Deletion of nonconserved helices near the 3' end of the rRNA intron of *Tetrahymena thermophila* alters self-splicing but not core catalytic activity

Elisabeth T. Barfod and Thomas R. Cech¹

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215 USA

The self-splicing rRNA intron of *Tetrahymena thermophila* contains two stem-loop structures (P9.1 and P9.2) near its 3' end that are not conserved among group I introns. As a step toward deriving the smallest active self-splicing RNA, 78 nucleotides encompassing P9.1 and P9.2 have been deleted. This deletion has no effect on the core catalytic activity of the intron, as judged by its ability to catalyze poly(C) polymerization and other related reactions. In contrast, reactions at the 3' splice site of the rRNA precursor—exon ligation and intermolecular exon ligation—take place with reduced efficiency, and exon ligation becomes rate-limiting for self-splicing. Moreover, intermolecular exon ligation with pentaribocytidylic acid is inaccurate, occurring primarily at a cryptic site in the 3' exon. A deletion of 79 nucleotides that disrupts P9, as well as removing P9.1 and P9.2, has more severe effects on both the first and second steps of splicing. P9, a conserved helix at the 5' edge of the deletion point, can form stable alternative structures in the deletion mutants. This aberrant folding may be responsible for the reduced activity and accuracy of reactions with mutant precursors. Analysis of the cryptic site suggests that choice of the 3' splice site may not only depend on sequence but also on proximity to P9. In the course of these studies, evidence has been obtained for an alternative 5' exon-binding site distinct from the normal site in the internal guide sequence.

[Key Words: RNA processing; group I intron; RNA structure; mutagenesis]

Received February 23, 1988; revised version accepted April 21, 1988.

Many RNA transcripts contain domains called introns (intervening sequences, IVSs) that are not found in the mature RNA but are removed by a process called RNA splicing. Some introns are capable of splicing in the absence of proteins or other macromolecular factors. Therefore, the information required for this self-splicing is contained within the native structure of the intron itself (Kruger et al. 1982).

Group I introns splice by a two-step transesterification mechanism that is initiated by nucleophilic attack of a guanosine cofactor (Cech 1987). The 60 reported members of this class are heterogeneous with respect to length, ranging from 258 to 2641 nucleotides (Michel and Cummings 1985; Trinkl and Wolf 1986), and show little primary sequence homology. However, at the secondary structure level, there is remarkable conservation. Three conserved base-paired elements (P3, P4, and P7) can be aligned so that all of the group I introns are superimposed in a core region (Davies et al. 1982; Michel et al. 1982; Been et al. 1987). Nucleotides unique to a particular intron are folded in additional helices or helix extensions, so that the linear or spatial distances between the conserved elements are maintained (Fig. 1A).

¹Corresponding author.

Self-splicing conditions have been found for 11 of the group I introns discovered to date (Cech 1987). Examination of the secondary structures of these molecules has not revealed additional conserved features beyond those found in all group I introns. Therefore, it should be possible to remove nonconserved helices from a self-splicing RNA and obtain a catalytic molecule that would be of a size more amenable to physical studies. Previous attempts at deleting sequences have met with some success (Been and Cech 1985; Price et al. 1985; Ehrenman et al. 1986; Szostak 1986). Here, we report that excision of nearly a fifth of the *Tetrahymena thermophila* rRNA intron leaves a molecule that is still capable of all of the reactions characteristic of a group I self-splicing RNA. In the course of characterizing the efficiency of this deletion mutant, we have found evidence of 3'-splice site influence on the first step of splicing and discovered a cryptic site of intermolecular exon ligation in the 3' exon. [Intermolecular exon ligation or *trans*-splicing is a reaction in which an exogenous oligonucleotide with a sequence resembling that at the end of the 5' exon becomes ligated to the 3' exon (Inoue et al. 1985)]. Analysis of the properties of the cryptic site suggests that spatial constraints, in addition to the known sequence constraints, are operant in 3' splice site choice.

Unexpectedly, evidence for an alternative 5' exon-binding site, which is not part of the well-characterized internal guide sequence (IGS; see Davies et al. 1982), has been found. Alternative conformations of the RNA that might explain the new site of intermolecular exon ligation are illustrated.

Results

Deletion mutants

Using *NheI* and *ScaI* restriction sites, 78 nucleotides encompassing the DNA coding for the two terminal non-

Figure 1. (See following page for legend.)

conserved helices, P9.1 and P9.2, were removed from the *Tetrahymena* rRNA intron. This mutant was named $\Delta 78$, and its sequence in the vicinity of the deletion is shown in Figure 1B. Another mutant with 79 nucleotides deleted, $\Delta 79$, was also found. The loss of the additional nucleotide disrupts the terminal base pair of conserved helix P9.

Synthetic DNA containing restriction endonuclease recognition sites (linkers) was inserted between the *NheI* and *ScaI* sites to increase the lateral distance between P9 and the 3' splice site so that it would resemble that in the wild-type secondary structure more closely. $\Delta 78 + 6$ contains six nucleotides of digested linker (Fig. 1B). $\Delta 78 + 14$ contains 14 nucleotides of one and one-half repeats of linker, and its 3'-splice site lines up with that of the wild-type in linear sequence. $\Delta 78 + 32$ has 32 nucleotides of sequence that resemble those of the linkers but are not identical to them or any other nucleotides present in pBGST7.

In vitro splicing activity

Internally radiolabeled precursor RNA was transcribed from the different deletion constructs, purified, and analyzed for self-splicing activity. An autoradiogram of the products separated on a polyacrylamide gel shows considerable variation in the reactivity of the mutants (Fig. 2).

$\Delta 78$ is the most active, producing about half as much excised IVS and ligated exons as wild type under splicing conditions. Under cyclization conditions, the difference in the level of ligated exons between wild type and $\Delta 78$ is reduced, indicating that increased magnesium ion and temperature increase the splicing efficiency of the mutant. (Neither increased magnesium ion nor temperature alone had this effect; data not shown.) The levels of free 5' exon and 3' exon are increased with

the $\Delta 78$ mutation, suggesting a block in exon ligation. There is more free 5' exon than free 3' exon and marked accumulation of IVS–3' exon, indicating that activity at the 3' splice site is impaired. Activity of the 3'-splice site toward hydrolysis is also low relative to wild type, as indicated by the reduced production of 3' exon. Circle formation, however, appears to be at normal levels.

$\Delta 79$, although only one nucleotide shorter than $\Delta 78$, is considerably less reactive. The amount of ligated exons and free IVS is about half that of $\Delta 78$ under splicing conditions, and IVS–3' exon and 5' exon are the major species that accumulate. Splicing activity increases dramatically under cyclization conditions, producing approximately the same amount of ligated exons as $\Delta 78$, again suggesting that the conditions of higher temperature and magnesium ion can compensate for the defect. As in $\Delta 78$, circular intron is formed at the normal level once the IVS is excised.

The reinsertion mutants, $\Delta 78 + 6$, $\Delta 78 + 14$, and $\Delta 78 + 32$, which were designed to restore spacing between the base of P9 and the 3'-splice site (see Fig. 1A), all splice less efficiently than $\Delta 78$. In these mutants the amounts of excised intron and ligated exons decrease with the number of nucleotides inserted. Activity at the 3' splice site still appears to be blocked, because IVS–3' exon accumulates. Accuracy of splicing may be impaired, as multiple species migrating near the positions expected for introns and circles appear under cyclization conditions.

Activity of the 3'-splice site and enzymatic activity of the core

The results given above suggest that the deletions affect certain reactions to a greater extent than others. This was directly tested by using the same substrate to perform two different reactions—intermolecular exon ligation

Figure 1. Deletion mutation sequences. (A) Secondary structure context of the deletion mutations. A model of the *T. thermophila* rRNA intron derived from structural studies (Cech et al. 1983; Inoue and Cech 1985) and comparative sequence analysis (Davies et al. 1982; Michel and Dujon 1983), drawn primarily according to convention (Burke et al. 1987). Helices that are potentially dispensable by phylogenetic criteria are outlined. Splice sites are indicated with thick arrows, and endpoints of the $\Delta 78$ deletion are indicated with thin arrows. (B) Sequences of the mutants described here aligned from nucleotide 327 in P9. (||) A deletion cut site; (|||) a deletion junction; (♦) the wild-type 3'-splice site; (◇) a cryptic 3'-splice site described in this work.

Figure 2. In vitro self-splicing of mutant precursor RNAs. Uniformly ^{32}P -labeled precursor RNAs prepared from *Nde*I-cut plasmid were incubated under various reaction conditions: (O) Incubated in the absence of magnesium ion; (S) splicing; (C) circularization; (H) hydrolysis. Reaction conditions are defined in Materials and methods. The bands are labeled as follows: (PRE) precursor; (CIR) circular IVS; (IVS-3'ex) IVS-3' exon; (LE) ligated exons; (IVS) linear IVS; (5'ex) 5' exon; (3'ex) 3' exon. The deletion mutants necessarily have different sized products for any species containing the IVS; they are marked $\Delta\text{IVS-3'ex}$, ΔIVS , etc.

tion and poly(C) polymerization. Intermolecular exon ligation, or *trans*-splicing, was used to test the activity of the 3'-splice site in the precursor. In this reaction, oligonucleotides that can bind to the 5' exon-binding site (IGS) are incubated with the precursor under splicing conditions in the absence of guanosine. The 5' exon equivalent displaces the intramolecular 5' exon and adds onto the 3' exon in a reaction analogous to exon ligation (Inoue et al. 1985). The poly(C) polymerization reaction was used to test the enzymatic proficiency of the molecule in the absence of exons. When appropriately bound to the 5' exon-binding site, pentaribocytidylic acid (C_5) is cleaved by the 3' terminal guanosine. One or more C_5 are thereby covalently added to the 3' end of the intron, creating a 3' exon equivalent. Another C_5 molecule can replace the cleaved C_5 and attack at the new 3' exon equivalent in a reaction analogous to exon ligation (Zaug and Cech 1986).

To compare the 3' splice site and catalytic core activities, 5' ^{32}P -labeled C_5 was reacted with tritiated precursor or IVS under identical conditions (Fig. 3). Polymerization is little affected by the mutations; the activity of $\Delta 78$ is close to that of the wild-type RNA, whereas $\Delta 79$ and $\Delta 78 + 6$ are each about half as active. In contrast, the addition of C_5 to the 3' exon is reduced dramatically. Moreover the major site of C_5 addition in the mutant RNAs is not the 3' splice site but an alternative site within the 3' exon (note bands at +8 and +20 in Fig. 3). This is an unexpected result, inasmuch as the polymerase mechanism requires C_5 attack at the same phosphodiester bond that is cleaved by C_5 in intermolec-

ular ligation. Circle reopening, another reaction that requires attack at that phosphodiester bond, also appears to be little affected by the deletion (see product labeled $\text{C}_5\text{-IVS}$ in Fig. 3).

Accuracy of 3'-splice site attack

The novel species produced by C_5 attack at the alternative sites in $\Delta 78$ were isolated and sequenced by partial ribonuclease cleavage (Fig. 4). The species labeled +8 was found to result from attack eight nucleotides into the 3' exon, following the sequence GUAG. The other product, labeled +20, has C_5 added to the normal 3'-splice site and ends after the G at nucleotide +8. This could result from site-specific hydrolysis at the cryptic site plus C_5 addition at the normal site, or from sequential C_5 addition at both the normal and cryptic sites.

The site of exon ligation used in the intramolecular self-splicing of the deletion mutants was examined. Splicing products comigrating with wild-type ligated exons were purified from gels and sequenced with di-deoxynucleotides and reverse transcriptase (data not shown). Within the limit of detection, the mutants showed only correct ligation of the 5' exon to the 3' exon.

The discrepancy in 3'-splice site between the intermolecular and intramolecular exon ligation reactions could reflect that C_5 is not an exact 5' exon equivalent. To test this possibility, precursors were reacted with GGCUCUCU (which ends in the same six nucleotides as the 5' exon) and CCCCC (PRE lanes in Fig. 5). The major site

Figure 3. C_5 addition to precursor at the 3'-splice site and C_5 polymerization by IVS species. Tritiated precursors or introns were incubated with 10 μ M 5'- 32 P-labeled C_5 for 30 or 60 min in a buffer favorable for both polymerization and intermolecular exon ligation. The doublets occurring at C_5 -3' exon and C_5 -3' exon + 8 are the result of heterogeneity at the 3' end of the T7 transcript (data not shown). The doublets seen in the higher order C_5 polymerization products are not due to impurities within the C_5 oligonucleotide preparation (data not shown) but probably result from reaction with the cyclization by-product, 15-mer. The prominent bands near the top of the gel (C_5 -IVS) are the result of circle reopening by C_5 , as cyclization of the IVS can occur under these conditions.

of GGCUCUCU attack in $\Delta 78$ is at the normal 3'-splice site, suggesting that the alternative specificity with C_5 is due to the sequence difference. There are two possible explanations for this sequence-specific alternative cutting. C_5 could be binding to the same 5' exon-binding site in a different conformation than GGCUCUCU, which is plausible, as there are many possible alignments of C_5 on a GGAGGG template. Alternatively, a different binding site, which is preferential for C_5 , could become accessible in the deletion mutants.

The latter hypothesis was tested directly by reacting oligonucleotides with precursors that had the 5' exon-binding site removed (–IGS lanes in Fig. 5). Under the conditions used, $\Delta 78$ did not show addition of C_5 in the absence of the 5' exon-binding site. However, the wild-type precursor lacking the 5' exon-binding site did show some reactivity for C_5 although at a different alternative site than $\Delta 78$. This demonstrates that an alternative binding site exists within the intron, although it is not seen in the wild-type precursor when the normal 5' exon-binding site is present.

Activity of the 5'-splice site

The results discussed above suggest that the loss of splicing efficiency in the mutants is due, at least in part, to a block in the second step of splicing, cleavage–ligation at the 3'-splice site. To measure independently the efficacy of the first step of splicing, guanosine addition, 3 H-labeled precursor RNAs were reacted under splicing conditions with [α - 32 P]GTP. An autoradiogram of the products resolved on an acrylamide–urea gel shows that the deletion mutants do not incorporate GTP at the same level as the wild-type precursor (Fig. 6A). As is shown in Table 1, the initial rates of GTP addition are more affected than the extent of reaction after 30 min, indicating that guanosine addition is not the major contribution to the reduced efficiency of mutant pre-rRNA splicing seen in Figure 1.

The diminished 5'-splice site activity of the Δ mutants could be due to the absence of sequences in P9.1–P9.2 or to an aberrant structure created by the deletion. To distinguish between these two effects, precursors missing different portions of the molecule were tested

Figure 4. Accuracy of 3'-splice site attack. Enzymatic sequencing of the products of C_5 addition to wild-type and mutant precursors. Labeled C_5 addition products were excised from a gel, purified, subjected to partial ribonuclease digestion, and run on an 18% polyacrylamide–8 M urea gel. The lanes are labeled as follows: (A) Alkaline ladder; (O) incubated in the absence of enzyme; (T) RNase T1 which cuts after G; (U) RNase U2, which cuts after A; (P) RNase PhyM, which cuts after U and A; (C) RNase CL 3, which cuts after C. The bands corresponding to the first five C residues at the bottom of the gel are not shown.

for guanosine addition as above. Plasmid linearized with *Bgl*II produces a precursor that ends at nucleotide 234 and therefore lacks many of the conserved helices (P3, P6, P7, P8; see Fig. 1A). This RNA shows no GTP addition (Fig. 6B) another precursor that contains all of the sequences up to the 5'-deletion point (position 335; see Fig. 1A) was made with plasmid linearized with *Nhe*I. It adds GTP at an initial rate that is less than wild type and also less than $\Delta 78$ (Table 1). The final precursor includes the helices deleted in the mutants, but not the nucleotides included past the 3'-deletion point; it was made with a *Sca*I run off, which ends at nucleotide 409 in the intron (see Fig. 1A). It adds GTP better than the 335 precursor does and at an initial rate comparable to that of $\Delta 78$. These results suggest that guanosine addition is enhanced by nucleotides proximal and distal to position 409 in the intron. These 3'-splice site interactions need not be directly with the 5'-splice site but may involve interactions elsewhere that alter binding to the catalytic core.

Discussion

At 413 nucleotides, the *Tetrahymena* rRNA intron is

the smallest natural self-splicing RNA and is at least as efficient as any of the other self-splicing introns. Previous deletion studies of this intron have shown that a nonconserved helix (P6b in Fig. 1A) can be truncated without changing the splicing activity (Price et al. 1985) and that nucleotides following position 335 are not required for the first step of splicing (Szostak 1986). In this work we have eliminated 78 nucleotides (between positions 331 and 410) and produced an intron still capable of catalyzing all of the transesterification reactions of the wild-type molecule.

Removal of the 78 nucleotides does not prohibit the catalysis of any of the reactions characteristic of this intron, but it does result in reduced efficiency for some of the reactions. There are several possible explanations for this decreased capacity. The simplest is that the intron has evolved such that the deleted helices are required for optimal stability of the precursor three-dimensional structure. Another possibility is that this particular deletion produces a suboptimal spacing between conserved helix P9 and the 3' splice site. Finally, the helices themselves might not be important, but in their absence refolding might occur and might disrupt catalytically required structures. These are not mutually exclusive pos-

Figure 5. GGCUCUCU and C_5 addition to wild-type and $\Delta 78$ precursors and to species with the 5' exon-binding site deleted. Tritiated precursors (PRE) and species lacking the 5' exon-binding site ($-IGS$) from wild-type and $\Delta 78$ were incubated with 5'- ^{32}P -labeled pGGCUCUCU or pCCCCC for 30 or 60 min under the same buffer conditions as those used for the C_5 reactions in Figure 3. (Lanes 0) Unreacted oligonucleotide.

sibilities but are most readily discussed separately.

The first proposed explanation—that there is a direct interaction of the deleted helices with the rest of the molecule—is not addressed by the experiments in this work and would require further mutagenesis to prove or disprove. Support for this idea comes from the observation that nucleotides 340–352 in P9.1 are complementary to nucleotides 75–87 in P2.1 (Been et al. 1987). The finding of proportionately greater reactivity under conditions of higher temperature and magnesium ion is consistent with a conformational change yielding increased catalytic capacity. Because reactions with the 3' exon are affected more than other reactions, a global loss of stability is probably less pertinent than a local structural defect.

The second possibility—that reactivity is reduced due to alteration of a critical spacing between P9 and the 3'-splice sites—although addressed by this work, cannot be excluded due to complicating factors (vide infra). The excised $\Delta 78$ intron is as capable as wild type in catalyzing polymerization reactions, indicating that the shorter distance between P9 and the 3'-splice site does not affect catalytic activity adversely. This is consistent with phylogenetic conservation as three group I introns have their 3'-terminal G immediately following P9;

these include *Schizosaccharomyces pombe* AI2a (Trinkl and Wolf 1986) and *S. cerevisiae* cob b4 (Michel et al. 1982). The excised intron of $\Delta 78 + 6$, however, is less reactive than $\Delta 78$, suggesting that either the sequence of the nucleotides inserted is affecting the structure of the enzyme or that certain distances can be tolerated better than others in a particular intron tertiary structure.

The final possibility—that the deletions cause re-folding of the molecule, which disrupts a catalytically important interaction—is entirely consistent with the results presented in this work. All of the reactions involving the 3' exon are reduced in activity: 3'-splice site hydrolysis, intermolecular ligation, and exon ligation. The polymerization and circle reopening reactions, which are mechanistically similar (involving cleavage of the phosphodiester bond 3' to G414) but have different sequences following G414, do not show the same dramatic reduction in activity. This suggests that 3'-splice site reactivity is no longer adversely affected after the 3' exon is removed. The reduction in the site-specific hydrolysis reaction indicates that the problem is probably one of 3'-exon orientation within the precursor. The sole requirement for site-specific hydrolysis should be proper alignment of the 3' exon within the active site of the molecule (Inoue et al. 1986). The difference in the ability

Figure 7. (See facing page for legend.)

deletion mutants, and the helices P9.1 and P9.2 may isolate P9 from alternative folding. Once the 3' exon is removed, the most stable foldings for the $\Delta 78 + 6$ P9 helices are very similar in shape and stability to the wild-type P9, which correlates with their restored activity in cyclization and poly(C) polymerization.

Alteration of P9 can also account for the reduced 3'-splice site activity of $\Delta 79$ relative to $\Delta 78$. The extra nucleotide deleted in $\Delta 79$ disrupts the terminal base pair of P9, so that its helix is 1 bp shorter in length. In addition, the spacing between P7 and P9 within the catalytic core is increased by one nucleotide. A point mutation in a different self-splicing group I intron, the phage T4 *td* intron, alters the nucleotide equivalent to the extra one removed in $\Delta 79$ and also has a phenotype of defective 3'-splice site activity (Hall et al. 1987). Thus, P9 may be involved in positioning the 3'-splice site.

Not only is the accuracy of reactions at the 3'-splice site affected in the deletion mutants, but the sequence of the cryptic 3'-splice site at GUAG/C is not one expected in light of previous work. Insertion of multiple linkers between U409 and A410 does not affect 3'-splice site choice (Price et al. 1985), so the sequences that define the 3'-splice site are presumably distal to A410. C413 and G414 have been shown to be important in the binding of the 3'-splice site (Tanner and Cech 1987; J.V. Price and T.R. Cech, in prep.). Thus, our prediction for a cryptic 3'-splice site would have been the sequence CUCG/U, which occurs in an ostensibly single-stranded region 20 nucleotides downstream from the normal CUCG/U 3'-splice site (see Fig. 1B). Because the GUAG/C site that is preferred occurs eight nucleotides from the correct site, proximity may also be a factor in 3'-splice site choice. In addition, interactions with the nucleotides downstream from the 3'-splice site may have some importance (Davies et al. 1982; Waring and Davies 1984; cf. Been and Cech 1985).

The novel site of intermolecular exon ligation (see Fig. 3) occurred upon reaction with C_5 but not with GGCUCUCU. Such specificity was unanticipated, as C_5 binds to the same site (the IGS) as the CUCUCU at the 3' end of the 5' exon (see Fig. 1A; Been and Cech 1986). This would suggest that C_5 can also bind to a second site in the molecule, a site that does not bind GGCUCUCU productively and is not normally available within the wild-type precursor. A potential binding site with these characteristics is the GGGAGA located in the P9 of the deletion mutants (Fig. 7B). Here, GGCUCUCU not only aligns differently than C_5 , but also forms more base pairs and could be sterically hindered from reaction at the GUAG/C site. This same binding site might also explain the C_5 addition site in the wild-type RNA lacking the

Table 1. Reactivity at the 5'-splice sites of mutant pre-rRNAs relative to that of wild-type pre-rRNA

RNA	Activity ^a	Initial rate
WT	100	100
$\Delta 78$	80	12
$\Delta 79$	22	4
$\Delta 78 + 6$	21	3
$\Delta 78 + 14$	11	2
$\Delta 78 + 32$	6	1
WT <i>NheI</i> *	10	1
WT <i>ScaI</i> *	58	8

Reactions were quantified by excising the bands from gels and measuring radioactivity in a liquid scintillation counter. RNA molecules labeled with an asterisk (*) were produced by runoff transcription of the wild-type (WT) template truncated at sites within the intron.

^a Amount of reaction after 30 min.

IGS, as it could form the same internal geometry (Fig. 7B). Finally, the location of the site is consistent with a three-dimensional model of group I active sites, as P1 and P9 are positioned in close proximity to one another (Kim and Cech 1987).

Although nucleotides beyond position 335 (the 5' terminus of the deletion) are not absolutely required for guanosine attack at the 5'-splice site (Szostak 1986), our results with the wild-type truncated precursors show that the efficiency of guanosine addition is markedly improved when at least some of these nucleotides are retained. This may reflect decreased structural stability of precursor truncated at position 335 (A. Zaug, unpubl.) or loss of particular interactions. The *ScaI* precursor that includes all nucleotides up to position 409 does not add guanosine at the initial rate of the wild-type RNA, which suggests that nucleotides immediately preceding the 3'-splice site and/or the first few nucleotides of the 3' exon contribute to the active structure at the 5'-splice site. The guanosine-binding site and the 3'-splice site should be spatially close, as hydrolysis at the 3'-splice site is inhibited by competitive inhibitors of guanosine binding (Bass and Cech 1986). Together, these results indicate that interactions of the 3'-splice site influence the first step of splicing.

Materials and methods

Materials

Restriction enzymes, linkers, and sequencing primers were purchased from New England Biolabs. Other primers were syn-

Figure 7. P9 structures potentially responsible for the reduced activity in the presence of the 3' exon and for the cryptic C_5 addition activities. The boxed P7 represents the sequences 5' to the refolded P9s and the filled square indicates the normal 3' splice site. (A) The deletion mutant P9 can be refolded with sequences in the 3' exon to give stable structures that hide the correct 3' splice site. Free energies of formation of the helices were calculated from published values (Freier et al. 1986; D.R. Groebe and U.L. Uhlenbeck, pers. comm.). The small helix in the wild-type 3' exon, which hides the cryptic splice site, is phylogenetically proven for 28S rRNAs (Clark et al. 1984). (B) P9 structures can be opened to provide an alternative binding site for C_5 . The $\Delta 78$ pairings (left) indicate how this site could accommodate C_5 and sterically occlude GGCUCUCU from productive binding. (Right) The analogous structure for the wild-type species missing the 5' exon-binding site, with the probable site of attack in the 3' exon indicated.

Barfod and Cech

thesized in the laboratory of Marvin Caruthers. T4 polynucleotide kinase, T4 DNA ligase, and radiolabeled nucleoside triphosphates were obtained from New England Nuclear. AMV reverse transcriptase came from Life Sciences. Pentaribocytidylic acid (C_5) and T4 RNA ligase were provided by Olke Uhlenbeck. Ribonucleases T1 and U2 were bought from Calbiochem, and mung bean nuclease from IBI. Ribonucleases CL 3, PhyM, and H came from BRL. T7 RNA polymerase, purified by a published procedure [Davanloo et al. 1984], was a gift from Art Zaug. GGCUCUCU was a gel purified, phage T7 RNA polymerase transcript of a synthetic DNA template and was a gift from Bert Flanagan.

Plasmid constructions

The IVS-deletion mutations in this work were derived from pBGST7, a plasmid containing the *T. thermophila* rRNA intron and short exons cloned downstream from a phage T7 promoter [Been and Cech 1986]. The DNA between the *NheI* and *ScaI* restriction sites at positions 332 and 410 within the intron was deleted. Plasmid pBGST7 linearized with *NheI* was digested with mung bean nuclease to remove the 3' single-stranded tail. This product was then treated with *ScaI* under partial digestion conditions, as the enzyme cuts at an additional site in the plasmid. After ligation and transformation into *Escherichia coli* JM83, the bacteria were plated on LB agar containing ampicillin and Xgal. The colonies were screened first by hybridization (after Gergen et al. 1979) to IP 350-15, a primer that binds within P9.1. DNA from minipreps [Holmes and Quigley 1981] of nonhybridizing clones was characterized by restriction endonuclease digestion and sequenced [Sanger et al. 1977]. In addition to the expected construction, $\Delta 78$, a deletion mutant of 79 nucleotides was also found. This mutant, $\Delta 79$, has deleted G231 in addition to the other 78 nucleotides and resulted presumably from single-stranded nuclease digestion of the first nucleotide of the double-stranded end of the restriction site, which became accessible from helix breathing.

Mutants that had sequences inserted into the $\Delta 78$ deletion were constructed as follows. An *XbaI* linker was chosen for its complementarity to the *NheI* sticky end. A *ScaI* partial digestion of pBGST7 was ligated with *XbaI* linkers, digested with *XbaI*, and finally digested with *NheI*. After ligation and transformation into JM83, the bacteria were plated on LB agar containing ampicillin and Xgal. DNA from minipreps was assayed by restriction endonuclease digestion and sequencing. The expected mutant, $\Delta 78 + 6$, resulting from the addition of one digested linker, was found. Two additional mutants were found: one ($\Delta 78 + 14$) resulting from the addition of an extra linker plus the digested one, and the other ($\Delta 78 + 32$), which has DNA of unknown origin between the *NheI* and *ScaI* sites.

RNA preparation

Precursor RNA was synthesized by in vitro transcription at 30°C, using T7 RNA polymerase. Plasmids were linearized with *HindIII*, *NarI*, *XbaI*, or *NdeI*, which when transcribed yielded unique precursors with different sized 3' exons. Ten micrograms of the DNA was ethanol precipitated and brought up in 250 μ l of buffer containing 4 mM each NTP, 15 mM $MgCl_2$, 1 mM spermidine, 5 mM dithiothreitol (DTT), 40 mM Tris (pH 9.1), 100 μ g/ml serum albumin, and 2500 units of T7 RNA polymerase. To radiolabel the RNA, either [α - ^{32}P]ATP or [3H]UTP was included without adjusting the concentration of unlabeled NTP in the transcription. To produce IVS and ligated exons, NaCl was added to 100 mM after 2 hr of RNA synthesis, and the transcription reactions were incubated at 37°C for an

additional 2 hr. The RNA was purified by 4% polyacrylamide–8 M urea gel electrophoresis, followed by excision of the appropriate band, elution by crush and soak, and column chromatography on Sephadex G50-150.

Species lacking the 5' exon-binding site were prepared by RNase H cleavage of wild-type and $\Delta 78$ precursor RNAs with a synthetic DNA oligonucleotide. Gel-purified precursors (30 pmole) were incubated with 164 pmole of primer IP32-14 (GCATGCCTGATAAC) in a buffer containing 50 mM Tris (pH 7.5) and 50 mM KCl. These mixtures were heated for 3 min at 95°C and slow-cooled to 37°C. $MgCl_2$ was then added to 10 mM, and the reaction started with 5 units of ribonuclease H. After 20 min at 37°C, the reactions were stopped with formamide dye mix containing 25 mM EDTA and loaded onto a 4% polyacrylamide–8 M urea gel. Ultraviolet irradiation of the ethidium bromide-stained gel revealed the bands expected from digestion of nucleotides 32–45 in the intron: 407 and 296 nucleotides for wild-type and 329 and 296 nucleotides for $\Delta 78$. The bands containing the introns beginning at nucleotide 46 were excised and purified as described above.

RNA sequencing

Ligated exons were sequenced by primer extension with reverse transcriptase in the presence of dideoxynucleotides, as described by Zaug et al. (1984), except that 42°C was used instead of 37°C in an attempt to reduce the effect of secondary structure in the RNA. Gel-purified C_5 –3'-exon species were sequenced by partial RNase digestion [Donis-Keller et al. 1977].

Reaction conditions

In vitro self-splicing of the mutant precursor RNAs was conducted under the following conditions (1) Zero: 5 mM EDTA, 30 mM Tris (pH 7.5), 100 mM $(NH_4)_2SO_4$, 30°C; (2) splicing: 0.05 mM guanosine, 5 mM $MgCl_2$, 30 mM Tris (pH 7.5), 100 mM $(NH_4)_2SO_4$, 30°C; (3) circularization: 0.05 mM guanosine, 10 mM $MgCl_2$, 30 mM Tris (pH 7.5), 100 mM $(NH_4)_2SO_4$, 42°C; (4) hydrolysis: 10 mM $MgCl_2$, 30 mM Tris (pH 9.0), 100 mM $(NH_4)_2SO_4$, 42°C. The reactions were stopped after 30 min with the addition of formamide dye mix containing 25 mM EDTA, and the samples were loaded onto a 4% polyacrylamide–8 M urea gel.

Reactions of C_5 with precursor and intron were performed in the following manner. Tritiated precursors prepared from *HindIII*–linearized plasmid were incubated in a buffer containing 50 mM Tris (pH 7.5), 100 mM NaCl, 20 mM $MgCl_2$, 0.001 mM precursor, and 0.01 mM C_5 with a trace quantity of 5'- ^{32}P -labeled C_5 . Reactions were stopped after 30 or 60 min and mixed with EDTA–formamide dye solution, and samples were loaded onto a 20% polyacrylamide–8 M urea gel. Reactions with GGCUCUCU were done identically.

GTP addition to the 5'-splice site was tested as follows. Tritiated precursors prepared from *HindIII*–linearized plasmid were incubated in a buffer containing 30 mM Tris (pH 7.5), 100 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, and 0.0005 mM [^{32}P]GTP (800 Ci/mole) at 30°C. Reactions were stopped on dry ice at 0, 2, 5, 10, 30, and 60 min and mixed with EDTA–formamide dye solution and the samples were loaded onto a 4% polyacrylamide, 8 M urea gel.

Acknowledgments

We are grateful to Ben Young for critical reading of the manuscript. This work was supported by grant GM28039 from the

National Institutes of Health. T.R.C. is an American Cancer Society Research Professor.

References

- Bass, B.L. and T.R. Cech. 1986. Ribozyme inhibitors: Deoxyguanosine and dideoxyguanosine are competitive inhibitors of self-splicing of the *Tetrahymena* ribosomal ribonucleic acid precursor. *Biochemistry* **25**: 4473–4477.
- Been, M.D. and T.R. Cech. 1985. Sites of circularization of the *Tetrahymena* rRNA IVS are determined by sequence and influenced by position and secondary structure. *Nucleic Acids Res.* **13**: 8389–8408.
- . 1986. One binding site determines sequence specificity of *Tetrahymena* pre-rRNA self-splicing, trans-splicing and RNA enzyme activity. *Cell* **47**: 207–216.
- Been, M.D., E.T. Barford, J.M. Burke, J.V. Price, N.K. Tanner, A.J. Zaig, and T.R. Cech. 1987. Structures involved in *Tetrahymena* rRNA self-splicing and RNA enzyme activity. *Cold Spring Harbor Symp. Quant. Biol.* **52**: 147–157.
- Burke, J.M., M. Belfort, T.R. Cech, R.W. Davies, R.J. Schweyen, D.A. Shub, J.W. Szostak, and H.F. Tabak. 1987. Structural conventions for group I introns. *Nucleic Acids Res.* **15**: 7217–7221.
- Cech, T.R. 1987. The chemistry of self-splicing RNA and RNA enzymes. *Science* **236**: 1532–1539.
- Cech, T.R., N.K. Tanner, I. Tinoco, B.R. Weir, M. Zuker, and P.S. Perlman. 1983. Secondary structure of the *Tetrahymena* ribosomal RNA intervening sequence: Structural homology with fungal mitochondrial intervening sequences. *Proc. Natl. Acad. Sci.* **80**: 3903–3907.
- Clark, C.G., B.W. Tague, V.C. Ware, and S.A. Gerbi. 1984. *Xenopus laevis* 28S ribosomal RNA: A secondary structure model and its evolutionary and functional implications. *Nucleic Acids Res.* **12**: 6197–6220.
- Davanloo, P., A.H. Rosenberg, J.J. Dunn, and F.W. Studier. 1984. Cloning and expression of the gene for bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci.* **81**: 2034–2039.
- Davies, R.W., R.B. Waring, J.A. Ray, T.A. Brown, and C. Scazzocchio. 1982. Making ends meet: A model for RNA splicing in fungal mitochondria. *Nature* **300**: 719–724.
- Donis-Keller, H., A.M. Maxam, and W. Gilbert. 1977. Mapping adenines, guanines, and pyrimidines in RNA. *Nucleic Acids Res.* **4**: 2527–2538.
- Ehrenman, K., J. Pedersen-Lane, D. West, R. Herman, F. Maley, and M. Belfort. 1986. Processing of phage T4 *td*-encoded RNA is analogous to the eukaryotic group I splicing pathway. *Proc. Natl. Acad. Sci.* **83**: 5875–5879.
- Freier, S.M., R. Kierzek, J.A. Jaeger, N. Sugimoto, M.H. Caruthers, T. Neilson, and D.H. Turner. 1986. Improved free-energy parameters for prediction of RNA duplex stability. *Proc. Natl. Acad. Sci.* **83**: 9373–9377.
- Gergen, J.P., R.H. Stern, and P.C. Wensink. 1979. Filter replicas and recombinant DNA plasmids. *Nucleic Acids Res.* **7**: 2115–2128.
- Hall, D.W., C.M. Povinelli, K. Ehrenman, J. Pedersen-Lane, F. Chu, and M. Belfort. 1987. Two splicing domains in the intron of the phage T4 thymidilate synthase (*td*) gene established by nondirected mutagenesis. *Cell* **48**: 63–71.
- Holmes, D.S. and M. Quigley. 1981. A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114**: 193–197.
- Inoue, T. and T.R. Cech. 1985. Secondary structure of the circular form of the *Tetrahymena* rRNA intervening sequence: A technique for RNA structural analysis using chemical probes and reverse transcriptase. *Proc. Natl. Acad. Sci.* **82**: 648–652.
- Inoue, T., F.X. Sullivan, and T.R. Cech. 1985. Intermolecular exon ligation of the rRNA precursor of *Tetrahymena*: Oligonucleotides can function as 5' exons. *Cell* **43**: 431–437.
- . 1986. New reactions of the ribosomal RNA precursor of *Tetrahymena* and the mechanism of self-splicing. *J. Mol. Biol.* **189**: 143–165.
- Kim, S.-H. and T.R. Cech. 1987. Three-dimensional model of the active site of the self-splicing rRNA precursor of *Tetrahymena*. *Proc. Natl. Acad. Sci.* **84**: 8788–8792.
- Kruger, K., P.J. Grabowski, A.J. Zaig, J. Sands, D.E. Gottschling, and T.R. Cech. 1982. Self-splicing RNA: Autoexcision and autocyclization of the ribosomal RNA intervening sequence of *Tetrahymena*. *Cell* **31**: 147–157.
- Michel, F. and D. Cummings. 1985. Analysis of class I introns in a mitochondrial plasmid associated with senescence of *Podospira anserina* reveals extraordinary resemblance to the *Tetrahymena* ribosomal intron. *Cur. Genet.* **10**: 69–79.
- Michel, F. and B. Dujon. 1983. Conservation of RNA secondary structures in two intron families including mitochondrial-, chloroplast-, and nuclear-encoded members. *EMBO J.* **2**: 33–38.
- Michel, F., A. Jacquier, and B. Dujon. 1982. Comparison of fungal mitochondrial introns reveals extensive homologies in RNA secondary structure. *Biochimie* **64**: 867–881.
- Price, J.V., G.L. Kieft, J.R. Kent, E.L. Sievers, and T.R. Cech. 1985. Sequence requirements for self-splicing of the *Tetrahymena thermophila* pre-ribosomal RNA. *Nucleic Acids Res.* **13**: 1871–1889.
- Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* **74**: 5463–5467.
- Szostak, J.W. 1986. Enzymatic activity of the conserved core of a group I self-splicing intron. *Nature* **322**: 83–86.
- Tanner, N.K. and T.R. Cech. 1987. Guanosine binding required for cyclization of the self-splicing intervening sequence ribonucleic acid from *Tetrahymena thermophila*. *Biochemistry* **26**: 3330–3340.
- Trinkl, H. and K. Wolf. 1986. The mosaic *cox I* gene in the mitochondrial genome of *Schizosaccharomyces pombe*: Minimal structural requirements and evolution of group I introns. *Gene* **45**: 289–297.
- Waring, R.B. and R.W. Davies. 1984. Assessment of a model of intron structure relevant to RNA self-splicing. *Gene* **28**: 277–291.
- Zaig, A.J. and T.R. Cech. 1986. The intervening sequence RNA of *Tetrahymena* is an enzyme. *Science* **231**: 470–475.
- Zaig, A.J., J.R. Kent, and T.R. Cech. 1984. A labile phosphodiester bond at the ligation junction in a circular intervening sequence RNA. *Science* **224**: 574–578.

Deletion of nonconserved helices near the 3' end of the rRNA intron of *Tetrahymena thermophila* alters self-splicing but not core catalytic activity.

E T Barfod and T R Cech

Genes Dev. 1988, **2**:

Access the most recent version at doi:[10.1101/gad.2.6.652](https://doi.org/10.1101/gad.2.6.652)

References

This article cites 32 articles, 10 of which can be accessed free at:
<http://genesdev.cshlp.org/content/2/6/652.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).

Focused on your science.

USC
SCIENTIFIC
SIDE BY SCIENCE