Activity of chimeric RNAs of U6 snRNA and (–)sTRSV in the cleavage of a substrate RNA

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ABSTRACT

U6 small nuclear RNA is one of the spliceosomal RNAs essential for pre-mRNA splicing. Discovery of mRNA-type introns in the highly conserved region of the U6 snRNA genes led to the hypothesis that U6 snRNA functions as a catalytic element during pre-mRNA splicing. The highly conserved region of U6 snRNA has a structural similarity with the catalytic domain of the negative strand of the satellite RNA of tobacco ring spot virus [(–)sTRSV], suggesting that the highly conserved region of U6 snRNA forms the catalytic center. We examined whether synthetic RNAs consisting of the sequence of the highly conserved region of U6 snRNA or various chimeric RNAs between the U6 region and the catalytic RNA of (–)sTRSV could cleave a substrate RNA that can partially base-pair with them and have a GU sequence. Chimeric RNAs with 70 to 83% sequence identity with the conserved region of *S. pombe* U6 snRNA cleaved the substrate RNA at the 5' side of the GU sequence, which is shared by the 5' end of an intron in a pre-mRNA. We found that the highly conserved region of U6 snRNA and the catalytic domain of (–)sTRSV are strikingly similar in structure to the catalytic core region of the group I self-splicing intron in cyanobacteria. These results suggest that U6 snRNA, (–)sTRSV and the group I self-splicing intron originated from a common ancestral RNA, and support the hypothesis that U6 snRNA catalyzes pre-mRNA splicing reaction.

INTRODUCTION

The small nuclear RNAs (snRNAs) are a class of metabolically stable small RNAs present in the nuclei of eukaryotic cells (1). They associate with proteins to form small nuclear ribonucleoprotein particles (snRNPs). Among snRNAs, U1, U2, U4, U5 and U6 snRNAs have been shown to be essential for pre-mRNA splicing as RNA components of the spliceosome (for review, see ref. 2). U1 and U2 snRNPs play important roles in the recognition of the 5' splice site and branch site, respectively. U5 snRNP was recently shown to be involved in the selection of the 5' splice site as well as the recognition of the 3' splice site (3, 4). U6 and U4 snRNAs are base-paired and are present

in the same snRNP. The U4/U6 interaction is thought to be destabilized prior to, or concomitant with, the 5' splice site cleavage (5, 6). The exact function(s) of U4/U6 snRNP in pre-mRNA splicing, however, has remained unknown.

To elucidate the function of U6 snRNA, we cloned the gene coding for U6 snRNA of fission yeast *Schizosaccharomyces pombe* (7). Interestingly, the U6 snRNA gene in *S. pombe* was found to have an intron with a characteristic structure for the nuclear pre-mRNA intron. It contains the consensus sequences identified at the 5' and 3' splice sites and branch site in nuclear pre-mRNA. Subsequent experiments using the polymerase chain reaction (PCR) revealed the existence of the mRNA-type introns in the U6 snRNA genes in two other yeasts, *Rhodotorula hasegawae* and *Rhodospiridium dacryoidum* (8). From phylogenetic sequence comparisons, U6 snRNA can be divided into four domains; the 5'-terminal domain, the central domain, the U4/U6 interaction domain, and the 3' terminal domain (9). Most of the mRNA-type introns were found in the highly conserved region containing the central domain and U4/U6 interaction domain of U6 snRNA. In addition, two of the mRNA-type introns in the U6 snRNA genes are located immediately adjacent to the nucleotides essential for the second step of the splicing reaction (10, 11). We found that the highly conserved region of U6 snRNA shows a structural similarity with the catalytic center of the negative strand of the satellite RNA of tobacco ring spot virus [(–)sTRSV] (8). The (–)sTRSV is able to undergo self-catalyzed cleavage during replication (12). The cleavage of RNA occurs at the 5' side of the GU sequence that is identical to the 5' end of mRNA-type introns. These findings suggest that U6 snRNA participates in the catalysis of pre-mRNA splicing and that the mRNA-type introns in the U6 snRNA genes were generated by insertion of an intron excised from pre-mRNA into U6 snRNA, followed by reverse transcription and integration of the cDNA copy into the genome (13). In that scenario, the sites of the intron insertion reflect the catalytic center of U6 snRNA. Accordingly, the highly conserved region of U6 snRNA is a most likely candidate for the catalytic center of the spliceosome. To gain support for this notion, we examined the catalytic activities of synthetic RNAs representing the highly conserved region of U6 snRNA and those of various chimeric RNAs between the conserved region of U6 snRNA and the catalytic domain of (–)sTRSV.

MATERIALS AND METHODS

Materials

T7 RNA polymerase, Klenow fragment of DNA polymerase I and T4 RNA ligase were purchased from Takara Shuzo Co., Ltd. Bacterial alkaline phosphatase and T4 polynucleotide kinase were obtained from Toyobo. α - 32 P-UTP and γ - 32 P-ATP were from Amersham and ICN Radiochemicals, respectively. Oligonucleotides were synthesized on an Applied Biosystems synthesizer (model 380A).

Preparation of RNA

Synthetic RNAs that have the desired sequences were prepared according to the method of Milligan et al. (14) with slight modifications. An 18-nucleotide oligonucleotide complement to the T7 promoter was hybridized on 65–68-nucleotide template oligonucleotides with the T7 promoter sequence. The hybrids were then treated with the Klenow enzyme to obtain double stranded DNAs. A reaction of transcription was performed in a 50 μ l solution containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 4 mM NaCl, 2 mM spermidine, 0.01% triton X-100, 4% polyethylene glycol 8000, 0.5 mM each of NTP, 10 mM DTT, 50 μ Ci of α - 32 P-UTP, 5 pmol of DNA template and 150 units of T7 RNA polymerase at 37°C for 120 min. The transcribed RNAs were then purified by fractionation on 20% acrylamide/7 M urea gels. The full-length synthetic U6 snRNA was prepared by transcription of the PCR-amplified cDNA of *S. pombe* mature U6 snRNA, which is linked with the T7 promoter.

Cleavage reaction

Unless otherwise indicated, the cleavage reactions were performed by incubating 1 pmol of a synthetic RNA to be tested with 1 pmol of a substrate RNA in 10 μ l solution containing 50 mM $MgCl_2$, 2 mM spermidine, 40 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl and 4% polyethylene glycol 8000 at 23°C for 5 h. As a control reaction, we used the catalytic RNA of (–)sTRSV (TRSV) whose sequence of the substrate binding region is designed to form base-pairings with the same substrate RNA. The reactions were terminated by adding 95% formamide/0.1% xylene cyanol. The products were then heated at 80°C for 5 minutes and electrophoresed on a 20% acrylamide/7 M urea gel. Autoradiography was carried out at –70°C with an intensifying screen. Quantitative analysis of the cleavage products was done using a Fuji Bio-Image Analyzer BAS2000.

End-Labeling of the cleavage products

The gel-purified 5' cleavage fragment was incubated in 10 μ l of 10 mM HCl at 25°C for 120 min to decyclize the cyclic phosphate (15). The sample was then mixed with 10 μ l of 0.1 M Tris-HCl (pH 8.0) and 0.7 u of bacterial alkaline phosphatase and was incubated at 60°C for 30 min. After treatment with phenol/chloroform/iso-amylalcohol (25:24:1), the RNA was recovered by ethanol precipitation. The RNA was then 3'-end labeled at 20°C for 4 h in a solution containing 10 u of T4 RNA ligase, 10 μ Ci of 32 P- pCp , 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 0.1 mM ATP and 10% DMSO.

Labeling of the 5'-end of the 3' cleavage fragment with 32 P was performed in a solution containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, 100 μ Ci of γ - 32 P-ATP and 20 u of T4 polynucleotide kinase at 37°C for 30 min.

Determination of the cleavage site

The substrate RNA was 3'-end labeled by T4 RNA ligase and 32 P- pCp , as described above and then subjected to the cleavage reaction with the chimeric RNA or the catalytic RNA of (–)sTRSV (TRSV). The products were electrophoresed on a 20% acrylamide/7 M urea gel. As markers, the substrate RNA labeled at 3'-end was partially alkaline hydrolyzed in a solution containing 50 mM $NaHCO_3/Na_2CO_3$ (pH 9.0), 1 mM EDTA, 0.25 mg/ml tRNA at 90°C for 20 minutes and the products were electrophoresed on the same gel.

RESULTS

Cleavage reaction of the synthetic RNAs

We prepared a 46-nucleotide synthetic RNA (U6-pom) consisting of a sequence of the highly conserved region of *S. pombe* U6 snRNA and examined whether it could cleave a substrate RNA *in vitro*. As a substrate RNA, we prepared a 17-nucleotide RNA

Fig. 1. (A) Presumed secondary structure of a 46-nucleotide part in U6 snRNA of *S. pombe* (U6-pom). Upper-case and lower-case letters indicate U6-pom and the presumed substrate RNA, respectively. The predicted cleavage site is shown by the arrow in parentheses. (B) Nucleotide sequences of synthetic RNAs tested. The bases differing from those of U6-pom are boxed. Four bases in the substrate RNA binding region of TRSV (underlined bases) are changed from the authentic sequence to anneal with the substrate RNA used. The presence or absence of the cleavage activity is indicated by + or – on the right.

that contains a GU sequence and can partially base-pair with U6-pom as the catalytic RNA of $(-)$ sTRSV can base-pair with its substrate RNA (Fig. 1A, see also Fig. 4c). Both RNAs were then incubated under various conditions (MgCl_2 concentrations from 12 to 800 mM, NH_4Cl concentrations from 100 mM to 2 M, presence or absence of 4% polyethylene glycol 8000, temperatures from 16 to 50°C, and incubation times up to 48 h). However, we observed no cleavage of the substrate RNA with U6-pom, whereas the catalytic RNA of $(-)$ sTRSV cleaved the same substrate RNA efficiently under the same conditions (Fig. 2, lanes 1 and 16 and data not shown). We prepared synthetic RNAs with sequences identical to that of the full-length *S. pombe* U6 snRNA or to the highly conserved region of *Saccharomyces cerevisiae* U6 snRNA. They did not show any cleavage activities either against the substrate RNA under the conditions tested.

We then asked whether or not the synthetic RNA having the highly conserved sequence of U6 snRNA can be converted to a catalytic RNA analogous to that of $(-)$ sTRSV by a slight modification of the sequence. We prepared a series of synthetic RNAs comprising the chimeric sequence between the highly conserved region of *S. pombe* U6 snRNA and the catalytic RNA of $(-)$ sTRSV, and examined whether these chimeric RNAs would cleave the substrate RNA *in vitro*. In the case of the catalytic RNA of $(-)$ sTRSV, sequences participating in the binding to the substrate RNA can be replaced by other sequences without a complete loss of cleavage activity (16). Therefore, we directed attention to differences between the presumed hairpin structure in the highly conserved region of U6 snRNA and that of the catalytic RNA of $(-)$ sTRSV when we designed chimeric RNAs. As shown in Fig. 1B, several bases in the presumed hairpin structure of U6-pom were replaced with corresponding bases of the catalytic domain of $(-)$ sTRSV. Among 14 kinds of chimeric RNAs synthesized, U6-2, U6-3, U6-5, U6-8, U6-12 and U6-13 cleaved the substrate RNA significantly at 23°C (Fig. 2). The products cleaved by these chimeric RNAs accumulated linearly according to the incubation time up to 7 h (data not shown). U6-2 shares a 70% sequence identity with U6-pom and had the highest activity among the chimeric RNAs synthesized. U6-3 has a single base deletion (A26) compared

with the sequence of U6-2. A26 is present in the catalytic RNA of $(-)$ sTRSV, but not at the corresponding position of U6-pom. Quantitative analysis of the cleavage products showed that efficiency of cleavage by U6-3 is approximately 4% of that by U6-2 (data not shown). U6-5, U6-8, U6-12 and U6-13 do not contain A at a position 26. However, efficiencies of cleavage by U6-5 and U6-8 are 1.6 and 5.7 fold higher than that by U6-3, respectively. Difference in the nucleotide sequence between U6-3 and U6-5 is 2 base substitutions at positions 32 and 33. The C residue at position 33 of U6-2 and U6-3 participates in stem formation. Therefore, U6-2 and U6-3 have a 3 bases hairpin loop resembling that of $(-)$ sTRSV while others have a 5 bases loop the sequence of which is identical to that of U6 snRNA in the hairpin structure (see Fig. 4, a, c and d). The difference between U6-5 and U6-8 is an A to U base substitution at position 20. All the chimeric RNAs that possess cleavage activity have AAA and AUU instead of GCC and GAC that are present in the internal loop region of the presumed hairpin structure of U6 snRNA. Of these, U6-12 has the highest sequence homology with the highly conserved region of U6 snRNA, it has an 83.3% sequence identity with U6-pom. U6-1, U6-4, U6-6, U6-7, U6-9, U6-10, U6-11 and U6-14 showed no cleavage activity. Most of them do not contain complete AAA or AUU sequences in the presumed internal loop. Although U6-9 and U6-14 contain AAA and AUU sequences in the loop region, they also have 2 base deletions (G and C residues) in the stem region, compared to the sequence of U6-8 and U6-12. Therefore, G and C residues that can form base-pairing in the presumed secondary structure also seem to be essential for cleavage activity, in addition to the AAA and AUU sequences in the internal loop region.

Characterization of the cleavage products

The catalytic RNA of $(-)$ sTRSV cleaves RNA to give a 2', 3' cyclic phosphate and 5' hydroxyl termini. As shown in Fig. 2, the fragments cleaved by the chimeric RNAs comigrated with

Fig. 2. Analysis of the cleavage activities of U6-pom, the catalytic RNA of $(-)$ sTRSV and the chimeric RNAs. One picomole of a synthetic RNA was incubated with an equal amount of the substrate RNA for 5 h at 23°C, as described under Materials and Methods. Lane 1 shows the reaction with U6-pom; lanes 2 to 15, the reactions with U6-1 to U6-14; lane 16, the reaction with TRSV; lane 17, the substrate RNA only. 5'F and 3'F indicate the positions of 5' cleavage products and 3' cleavage products, respectively. Since the synthetic RNAs were prepared at different times, their specific radioactivities of ^{32}P -UMP incorporated varied.

Fig. 3. Determination of the cleavage site. The substrate RNA was 3'-end labeled with ^{32}P -pCp and T4 RNA ligase, incubated with U6-8 chimeric RNA or the catalytic RNA of $(-)$ sTRSV (TRSV) for 2 h, then electrophoresed on a 20% acrylamide/7 M urea gel with the alkaline-hydrolyzed products of the same substrate RNA as markers. Minor bands observed above the bands corresponding to the fragments cleaved at the 5' side of the GU sequence possibly derived from cleavage of a contaminated RNA with an extra base at the 3' end, which attached during transcription.

those cleaved by TRSV on the gel. This would suggest that the chimeric RNAs cut the substrate RNA at the same site where the catalytic RNA of (–)sTRSV does, and that the cleavage products have terminal structures identical to those of the cleavage products by the catalytic RNA of (–)sTRSV.

To confirm the terminal structures of the cleavage products by the chimeric RNA, we carried out following experiments. The non-labeled 5' cleavage fragment was gel-purified and subjected to 3'-end labeling with T4 RNA ligase and ^{32}P -pCp. The 5' cleavage fragment was not labeled by this treatment, even after extensive incubation with an alkaline phosphatase (data not shown). Thus, the 3' terminus of the 5' cleavage fragment was neither hydroxyl nor phosphomonoester groups. Labeling of the 3' end was, however, possible after the 5' cleavage fragment was pre-incubated in 10 mM HCl and then treated with the alkaline phosphatase, indicating that the 3' terminal has a 2', 3' cyclic phosphodiester group (15). On the other hand, the 5' end of the 3' cleavage fragment could be labeled with γ - ^{32}P -ATP and T4 polynucleotide kinase without pre-treatment with the alkaline phosphatase, indicating that the 5' terminus of the 3' cleavage fragment has a hydroxyl group.

To determine the cleavage site by the chimeric RNA, 3'-end labeled substrate RNA was cleaved by U6-8 or the catalytic RNA of (–)sTRSV (TRSV). The products were then fractionated on

a 20% acrylamide/ 7M urea gel (Fig. 3). Alkaline hydrolyzed products of the same substrate RNA were used as markers. The 3' cleavage fragments produced by alkaline hydrolysis have 5'-hydroxyl termini that are identical to the terminal structure of the 3' fragments generated by cleavage with the catalytic RNA of (–)sTRSV or chimeric RNAs. U6-8 cleaved the substrate RNA at the 5' side of the GU sequence that is the same site cleaved by the catalytic RNA of (–)sTRSV.

DISCUSSION

We have found that the highly conserved region of U6 snRNA is similar in structure to the catalytic domain of (–)sTRSV that undergoes self-catalyzed cleavage at the 5' side of the GU sequence and ligation. This finding strongly suggests that U6 snRNA has a catalytic role in pre-mRNA splicing which involves cleavage at the 5' side of the GU sequence. RNA cleavage reaction by the catalytic domain of (–)sTRSV absolutely requires a G residue immediately 3' to the cleavage site, and GUC is apparently the best target sequence for optimal cleavage efficiency (17). Compared with other ribozymes, the G requirement 3' to the cleavage site in (–)sTRSV is unique and is similar to the

Fig. 4. Structural similarity among U6-12, the catalytic core region of the group I self-splicing intron in cyanobacteria, the catalytic domain of (–)sTRSV and the highly conserved region of U6 snRNA. Sequences homologous with that of the catalytic core region of the group I self-splicing intron are boxed. In case of the group I intron, sequences homologous with U6-12 are boxed. The outlined letters in U6-12 are bases that we substituted or inserted. The sequence and presumed secondary structure of the highly conserved region of U6 snRNA is taken from *S. pombe* U6 snRNA (7). The arrows in (a) and (c) indicate the cleavage site. P, Q, R and S regions with the bold letters in (b) represent the conserved primary sequence elements of the group I intron. Numbering of nucleotides in the catalytic domain of (–)sTRSV is according to that of a plus strand of sTRSV (28). Numbering of nucleotides in the catalytic core region of the group I self-splicing intron in cyanobacteria and *S. pombe* U6 snRNA is according to Xu et al. (23) and Tani and Ohshima (7), respectively.

G requirement of the 5' splice site in pre-mRNA splicing. A hammerhead ribozyme does not require G at the cleavage site (18).

To test the hypothesis that U6 snRNA functions as a catalytic element in pre-mRNA splicing, we prepared synthetic RNAs comprising the sequence of the highly conserved region of U6 snRNA or full length *S. pombe* U6 snRNA and tested their cleavage activities against the substrate RNA containing the GU sequence. Neither synthetic RNAs showed cleavage activity under the conditions tested, indicating that U6 snRNA may not function in the same manner as the catalytic domain of (–)sTRSV does.

In the case of (–)sTRSV, it is thought that the catalytic RNA abstracts a proton from the 2'-hydroxyl group adjacent to the susceptible phosphodiester bond in the substrate RNA and cleaves the bond to give a 2', 3'-cyclic phosphate and 5'-hydroxyl termini. This is not the case in pre-mRNA splicing which takes place in a two step reaction. In the first step of the pre-mRNA splicing, the cleavage at the 5' side of the GU sequence and the formation of the 2', 5' phosphodiester bond at the branch site occur as a concomitant reaction. In the second step of the reaction, 3' splice site cleavage occurs concomitant with exon ligation. Therefore, even if U6 snRNA has a catalytic role in pre-mRNA splicing, it might not cleave the substrate RNA in the same manner seen with (–)sTRSV. We proposed the following model as a role of U6 snRNA in the catalysis of pre-mRNA splicing (8). In the first step of the reaction of pre-mRNA splicing, U6 snRNA abstracts a proton from the 2'-hydroxyl group of adenosine in the branch site. This leads to cleavage of the 5' splice site and formation of 3'-hydroxyl and 2', 5' phosphodiester bond. In the second step of the reaction, U6 snRNA abstracts a proton from the 3'-hydroxyl group of the 5' exon, leading to excision of a lariat intron and ligation of the exons. The 3' region of U6 snRNA was shown to be base-paired with 5'-end of U2 snRNA, which interacts with the branch site (19). This implies that U6 snRNA is in close proximity to the branch site. The U6/U2 interaction proved to be essential for pre-mRNA splicing in vertebrates (20, 21). In addition, Sawa and Shimura recently reported that U6 snRNA is cross-linked with the 5' splice site when the precursor RNA in HeLa nuclear extracts is irradiated by UV under splicing conditions, suggesting that U6 snRNA is positioned closely to the 5' splice site during pre-mRNA splicing (22). The close positioning of U6 snRNA, the branch site and the 5' splice site during the reaction of pre-mRNA splicing is consistent with our hypothesis described above. In our model, the catalysis by U6 snRNA requires the intimate interaction of U6 snRNA with other snRNAs and substrate RNA.

We found that minor alterations in the sequence in the presumed hairpin structure in U6 snRNA resulted in acquisition of (–)sTRSV-like cleavage activity. Among the chimeric RNAs having the cleavage activity, U6-12 has only an 8 nucleotide difference from the original U6 domain. Therefore, U6-12 has an 83.3% sequence homology with the conserved region of *S. pombe* U6 snRNA while it has only 66% sequence homology with the catalytic domain of (–)sTRSV. In this respect, U6-12 is closer to U6 snRNA than the catalytic domain of (–)sTRSV. The replacement of GCC and GAC located in the internal loop region of the presumed hairpin structure in U6 snRNA to AAA and AUU, and G/C insertions into the stem region, are sufficient for obtaining the cleavage activity. Thus, the internal loop region in this hairpin structure may have an important role, such as selection of a hydroxyl group whose proton is abstracted by the catalytic RNAs, in the catalysis of (–)sTRSV-like cleavage.

Interestingly, we found that the hairpin structures in the catalytic domain of (–)sTRSV, U6 snRNA and chimeric RNAs such as U6-12 have a remarkable sequence homology with the conserved elements P and Q in the catalytic core region of the group I self-splicing intron found in cyanobacteria (23, 24) (Fig. 4). A sequence CAGAGA that is close to the cleavage site in U6-12 and the catalytic domain of (–)sTRSV was also found in the conserved element R of the catalytic core region of the group I self-splicing intron in cyanobacteria. The second G residues in the CAGAGA sequence in the conserved element R is thought to be involved in the binding of guanosine which is essential for the self-splicing reaction (25). The identical CAGAGA sequence is present at the 5' side of the presumed hairpin structure of U6 snRNA. The CAGAGA sequence in U6 snRNA was shown to be essential for pre-mRNA splicing, in the *in vitro* and *in vivo* experiments in yeast (10, 11). Although the sequence homology of U6 snRNA or the catalytic domain of (–)sTRSV with the catalytic core region of the group I intron is generally found, it is most striking when we compared them with the group I intron in cyanobacteria or chloroplasts. Cyanobacteria have been considered to be the progenitors of chloroplasts (26). It is noteworthy that the group I introns in cyanobacteria and chloroplasts may maintain the structure of the ancient group I intron (24). The finding of sequence homology among the highly conserved region of U6 snRNA, the catalytic RNA of (–)sTRSV and the catalytic core region of the group I self-splicing intron suggests that these three RNAs might be derived from a common ancestral RNA and that they have an analogous function, that is, a catalytic role. This finding supports further the idea that U6 snRNA functions as a catalytic element in pre-mRNA splicing.

Although we did not detect (–)sTRSV-like cleavage activity in U6 snRNA *in vitro*, we can not exclude the possibility that U6 snRNA can express (–)sTRSV-like cleavage activity under certain conditions during the splicing reaction. Recently, Lund and Dahlberg reported that U6 snRNA has at their 3' ends a 2', 3'-cyclic phosphate, a terminal structure identical to that generated in the cleavage by the catalytic RNA of (–)sTRSV (27). As suggested by them, it is possible that U6 snRNA forms covalent linkage with another spliceosomal RNA to set up the correct architecture of the spliceosome and that the transient linkage is cleaved by U6 snRNA itself in the manner similar to that for the catalysis by (–)sTRSV.

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