Determinants of the 3' splice site for self-splicing of the *Tetrahymena* pre-rRNA

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Tetrahymena preribosomal RNA undergoes self-splicing in vitro. The structural components involved in recognition of the 5' splice site have been identified, but the mechanism by which the 3' splice site is recognized is not established. To identify some components of 3' splice site recognition, we have generated mutations near the 3' splice site and determined their effects on self-splicing. Alteration of the 3'-terminal guanosine of the intervening sequence (IVS), a conserved nucleotide in group I IVSs, almost eliminated 3' splice site activity; the IVS-3' exon splicing intermediate accumulated, and exon ligation was extremely slow. These mutations do not result in recruitment of cryptic 3' splice sites, in contrast to mutations that affect the 5' splice site. Alteration of the cytidine preceding the 3'-terminal guanosine or of the first two nucleotides of the 3' exon had similar but less severe effects on exon ligation. Most of the mutants showed some reduction (less than threefold) in GTP addition at the 5' splice site. A mutation that placed a new guanosine residue just upstream from the 3'-terminal guanosine misspliced to produce ligated exons with one extra nucleotide between the 5' and 3' exons. We conclude that multiple nucleotides, located both at the 3' end of the IVS and in the 3' exon, are required for 3' splice site recognition.

[Key Words: Self-splicing; group I introns; intervening sequences; *Tetrahymena*; pre-rRNA; 3' splice site]

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The intervening sequence (IVS) found in the preribosomal RNA of *Tetrahymena* is removed from the pre-rRNA in a self-splicing reaction, in the absence of proteins (Kruger et al. 1982). The splicing activity resides in the folded structure of the intervening sequence (Zaug et al. 1983, 1986; Zaug and Cech 1986; Price et al. 1987). A model for the basic chemical mechanism of self-splicing (Fig. 1A,B; Cech et al. 1981; Zaug et al. 1983) has proved to be extremely useful in predicting the outcome of new experiments or in understanding new results with the *Tetrahymena* IVS (Inoue et al. 1986; Zaug and Cech 1986; Price et al. 1987) and related group I IVSs (Ehrenman et al. 1986; Tabak et al. 1987). The mechanism involves nucleophilic attack of a guanosine cofactor at the 5' splice site. The guanosine becomes covalently attached to the 5' end of the IVS, leaving the 5' exon with a free 3' hydroxyl. The next step is nucleophilic attack of the 3' hydroxyl of the 5' exon at the 3' splice site. This results in exon ligation and release of the IVS.

One key feature of the reaction is its specificity. The in vivo precursor RNA is over 6000 nucleotides long. The splice sites must be selected unambiguously for ac-

curate processing to occur. Because splicing occurs accurately in a truncated precursor RNA (Kruger et al. 1982; Price et al. 1987) the specificity for the splicing reaction must be derived from recognition of the splice sites by structures within, or close to, the IVS. Davies et al. (1982) proposed that a sequence element within the IVS binds both the 5' and 3' exon sequences to bring them into proper alignment for exon ligation. The interaction of this internal guide sequence (IGS) with the 5' exon has been tested rigorously and has been found to provide the specificity for recognition of the 5' splice site (Been and Cech 1985, 1986; Waring et al. 1986; Price et al. 1987). On the other hand, there is no biochemical evidence that any interaction between the IGS and the 3' exon is required for recognition of the 3' splice site. Deletion of the portion of the IGS proposed to interact with the 3' splice site has little or no effect on the efficiency of the splicing reaction (Been and Cech 1985). A 2-base mutation in this same portion of the IGS decreases the overall rate of splicing but does not appear to specifically affect the second step of splicing—exon ligation (Davies et al. 1987). Thus, little is known about what structure or structures of the IVS are involved in recognition of the 3' splice site.

In addition, very little is known about what features of the 3' splice site are important for it to be recognized as

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Figure 1. Mechanism of splicing of wild-type RNA and missplicing of 413G RNA. (A) Normal splicing reaction. During the first step of splicing, guanosine-dependent cleavage at the 5' splice site (●) releases the 5' exon with a 3' hydroxyl group. (B) In the second step of splicing, the 3' hydroxyl group of the 5' exon undergoes nucleophilic attack at the phosphate at the 3' splice site (◆) and becomes ligated to the 3' exon. The ligated exons are released from the IVS. (C) In 413G RNA, the 3' hydroxyl group of the 5' exon attacks the phosphate that follows the new guanosine residue introduced at position 413 (◇) and becomes ligated to this position, generating a ligated exon species with an extra guanosine between the two normal exons. Lowercase letters indicate exon sequences; uppercase letters indicate IVS.

such. It seems likely that a guanosine residue at the 3' end of the IVS is one important component. The 3'-terminal guanosine is phylogenetically conserved in group I IVSs (Waring and Davies 1984). In a recent compilation, of 66 group I IVSs, all terminate in G (Cech 1988). Biochemical evidence also suggests a role for this guanosine residue. Kay and Inoue (1987) showed that dinucleotides of the form GpN (but not ApN, CpN, or UpN) could be attacked by CpU_{OH} in an intermolecular version of exon ligation, implying that a 3' splice site must be preceded by a G. Tanner and Cech (1987) inferred that the 3'-terminal guanosine and the preceding cytosine are important for reactivity in cyclization of the IVS, a reaction that appears to be mechanistically similar to splicing.

Another clue as to the extent of the domain recognized as the 3' splice site is the fact that insertions and deletions at a position 5 nucleotides upstream from the 3' splice site do not affect accuracy of 3' splice site usage (Price et al. 1985; Barford and Cech 1988). Thus, the 3' splice site is not chosen by its distance from a fixed point within the IVS, and sequences distal to position

409 (the last 5 nucleotides of the IVS, or the 3' exon) are implicated in defining the 3' splice site. The 3' exon sequences have been truncated to within 23 nucleotides of the 3' splice site without apparent effect on the splicing reaction (Price et al. 1987).

To characterize further the determinants involved in recognition of the 3' splice site, we have constructed mutations at the splice site and studied their effects on the splicing reaction.

Results

Plasmid constructions

Plasmid pBGST7 was chosen as the parent vector for site-directed mutagenesis because it allows direct in vivo screening for mutations that affect self-splicing activity (Fig. 2; Been and Cech 1986). The plasmid confers β-galactosidase activity to an appropriate host, such as *Escherichia coli* strain JM83 (see Vieira and Messing 1982; Price and Cech 1985). Mutations in the IVS that

Figure 2. Transcript structures and 3' splice-site mutations. A fragment containing the *Tetrahymena* self-splicing IVS was inserted between the *EcoRI* and *HindIII* restriction sites of pUC18 such that after splicing, the reading frame for the β -galactosidase α -fragment is uninterrupted. In *E. coli*, precursor RNA is transcribed from the *lac* promoter. This arrangement allows in vivo screening for mutations affecting self-splicing by monitoring β -galactosidase expression. A promoter for bacteriophage T7 RNA polymerase located ~250 nucleotides upstream from the *lac* promoter allows in vitro transcription of precursor RNA for biochemical studies. The region of the 3' splice site is expanded to indicate the mutations introduced. (Open box) Plasmid sequences; (solid boxes) *Tetrahymena* exon sequences; (wavy line) IVS; (hatching) β -galactosidase-coding sequences; (lowercase letters) exon sequences; (uppercase letters) IVS; (solid diamond) 3' splice site.

eliminate self-splicing activity also eliminate β -galactosidase expression. Thus, a preliminary indication of splicing activity can be obtained using 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) indicator plates. pBGST7 also carries a promoter for bacteriophage T7 RNA polymerase. This promoter allows in vitro transcription of a 716-nucleotide precursor RNA in quantities suitable for biochemical studies. This precursor RNA contains 258 nucleotides of plasmid sequences followed by 16 nucleotides of the natural *Tetrahymena* 5' exon, the 413-nucleotide IVS, 23 nucleotides of the natural 3' exon, and 6 additional nucleotides of plasmid sequence at the end of the transcript.

Plasmids with site-directed substitutions were constructed by substituting synthetic DNA fragments for a *ScaI*–*HindIII* restriction fragment that spans the 3' splice site (Fig. 2). Two plasmids carrying mutations that alter the C at position 413 of the IVS are called pBG413A and pBG413G, to denote the position of the change and the new base that has been substituted. These two plasmids confer a light blue color to bacterial colonies on X-gal plates, indicating a partial reduction in splicing activity. Two plasmids carrying mutations that alter the G at position 414 of the IVS are called pBG414U and pBG414C. Bacterial colonies bearing these plasmids remain white on X-gal plates, indicating a substantial reduction in splicing activity. In addition, a plasmid with altered bases at the first two positions of the 3' exon was obtained from M. Been. This plasmid, which contains a TA $\rightarrow$ GC double substitution, is called pBG + 1c: + 2g. It confers a light blue color to bacteria growing on X-gal plates.

In vitro splicing assays

The five mutated plasmids and pBGST7 were transcribed in vitro, and precursor RNAs were gel purified.

Each RNA was subjected to three assay conditions: splicing, which optimizes yield of linear IVS; cyclization, which optimizes yield of circular IVS and produces ligated exons more efficiently than splicing conditions; and hydrolysis, which reveals the guanosine-independent products of site-specific hydrolysis at the 3' and 5' splice sites (Zang et al. 1984; Inoue et al. 1986).

All of the RNAs show some degree of reactivity (Fig. 3). RNAs transcribed from pBG + 1c: + 2g, pBG413A, and pBG413G produce all of the reaction products produced by wild-type RNA transcribed from pBGST7, although the mutated RNAs show reduced accumulation of ligated exons and increased amounts of side products. 414U and 414C precursor RNAs primarily produce the splicing intermediates generated by cleavage at the 5' splice site. Only very small amounts of linear and circular IVS are produced by these two mutants. Ligated exons are produced by 414U and 414C RNAs but only in minute quantities.

Sequence of the ligated exons

Ligated exon RNA species from each of the five mutants and from pBGST7 were gel purified and sequenced by extension of a 32 P-labeled primer with reverse transcriptase in the presence of dideoxynucleotides (Fig. 4 and data not shown). With one exception, all of the ligated exon species gave the sequence expected for accurate joining of the two splice sites. The exception was 413G RNA which, surprisingly, produced ligated exons with a guanosine residue inserted between the two exons. Apparently, when a guanosine is substituted for the cytidine at position 413, the new guanosine is preferentially recognized at the 3' end of the IVS and splicing is directed to its phosphodiester bond (Fig. 1C).

Figure 3. In vitro splicing assays of mutant RNAs. Precursor RNAs were transcribed in vitro in the presence of [3 H]UTP, gel purified, and subjected to a variety of reaction conditions. Reaction products were fractionated on 4% polyacrylamide gels containing 8 M urea. Reaction conditions were as follows: (-) 200 mM $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$, 30 mM Tris (pH 7.5), 10 mM EDTA at 42°C; (H) hydrolysis; (S) splicing; (C) cyclization (see Materials and methods). All reactions were for 1 hr. (C° IVS) Large form of the circular IVS resulting from cyclization to the 5' splice site (Inoue et al. 1986); (C IVS) circular IVS; (L IVS) linear IVS; (L-15 IVS) a shortened form of the linear IVS resulting from site-specific hydrolysis of the circular IVS at the cyclization site (Zaug et al. 1984).

Effect of mutations at the 3' splice site on reactivity at the 5' splice site

Precursor RNA from each plasmid was incubated with ^{32}P -labeled GTP in splicing conditions. Guanosine labeling was observed in RNA species of three different electrophoretic mobilities, corresponding to the linear IVS, the IVS + 3' exon splicing intermediate originally described by Inoue et al. (1986), and the 15-mer released from the 5' end of the IVS during cyclization (Zaug et al. 1983). Only the IVS and IVS + 3' exon are included in the sample data shown in Figure 5A. The amount of 5' splice site activity, as determined by total GTP addition to all three species, is roughly equivalent in all six precursor RNAs (Fig. 5B). RNA from +lc: +2g is the least active, producing about 40% as much GTP labeling as wild-type RNA. 4l3G RNA actually produces slightly more

GTP-labeled material than wild-type RNA. The differences between the various RNAs become apparent when the levels of IVS + 3' exon splicing intermediate are compared (Fig. 5C). In the reaction conditions used for this experiment, wild-type RNA does not accumulate detectable levels of GTP-labeled IVS + 3' exon. In reactions containing 4l3A or 4l3G precursor RNAs, the IVS + 3' exon species represents 20–30% of the GTP-labeled material produced initially, but this material appears to undergo a subsequent reaction to produce the linear IVS at later time points. The GTP-labeled reaction products of +lc: +2g RNA consist of ~50% splicing intermediate. This intermediate accumulates at the same rate as the GTP-labeled IVS and appears to be a fairly stable product. 4l4U and 4l4C precursor RNAs produce only the splicing intermediate; no detectable levels of IVS are observed.

Figure 4. Sequencing gel of wild-type and 4l3G ligated exons. Precursor RNA samples were incubated for 1 hr in cyclization conditions (see Materials and methods). Ligated exon RNAs were gel purified and sequenced by primer extension with reverse transcriptase in the presence of dideoxynucleotides. (Top) A, G, C, and U indicate the nucleotide complementary to the dideoxynucleotide added; O indicates no dideoxynucleotide added. The sequences of the RNAs are indicated next to the gel. (Open arrow) Normal splice junction; (solid arrow) extra guanine in 4l3G ligated exons.

Reactivity at the 3' splice site

Relative activity at the 3' splice site was determined for each of the six precursor RNAs by measuring the amount of ligated exons produced by each precursor at various times (Fig. 6). Each precursor shows a different rate of accumulation of ligated exons. 4l3G RNA produces ligated exons nearly as fast as wild-type RNA, even though the ligation reaction is inaccurate in this mutant (see above). 4l3A and +lc: +2g RNAs produce ligated exons at reduced rates, but the decrease in the rate of exon ligation is not substantial (~60% and ~20% of the rate of wild-type RNA, respectively). 4l4U and 4l4C RNAs produce a very small amount of ligated exon RNA species. These are only detectable at the longest time points where they represent ~4% and ~6%, respectively, of the amount of ligated exons generated by wild-type RNA.

Discussion

One main conclusion from the present work is that the guanosine at the 3' end of the IVS (G_{414}) greatly enhances the rate of the second step of self-splicing. Although the 100% phylogenetic conservation of this nucleotide gave

Figure 5. 5' splice-site activity, as measured by auto addition of [^{32}P]GTP. Precursor RNAs (40 nM) were incubated in the presence of 2 μM [^{32}P]GTP (800 Ci/mmol) under standard splicing conditions. At time points 5, 10, and 60 min, samples were transferred to an equal volume of 97% formamide, 10 mM EDTA, and 0.04% bromophenol blue and separated in a polyacrylamide gel containing 8 M urea. After autoradiography, RNA species were excised from the gel and quantified by Cerenkov counting. (A) Autoradiograph of a 6% polyacrylamide gel showing accumulation of the [^{32}P]GTP-labeled IVS and IVS+3' exon species in wild-type and mutant RNAs. (B) Accumulation of the [^{32}P]GTP-labeled RNA for wild-type RNA and each of the 5 mutant RNAs. Quantities plotted are the combined values of ^{32}P -labeled IVS, IVS+3' exon, and 15-mer species at each time point. (C) Percent of [^{32}P]GTP-labeled material found in the IVS+3' exon splicing intermediate. (○) Wild type; (●) +1c:+2g; (□) 413A; (■) 413G; (△) 414U; (▲) 414C.

a priori evidence of its importance, the biochemical analysis presented here established a number of points that could not be predicted. These include (1) the severity of the phenotype of nucleotide substitutions at position 414 (a small advantage might be enough to explain selection for a G, but the biochemical data show

that a G is obligatory for efficient splicing); (2) the persistence of a low level of accurate exon ligation in G_{414} mutants, which is evidence for determinants of the location of the 3' splice site in addition to G_{414} ; and (3) the lack of activation of cryptic 3' splice sites, a result opposite to that observed with the 5' splice site of the same IVS. The preceding nucleotide (C_{413}) is not phylogenetically conserved, yet mutations at that position decrease the rate of the second step of splicing substantially. Thus, a second conclusion is that the nucleotide in the IVS preceding the terminal G helps determine 3' splice site reactivity, in agreement with an earlier suggestion (Tanner and Cech 1987). Finally, the 413G mutant provides the first example of an accuracy mutant at the 3' splice site of a self-splicing RNA.

Guanosine plays an important role in both the first and the second steps of the splicing reaction. Free guanosine or GTP from solution is specifically bound and covalently added to the 5' end of the IVS during the first step of splicing (Cech et al. 1981; Bass and Cech 1984). The results presented here argue that the guanosine residue at the 3' end of the IVS is important for recognition of the 3' splice site for the second step of splicing. The exogenous guanosine and the 3' guanosine could be recognized by two distinct binding sites or they could compete for the same binding interactions. If there were only one guanosine binding site, elimination of the guanosine residue at the 3' splice site may have eliminated binding competition and thus led to an increase in the reactivity of the 5' splice site, as measured by [^{32}P]GTP addition. Because this is not observed, our results may be considered to favor the concept of two distinct binding sites. A more detailed proposal for two G-binding sites has been presented recently by Kay et al. (1988).

The relative splicing efficiencies of wild-type and mutant RNAs were determined by examining the rate of exon ligation (Fig. 6). Although both the accumulation of IVS+3' exon and the rate of exon ligation indicate reduced 3' splice site reactivity in the mutant RNAs, these methods are indirect because the availability of the 5' exon as a cosubstrate profoundly affects the rate of the 3' splice site reaction (Inoue et al. 1985). Therefore, the 3' splice site reaction rate can be inferred more accurately by normalizing for the total release of 5' exon. Calculation of (moles ligated exon)/(moles ligated exon + moles 5' exon) for each time point in Figure 6 represents the fraction of 5' exon released that was subsequently involved in a ligation at the 3' splice site. The ratio does not vary with time but represents the efficiency of 3' splice site usage. The average value for each RNA species is ST7 (wild type), 0.73; +1c:+2g, 0.17; 413A, 0.41; 413G, 0.60; 414U, 0.01; and 414C, 0.04. The basic conclusions about relative 3' splice site activity of the different mutant RNAs are unaffected by this correction for the differences in 5' splice site activity.

It is evident that the guanosine residue at the 3' end of the IVS is extremely important for the accuracy of exon ligation, as well as the activity of 3' splice site. Mutation 413G redirects the specificity of 3' splice site selection due to substitution of a guanosine for a cytidine near the

Figure 6. Production of ligated exons by wild-type and mutant RNAs. Precursor RNA was labeled uniformly with [α - 32 P]GTP during transcription. Gel-purified precursor RNA was incubated in 60 μ l 1 mM GTP, 5 mM MgCl₂, 200 mM NH₄C₂H₃O₂, and 30 mM Tris (pH 7.5) at 30°C. At times 2, 6, 20, 60, and 300 min after reaction was started, a 10- μ l aliquot was transferred to a tube containing 1 μ l of 100 mM EDTA. Control samples were mixed with the EDTA stop solution before addition of 10 $\times$ splicing buffer. The control samples were incubated for 1 hr at 30°C. Samples were separated on 4% polyacrylamide gels containing 8 M urea. After autoradiography, the bands were excised from the gel and quantified by Cerenkov counting. Values for ligated exons were expressed as a percent of the total number of cpm in each sample and corrected for background by normalizing against a corresponding gel slice from the control lane. If 100% of the precursor RNA were spliced productively, the expected yield of ligated exons would be 41% of the total RNA. (○) Wild type; (●) +1c:+2g; (□) 413A; (■) 413G; (△) 414U; (▲) 414C.

3' splice site.¹ Of 66 group I IVSs, only 4 have a guanosine residue preceding the 3'-terminal guanosine (Cech 1988); there is a phylogenetic preference for anything but G at this position.

Mutations 414U and 414C, which eliminate the guanosine residue, are greatly reduced in 3' splice site activity, although small amounts of accurately ligated exons are still produced; no missplicing to other nearby guanosine residues is observed. A similar result is observed in yeast pre-mRNA splicing, which does not

occur by self-splicing. Vijayraghavan et al. (1986) generated a G $\rightarrow$ C substitution in the 3'-terminal guanosine of a yeast nuclear mRNA IVS. Their mutation caused a 97% reduction in in vivo splicing activity, affected only the 3' cleavage and ligation step of splicing, and produced a small amount of ligated exons. It is perhaps surprising that in both this yeast mRNA example and in the present case no cryptic splice sites are activated by inactivation of the normal 3' splice site, as has been described for inactivation of the 5' splice site of the *Tetrahymena* IVS (Been et al. 1987; Price et al. 1987). In the present example, there are several guanosine residues nearby (at position 408, 6 nucleotides upstream and at position +4, 4 nucleotides downstream). There is even a UCGU sequence 20 nucleotides downstream in the 3' exon, although this sequence is partially concealed in a helix that is normally present in the rRNA (nucleotides +3 to +7 paired with nucleotides +14 to +18; Clark et al. 1984; Noller 1984).

The fact that a low level of accurate exon ligation still occurs in the absence of a guanosine residue at the 3' splice site suggests that other determinants must also be

¹In light of this missplicing event, it is surprising that plasmid pBG413G confers an intermediate amount of β -galactosidase activity to bacterial colonies, as judged by a light-blue phenotype on X-gal plates. The frame-shift induced by missplicing is expected to cause termination of β -galactosidase translation at a UAA codon at position +2 of the 3' exon. Perhaps, some of the precursor RNA is spliced normally in vivo. An alternative possibility is that a small amount of translation is initiated at an AUG codon 10 nucleotides upstream of the splice site, in the 5' exon. This codon is preceded by a GGGU sequence at positions -18 to -15 of the 5' exon, which may serve as a ribosome binding site. This AUG codon is normally out of frame relative to the β -galactosidase fragment, but the insertion of the extra G residue at the splice junction brings it into register.

involved in selection of this site. Other lines of evidence that support this idea include the fact that guanosine residues introduced 2 nucleotides downstream from the 3' splice site in +1c:+2g RNA (this paper) or 5 nucleotides upstream from the 3' splice site in the case of a *Bam*HI linker inserted at position 409 of the IVS (Price et al. 1985) are not used as alternative 3' splice sites. The cytosine residue at position 413 of the IVS and the U and A residues at positions 1 and 2 of the 3' exon are good candidates for additional determinants because mutations 413A and +1c:+2g cause a reduction in 3' splice site activity. The *Bam*HI linker insertion also allows us to discount the possibility that the splice site is identified as the first guanosine residue following some internal structure such as the P9.2 helix (which is the last secondary structure element in the IVS; see Burke et al. 1987).

In the IGS model of Davies et al. (1982, 1987), bases +2 through +8 of the 3' exon pair with the IGS to align the 3' splice site. The +1c:+2g mutant examined here would have some effect on this proposed interaction, substituting a g·U base pair for the normal a·U base pair between position +2 of the 3' exon and the U20 in the IGS. Evaluating whether or not such a change would give the observed large reduction in 3' splice site activity would be conjectural. Thus, the present work provides no rigorous test of the IGS model. We can conclude, however, that IGS·3' exon pairing is neither necessary (Been and Cech 1985) nor sufficient (this work) to specify the 3' splice site.

We have presented evidence that there are determinants of the 3' splice site both at the 3' end of the IVS and in the adjacent 3' exon. Future models for the mechanism of 3' splice site recognition should accommodate these findings.

Materials and methods

Materials

Nucleoside triphosphates were purchased from P-L Biochemicals. Radionucleotides, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Nuclear, reverse transcriptase from Life Sciences, and restriction enzymes from New England Biolabs. Bacteriophage T7 RNA polymerase was purified by A. Zaug, using the method of Davanloo et al. (1984).

Deoxyoligonucleotide JP2 (5'-AGCT(-TGTGACG(-AGG-CATT(-TGGCTACCTTACGAGT-3')) was synthesized on an Applied Biosystems DNA synthesizer, using Applied Biosystems reagents. Deoxyoligonucleotides JP3 (5'-ACT[A/G/T]GTAAGGTAGCCAAATGCCTCGTCACA-3'), JP4 (5'-AGCTTGTGACGAGGCATTTGGCTACCTTAC[A/C/T]AGT-3'), and JP5 (5'-ACTC[A/C/T]TAAGGTAGCCAAATGCCTCGTCACA-3') were synthesized on a Biosearch DNA synthesizer using Biosearch reagents.

Plasmid construction

Plasmid pBGST7 (Been and Cech 1986) was obtained from M. Been. Oligonucleotides were synthesized to replace the 30-nucleotide top strand and the 34-nucleotide bottom strand of a *Sca*I/*Hind*III restriction fragment encompassing the 3' end of the IVS (Fig. 2). Substitutions were made at position 414 where

A, C, and T were incorporated instead of the G which corresponds to the 3'-terminal guanosine of the IVS (oligonucleotide JP5). Substitutions were also made at position 413 where A, G, and T were incorporated instead of the C which corresponds to a position 1 base upstream from the 3' end of the IVS (oligonucleotides JP3 and JP4). pBGST7 was digested partially with restriction endonuclease *Sca*I. Full-length linear molecules were gel purified and ligated to a 50-fold excess of phosphorylated oligonucleotides JP3 and JP4 or JP2 and JP5. The ligated material was digested with an excess of restriction endonuclease *Hind*III and full-length linear molecules were again gel purified. These molecules were ligated and used to transform *E. coli* strain JM83 (Vieira and Messing 1982). Transformed bacteria were plated on X-gal indicator plates. Plasmids from individual colonies (ranging from blue to white) were purified by the method of Holmes and Quigley (1981), and the sequence in the region of mutagenesis was determined as described below. Of the plasmids mutagenized at position 414, 19 were sequenced; 4 of these were substitutions of G₄₁₄ → C, 3 were G₄₁₄ → T, 4 were unmutated, and 8 were various kinds of construction artifacts. No examples of a G₄₁₄ → A substitution were identified. Twenty-four plasmids mutagenized at position 413 were sequenced: 12 were found to be C₄₁₃ → G substitutions, 3 were C₄₁₃ → A substitutions, 4 were unmutated, and 5 were construction artifacts. No examples of a C₄₁₃ → T substitution were found.

Plasmid pBG+1c:+2g was constructed by M. Been, using oligonucleotide-directed mutagenesis as described in Been and Cech (1986).

Nomenclature for these mutations is based on the numbering system for the excised IVS RNA, which is 414 nucleotides long due to the addition of guanosine to the 5' end of the IVS during splicing. Thus, the sequence of the IVS in the DNA begins with position 2 and ends with position 414. Nucleotides in the exons are indicated with lowercase letters and are counted from the splice sites. They are designated with a minus (-) to denote the number of bases before the 5' splice site or a plus (+) to denote the number of bases after the 3' splice site (Been et al. 1987).

Nucleic acid sequencing

Dideoxynucleotide sequencing of RNA and DNA was performed as described by Inoue and Cech (1985) and modified by Price et al. (1987). Plasmids were sequenced using New England Biolabs M13 sequencing primer 1200 (5'-TCCCAGTCACGACGT-3'). RNA was sequenced using primer EP3P (5'-AGCTTGTGACGAGGCAT-3'), obtained from M. Been.

Transcription reactions

Plasmid DNA was digested with restriction endonuclease *Hind*III, extracted once with phenol, once with chloroform, and precipitated with 2.5 volumes of ethanol. Transcriptions contained 5 µg plasmid DNA, 15 mM MgCl₂, 40 mM Tris (pH 7.5), 1 mM NTPs, 2 mM spermidine, 5 mM dithiothreitol, and 2500 units of T7 RNA polymerase for a 1-ml reaction. Uniformly labeled RNA was produced by including either 400 µCi/ml [³H]UTP or 270 µCi/ml [α-³²P]GTP in the transcription reaction. Transcriptions were incubated at 37°C for 2 hr and terminated by addition of EDTA to a concentration of 20 mM. NaCl was added to a concentration of 0.1 M and the RNA was precipitated with 2.5 volumes of absolute ethanol. Full-length transcription products were gel purified in 4% polyacrylamide gels containing 8 M urea.

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Gel purification

RNA was recovered from polyacrylamide gels by excising a gel slice containing the sample. The gel slice was crushed and soaked in 0.5 M $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$, 1 mM EDTA, and 0.1% SDS overnight at 22°C. The supernatant was filtered with a Quick-Sep filter (Isolabs, Inc.) and precipitated with 2.5 volumes of absolute ethanol.

DNA was recovered from agarose gels using an IBI electroelution apparatus according to the manufacturer's instructions.

Splicing reactions

Splicing reactions were performed in 1 mM GTP, 5 mM MgCl_2 , 200 mM $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$, and 20 mM Tris (pH 7.5) at 30°C. Splicing reactions were terminated by addition of EDTA to a concentration of 10 mM. Where [^{32}P]GTP was used as the cofactor in splicing reactions, the total concentration of GTP was reduced to 2 μM . Hydrolysis reactions were performed in 10 mM MgCl_2 , 200 mM $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$, and 30 mM Tris (pH 7.5) at 42°C. Cyclization reactions were performed in 1 mM GTP, 10 mM MgCl_2 , 200 mM $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$, and 30 mM Tris (pH 7.5) at 42°C. These reactions were terminated by addition of EDTA to a concentration of 20 mM. Reactions were for 1 hr unless otherwise stated.

Quantitation of ^{32}P -labeled RNA species

RNA samples labeled with ^{32}P were excised from the gel. The gel slices were covered with water and quantitated by Cerenkov counting in a Beckman LS-7000 scintillation counter.

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