6-10x10^4 cpm just after synthesis), 100-fold wt. of E. coli RNA (Sigma) over the labeled RNA, 10 mM Tris. HCl (pH 7.5), 10 mM KCl, 2 or 3 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol and 50 µg/ml bovine serum albumin (BRL). In some indicated cases, 100 µl reaction mixture was used. After incubation at 30° C for 10 min, the reaction mixture was filtered in about 5 sec through a Millipore HAWP filter using a microfiltration assembly which has 96 holes, each of 7mm diameter (Ikeda Rika, Inc., Tokyo). The filter was washed with 200 µl of the same medium but lacking RNA, test sample and albumin. After the filter had been dried, it was cut into pieces which were then measured for radioactivity in a liquid scintillation counter. The non-specific binding of the RNA to the filter (about 1% of the input) has been subtracted to obtain the result.

RESULTS

Preparation of small RNA transcript for the binding study

In our previous investigation of a factor that binds to a splice junction sequence(5), we took advantage of a rapid and sensitive filter-binding assay. In that assay, we used chemically synthesized single— stranded DNA of 16-21 nucleotides carrying a consensus or altered splice junction sequence. We also used RNA of about 80 nucleotides which was transcribed in vitro from a double-stranded synthetic DNA that was inserted downstream of a promoter fragment of the E. coli lac operon (5). We are now using a much shorter RNA (about 30 nucleotides) to improve the specificity of the binding. The RNA is transcribed from the promoter of bacteriophage SP6 (8)(Fig. 1), and purified by gel electrophoresis. The sequence used as the consensus 5' or 3' splice junction (9) is the same as that used previously (5). The control sequence in Fig. 1 is complementary to a part of U2-RNA (5).

Fig. 1. Nucleotide sequence of the SP6 promoter/polylinker region of plasmid pSP64 (8), and inserted synthetic DNA that contains a consensus 5' splice junction (9), consensus 3' splice junction (9), branch point (14) or an unrelated control sequence. Each recombinant plasmid was linearized by XbaI and then used as a template for transcription in vitro by SP6 RNA polymerase.

Recently, formation of a branched structure, or lariat, including a region within an intron has been implicated as an essential step in mRNA splicing (10-13). CUGAC, CUPuAPy and PyNPyPyPuAPy have been proposed as consensus sequences for the branch point (10, 13, 14). We have also prepared a 37 nucleotide RNA that contains CUGAC, probably a most standard branch point sequence, surrounded by an unrelated sequence (Fig. 1).

RNA binding activities in the snRNP fraction

We have fractionated a nuclear extract from HeLa cells on a DEAE-Sepharose column (Fig. 2A) and surveyed the RNA binding activities among the fractions. The binding activity for a 5' splice junction RNA was found only in the fractions containing U1, U2, U5 and U4/U6 RNAs, namely snRNP fractions (Fig. 2B, -O-). In some other cases, binding activity for the RNA, although much weaker, was also found in the flow-through fraction where no small nuclear RNA (snRNA) was detected by the method used(data

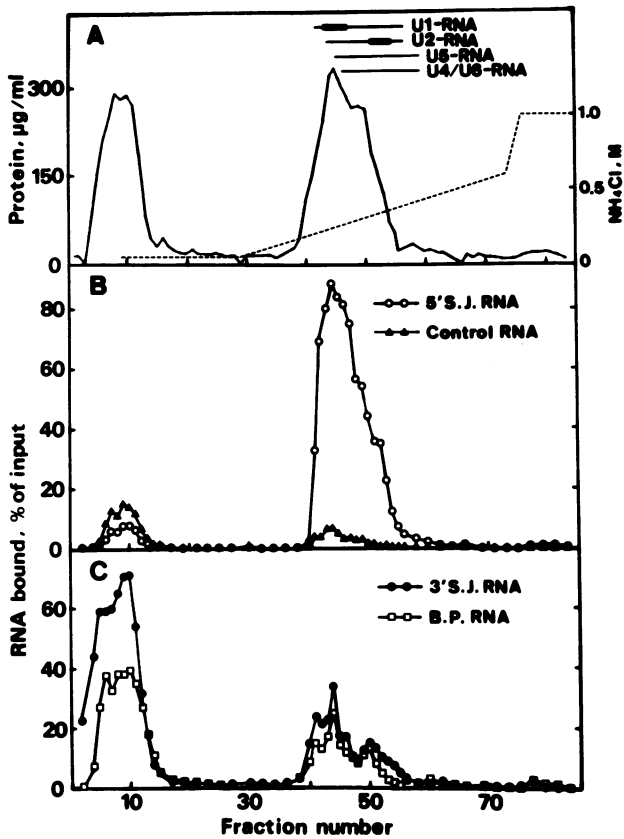

Fig. 2. A, Fractionation of a nuclear extract from 3.5×10^9 HeLa cells by chromatography on DEAE-Sepharose. The size of the column was 2.5 cm x 12 cm, and 6 ml fractions were collected with the flow rate of 15 ml/hr. Protein concentration is shown by the solid line and the NH_4Cl concentration by the broken line. The fractions in which a small nuclear RNA was detected are indicated, the peak fractions being shown by a solid box. B and C, Binding activities assayed using 10 µl of each fraction for the RNAs described in Fig. 1: 5' splice junction RNA (-O-), 3' splice junction RNA (-●-), branch point RNA (-□-) and control RNA (-▲-).

not shown). In the snRNP fractions, binding activity for a 3' splice junction RNA was also found (Fig. 2C, -●-), which is consistent with our previous results (5). The most prominent RNA component in the snRNP fractions is usually U1-RNA. Furthermore, the distribution of the RNA binding activities in the snRNP

fractions is most similar to that of U1-RNA among the snRNAs found. Therefore, we called a similar snRNP fraction as U1-RNP fraction in the previous report (5). Remarkably, more prominent binding activity for the 3' splice junction RNA was found in the flow-through fraction (Fig. 2C, -●-). Similarly, a marked binding activity for the branch point RNA was found in the flow-through fraction and a weaker activity in the snRNP fraction (Fig. 2C, -□-).

That the snRNP fraction has specific binding activities for 5' and 3' splice junction sequences is suggested by the following results. First, binding of a similar snRNP (U1-RNP) fraction to single-stranded DNA carrying a 5' or 3' splice junction sequence was significantly reduced when conserved GT or AG was altered (5). Second binding to a control RNA is negligible (Fig. 2B, -▲-). Third, binding to the 5' splice junction RNA is negligible in the flow-through fraction while it is much higher than that to the 3' splice junction RNA in the snRNP fraction (Fig. 2B & 2C). Fourth, the binding is not likely to depend on any secondary structure of the RNA since no significant intra-molecular complementarity is found in any RNA. Finally, 100-fold excess of *E. coli* RNA and several hundred-fold tRNA over the labeled target RNA is present in the binding reaction. We have established sequence specific binding of the U1-rich snRNP fraction to a splice junction sequence by comparing the binding activities for RNA carrying various sequences. The results will be published elsewhere.

The RNA binding activities exhibited by varied amounts of the U1-rich snRNP fraction, which corresponds to the binding peak fraction in Fig. 2, is shown in Fig. 3A. Since ATP is required for mRNA splicing (15, 16), the binding activity was also assayed in the presence of 0.5, 1 and 1.5 mM ATP. The activity was not affected or suppressed slightly by ATP (data not shown). U1-RNP was highly purified from the U1-RNA rich fractions by chromatography on heparin-agarose and Blue-Sepharose (7). The purified U1-RNP fraction showed binding activity for the 5' splice junction RNA, but no binding activity for the 3' splice junction or branch point RNA (Fig. 3B). The result is consistent with our previous report (5).

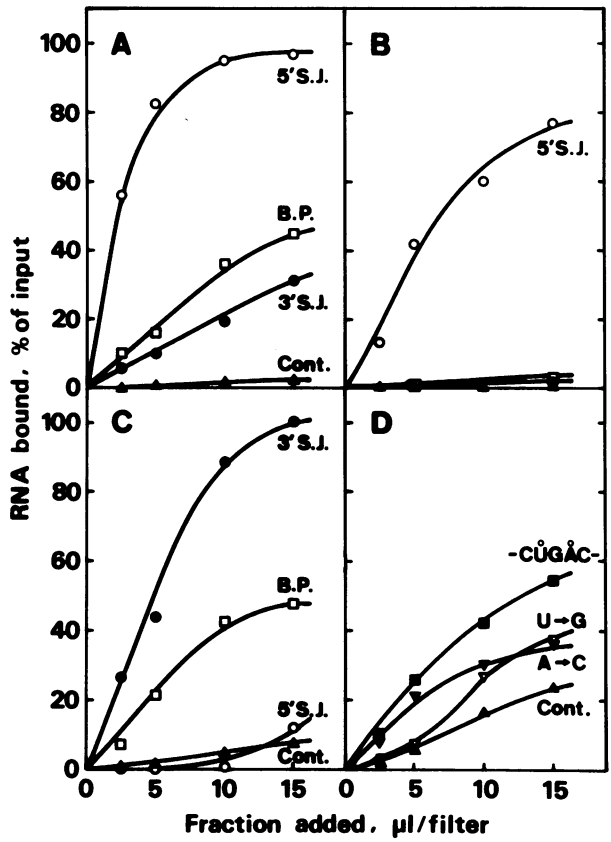

Fig. 3. A, B and C, Binding to the RNAs shown in Fig. 1 and Fig. 2 by varied amounts of U1-rich snRNP fraction (#44 of Fig. 2) (A), by a purified U1-RNP fraction (B) or by a flow-through fraction (#10 of Fig. 2) (C). D, Binding by the same flow-through fraction to 27 nucleotide RNA containing -CUGAC- (-■-), -CUGCC- (-▼-), -CGGAC- (-▽-), or to the 35 nucleotide control RNA used in other experiments (-▲-). The 27 nucleotide RNA was transcribed from the plasmid to which AGCTTCTGACT (AM-90), AGCTTCTGCCT (AM-92) or AGCTTCGGACT (AM-94) was inserted together with a complementary strand to each (AM-91, 93 or 95) as described in Fig. 1.

RNA binding activities in the flow-through fraction

Fig. 3C shows the RNA binding exhibited by varied amounts of a DEAE flow-through fraction. Fig. 3D (-■-) shows binding of the same flow-through fraction to a 27 nucleotide RNA that carries the same -CUGAC- but different surrounding sequences from those of the branch point RNA in Fig. 1. It is quite similar to the

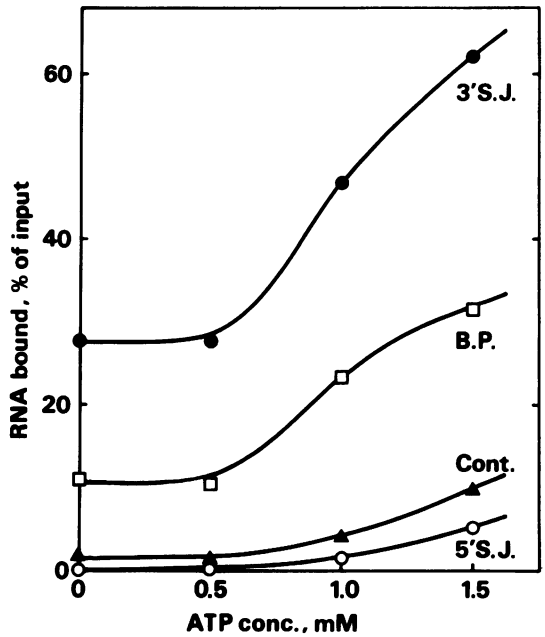

Fig. 4. Effect of ATP on the RNA binding activity by a flow-through fraction. 2.5 µl of a flow-through fraction which is similar to that shown in Fig. 2 was assayed for the RNA binding activity in the presence of the indicated concentration of ATP and 3mM $MgCl_2$.

binding to the longer branch point RNA shown in Fig. 3C (-□-). Furthermore, binding to a 27 nucleotide RNA containing a single base substitution for A or U in the CUGAC sequence is decreased significantly (Fig. 3D). These results indicate that A and U are important in the binding shown by the flow-through fraction. Since these nucleotides seem to be significantly conserved at a branch point (10, 13, 14), the factor responsible for this binding may be a good candidate for a factor recognizing a branch point. Considerable variety has been observed for cryptic branch point sequences (17, 18), and therefore further study into the sequence specificity in the binding by the factor(s) will be needed. As for the binding to the 3' splice junction RNA by the flow-through fraction, the sequence specificity is under study (see Discussion).

Fig. 4 shows the effect of ATP on the RNA binding activity of

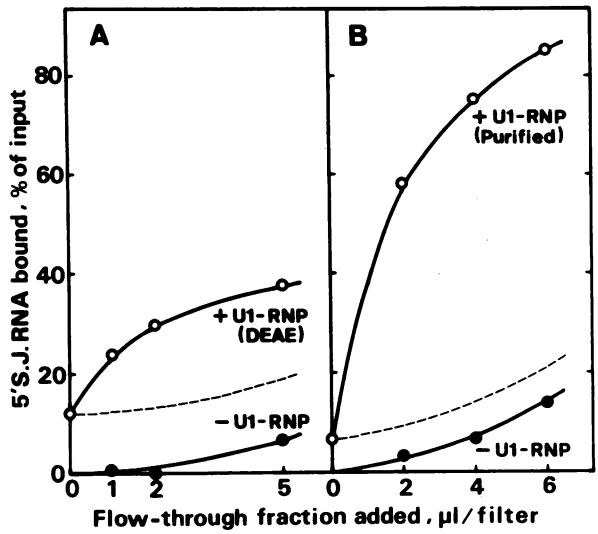

Fig. 5. Enhancement of the binding activity for the 5' splice junction RNA by mixing the flow-through fraction with the U1-rich snRNP fraction (A) or with a purified U1-RNP fraction (B). The indicated amount of the flow-through fraction (#10 in Fig. 2) was mixed with 0.5 µl of the U1-rich snRNP fraction (#43-45 in Fig. 2) or 3 µl of the purified U1- RNP fraction, and the mixture was kept on ice for about 2hr during preparation of the assay for RNA binding in 100 µl of reaction mixture (-O-). The binding activity of the flow-through fraction alone was also assayed in exactly the same manner (-●-). The broken line indicates the simple sum of the binding activities by each fraction alone.

a flow-through fraction. In contrast to the case of the snRNP fraction, the binding activity for the RNA carrying a 3' splice junction or branch point was enhanced significantly with increasing concentrations of ATP.

Interaction of the flow-through fraction with U1-RNP

We have tried to ascertain whether the DEAE flow-through fraction interacts with U1-RNP which is the established factor involved in mRNA splicing (1-3). As shown in Fig. 5A, when a flow-through fraction was mixed with a U1-rich snRNP fraction, the binding to the 5' splice junction RNA was much higher than the simple sum of the binding by each fraction alone. When it was added to a purified U1-RNP fraction (Fig. 5B), the enhancing effect was more pronounced. Little or no effect was produced on the binding to the 3' splice junction RNA or control RNA by mixing the two

fractions (data not shown). These results reveal an either direct or indirect interaction between the factor(s) in the flow-through fraction and U1-RNP. It is plausible that the flow-through factor(s) stimulates the binding of U1-RNP to a 5' splice junction although other explanations might also be possible.

DISCUSSION

We have investigated factors that can bind to a splice junction or branch point sequence by using a small RNA transcript and filter binding assay. We have confirmed our previous results that a partially purified U1-RNP fraction or U1-rich snRNP fraction binds to both 5' and 3' consensus splice junction sequences whereas highly purified U1-RNP binds selectively to a 5' splice junction sequence. Recently, Chabot *et al.* (19) have reported selective and ATP independent binding of an snRNP to the 3' splice site of a human β-globin transcript and suggested that the snRNP is U5-RNP. U5 RNA was usually detected in our snRNP fractions although it is much less abundant than U1-RNA. Therefore the factor in the snRNP fraction that is responsible for the binding activity for the 3' splice junction RNA could be U5-RNP. It should be elucidated whether this is the case or not. We have newly found binding activity for the RNA carrying a branch point sequence in the snRNP fraction. However sequence specificity of this binding remains to be studied.

We have also found three types of activities possibly involved in mRNA splicing in the DEAE flow-through fraction where no snRNAs were detected by the method used. These are 1) the binding activity for RNA carrying a 3' splice junction sequence, 2) the binding activity for RNA carrying a branch point sequence and 3) the activity to interact with U1-RNP resulting in the stimulation of the binding activity for the 5' splice junction RNA. It is not clear at present whether the factors in the flow-through fraction which are responsible for the binding to the 3' splice junction RNA and the branch point RNA are identical to those in the snRNP fraction. A possible explanation is that the factors in the flow-through fraction were complexed with snRNPs *in situ* and have partially dissociated from them during isolation. Since the flow-through fraction may contain many

components, it is possible that more than one components are responsible for any one of the activities found in the fraction. Purification of factors from the flow-through fraction and their characterization have suggested that there is at least one pyrimidine stretch binding component in the flow-through fraction.

Krainer & Maniatis (20) and Black *et al.* (6) have reported that U2-RNP is required for *in vitro* splicing. Black *et al.* (6) have also stated that U2-RNP associates with a region in mRNA precursor including a branch point and that the association is not observed with isolated U2-RNP. They suggest that some factor other than U2-RNP must bind to the pre-mRNA or to U2-RNP before the observed interaction between the branch point region and U2-RNP (6). Therefore the factor(s) binding to the branch point RNA in this report may be a candidate for the factor which has been postulated by them. Ruskin and Green (21) have recently reported ATP dependent and nuclease sensitive binding of a factor (or factors) in the splicing extract to a branch point region. The binding of our flow-through factor(s) to the branch point RNA is stimulated by, but is detected without, ATP (Fig. 4). Furthermore, it is not sensitive to micrococcal nuclease (data not shown). Therefore, the factor(s) in the flow-through fraction which is responsible for the binding to the branch point RNA does not seem to be identical to that found by Ruskin and Green (21). However, the factor(s) in our flow-through fraction might be a nuclease insensitive component which is involved in the binding to a branch point region reported by them.

The probability that at least one of the factors found in the flow-through fraction is involved in mRNA splicing is further supported by the recent report of Furneaux *et al.* (22). They indicated that a DEAE cellulose flow-through fraction is required for *in vitro* splicing as well as a DEAE-bound, nuclease -sensitive fraction which is supposed to contain U1- and U2-RNP. Recently it has also been reported that even the first detectable step in mRNA splicing, cleavage at the 5' splice junction, requires a 3' splice junction or a polypyrimidine stretch (13, 23, 24) and that a large complex is required for splicing reactions *in vitro* (23, 25, 26). Thus the splicing complex is pre-

sumed to contain all the factors which are necessary to recognize the 5' splice junction, 3' splice junction and the branch point. We propose that the factors which are responsible for the three types of activities found in the flow-through fraction are involved in mRNA splicing as components of the splicing complex.

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