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Compensatory mutations demonstrate that P8 and P6 are RNA secondary structure elements important for processing of a group I intron

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ABSTRACT

Compensatory mutations have been constructed which demonstrate that P8 and P6, two of nine proposed base-pairing interactions characteristic of group I introns, exist within the folded structure of the *Tetrahymena thermophila* rRNA intervening sequence, and that these secondary structure elements are important for splicing in *E. coli* and self-splicing *in vitro*. Two-base mutations in the 5' and 3' segments of P8 are predicted to disrupt P8 and a strong splicing-defective phenotype is observed in each case. A compensatory four-base mutation in P8 is predicted to restore pairing, and results in the restoration of splicing activity to nearly wild type levels. Thus, we conclude that P8 exists and is essential for splicing. In contrast to the strong phenotypes generally exhibited by mutations which disrupt RNA secondary structure, a two-base mutation in L8, the loop between P8[5'] and P8[3'], results in only a slight decrease in splicing activity. We also tested P6, a pairing which is proposed to consist of only two base-pairs in this intron. A two-base mutation in P6[3'] reduces splicing activity to a greater extent than does a two-base mutation in P6[5']. Comparison of the activities of these mutants and a compensatory P6 four-base mutant support the existence of P6, and suggest that the P6 pairing may be particularly important in the exon ligation step of splicing.

INTRODUCTION

The precise excision of introns and covalent joining of exons is a key feature of the processing of RNA transcripts from a wide variety of genes. Based on intron structure, the stepwise pathway of splicing and the biochemical apparatus required to carry out the splicing reactions, there exist a minimum of three groups of introns -- nuclear pre-tRNA introns, group I introns, and a third group consisting of nuclear pre-mRNA introns and group II introns (see reference 1 for review).

Group I introns are of interest since precursor RNAs (pre-RNAs) containing 12 of the 64 group I introns characterized to date have been shown to be self-splicing *in vitro* (2). Splicing of group I introns proceeds by two sequential phosphate ester transfer reactions (see reference 3 for review). Guanosine attack on the 5' splice site is followed by 5' exon attack on the 3' splice site. In marked contrast to nuclear pre-mRNA introns, the requirements for *in vitro* self-splicing of group I introns are very simple. Guanosine is required to initiate the splicing reactions; magnesium ions and monovalent salts are also required. Activities of catalytic RNA molecules are lost upon thermal or chemical denaturation (4). As is the case for protein enzymes, RNA enzymes require a specific secondary and tertiary structure for activity.

The *Tetrahymena thermophila* nuclear pre-rRNA contains the best characterized group I intron. Our work involves a genetic approach to the structure-function relationships of intron sequences within the *Tetrahymena* pre-rRNA. In 1982 Michel, Davies and coworkers independently noted that group I introns contain four conserved sequence elements, and they proposed similar secondary structure models for group I introns (5, 6). In the secondary structure models, the introns are proposed to fold using nine short base-pairing regions, termed P1 - P9 (see reference 7 for a summary of intron structure and nomenclature). These models have been generally well-supported by comparative DNA sequence data on group I introns which has accumulated since that time (2). The secondary structure models can be tested by the isolation of *cis*-acting mutants and second site revertants *in vivo*, or by the construction and splicing analysis of variant pre-RNA molecules containing individual and compensatory base substitutions *in vitro* (see reference 8 for review). In addition to testing the predictions of the models, the *in vitro* approach allows one to probe the functional importance of specific sequences and base-pairing interactions for individual intron processing reactions.

Previously, we used compensatory mutations to demonstrate that base pairing of secondary structure elements P7 (the R-S pairing) and P3 are essential for self-splicing of the *Tetrahymena* rRNA intervening sequence (IVS; 9,10). Other workers have used a similar approach to show that P1, the 5' splice site helix, is essential for processing of the same intron (11,12). In the yeast mitochondrial system, *in vivo* isolation and characterization of second site revertants of *cis*-acting intron mutations has provided strong evidence for an essential role of P1, P3 and P7 in pre-mRNA processing (13-15). In this paper, we report the use of in vitro mutagenesis and splicing to demonstrate that two additional secondary structure elements, P8 and P6, exist and are functionally important for RNA-catalyzed processing of the *Tetrahymena* rRNA intron.

MATERIALS AND METHODS

General Methods and Materials

Cloning, purification, and enzymatic manipulations of DNA were carried out as described (16), unless otherwise noted. T7 RNA polymerase was a generous gift of A. Zaug. DNA ligase was obtained from New England Biolabs. All other enzymes and vector pTZ18U were from United States Biochemical. Radioisotopes were from NEN (α-^{35}S dATP) and ICN (^{3}H-UTP). Unlabeled nucleotides were from Pharmacia. Kodak XAR-5 film was used for fluorography and autoradiography.

Phagemid Expression Construct pTZIVSU

Plasmid pBGST7 (11) was a generous gift of M. Been. The EcoRI-HindIII restriction fragment containing the *Tetrahymena* rRNA intron and translatable exon sequences of pBGST7 was subcloned into the EcoRI and HindIII sites of phagemid vector

pTZ18U (17, Fig. 1). Following transformation of *E. coli* strain NM522 (18), cells were plated on LM agar (18) containing ampicillin (50 μg/ml), X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 40 μg/ml) and IPTG (isopropylthiogalactoside, 150 μg/ml). Plasmid DNA was prepared from blue colonies, and the presence of the correct insert was confirmed by restriction analysis and DNA sequencing.

Chemical Synthesis of DNA Oligonucleotides

DNA oligonucleotides were synthesized manually using the phosphoramidite method on a Cruachem PS100 synthesizer according to the manufacturer's instructions. Deprotected oligonucleotides were purified by electrophoresis through a 20% polyacrylamide gel, visualized by UV shadowing, eluted and purified on a Sep-Pak C18 column (Waters Associates) as described (19). Primers were 5'-phosphorylated as described (9). Oligonucleotide sequences are as follows, with mutagenic sites underlined. Note that oligonucleotide sequences are complementary to the IVS RNA sequence. P6[5'] mutant, 5' GGACTTGTGTGCGTGG 3'. P6[3'] mutant, GTCTGTGACATGCATC. P8[5'] mutant, ACATCTTACGCGACCGA. P8[3'] mutant, TATCTTATCATAAGAATAC. L8 mutant, GAAGAAGACGTCTTCCCCGA.

Site-Directed Mutagenesis

Oligonucleotide-directed mutagenesis was carried out using deoxyuridine-substituted single-stranded templates according to the method of Kunkel et al. (20). Deoxyuridine-substituted single-stranded pTZIVSU DNA was prepared as described (20,21). For mutagenesis, 10 picomoles of 5'-phosphorylated mutagenic oligonucleotide were mixed with 1 pmol template DNA in 12.5 μl of 20 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol. The mixture was heated at 90°C for 3 min and cooled slowly to room temperature. The extension-ligation reaction was initiated by adding to the DNA solution 10 μl of an ice cold mixture containing 20 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 5 units T4 DNA polymerase, 3 units T4 DNA ligase, 1 mM ATP, and 0.5 μl dNTP mix (containing 25 mM dATP, dGTP, dCTP and dTTP), and the mixture was incubated on ice for 5 min., at room temperature for 5 min, and finally at 37°C for 2 hr. The reaction mixture was then used to transform *E. coli* NM522, and cells were plated on LM agar plates (18) supplemented with ampicillin, IPTG and X-gal. The plates were incubated at 37°C for 24 hrs, then placed at 4°C for further color development. Color scoring was done after 24, 36 and 48 hr.

The single-stranded template used for mutagenesis reactions was pTZIVSU, except that the P6[3'] mutant DNA was used as the template for the construction of the compensatory P6 mutant and the P8[3'] mutant DNA was used as the template for the construction of the compensatory P8 mutant.

Screening for Mutants by DNA Sequencing of Plasmid DNA

Mutants were identified by dideoxy sequencing of double-stranded plasmid DNA.

DNA was prepared and sequenced as described (22) using intron-specific oligonucleotide primers. Generally 70-90% of transformants were the desired mutants.

Transcription and RNA splicing

Plasmid DNA was linearized by digestion with restriction endonuclease BglI. Transcription of ^{3}H-labeled pre-RNAs with T7 RNA polymerase and RNA processing reactions were carried out as described (see references 9,10 and legends to Figs. 4 and 5).

RESULTS

Phagemid pTZIVSU

In *E. coli*, lacZα pre-mRNAs containing the *Tetrahymena* rRNA IVS can be properly spliced (23,24). The phagemid construct used for this work contains the *Tetrahymena* intron together with short translatable exon sequences cloned in-frame with respect to the polylinker sequence of vector pTZ18U (Fig. 1). Clones containing wild type and splicing-proficient variants of the intron produce functional β-galactosidase α-complementing fragment. When expressed in a host strain which produces the remainder of the enzyme, blue colonies are formed on media containing X-gal. Clones

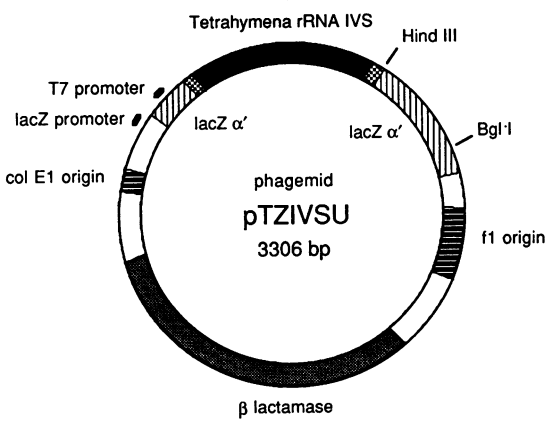

Fig. 1. Phagemid construct pTZIVSU. The *Tetrahymena* rRNA intron (black) and short translatable rRNA exon sequences (dotted) were subcloned from plasmid pBGST7 (11) into the polylinker of the lacZα region (light crosshatched) of phagemid vector pTZ18U (12). When the intron is spliced functional β-galactosidase α-complementing fragment is synthesized in *E. coli*. Double-stranded or single-stranded DNA can be prepared when DNA replication is driven by the colE1 plasmid replication origin or the phage f1 replication origin, respectively.

containing splicing-deficient mutants give rise to light-blue or white colonies, according to the degree of splicing inhibition.

Mutants

The current secondary structure model of the *Tetrahymena* intron is shown in Fig. 2. Mutants constructed for this work are shown in Fig. 3. Mutations designed to destabilize putative secondary structures P8 and P6 were constructed (P8[5'] mutant, G280C:G282U; P8[3'] mutant, C296A:C298G; P6[5'] mutant, G215C:C216A; P6[3'] mutant, G257U:U258G). Multiple-base transversions were chosen in order to induce significant destabilization of P8 and P6. Compensatory mutations were designed to

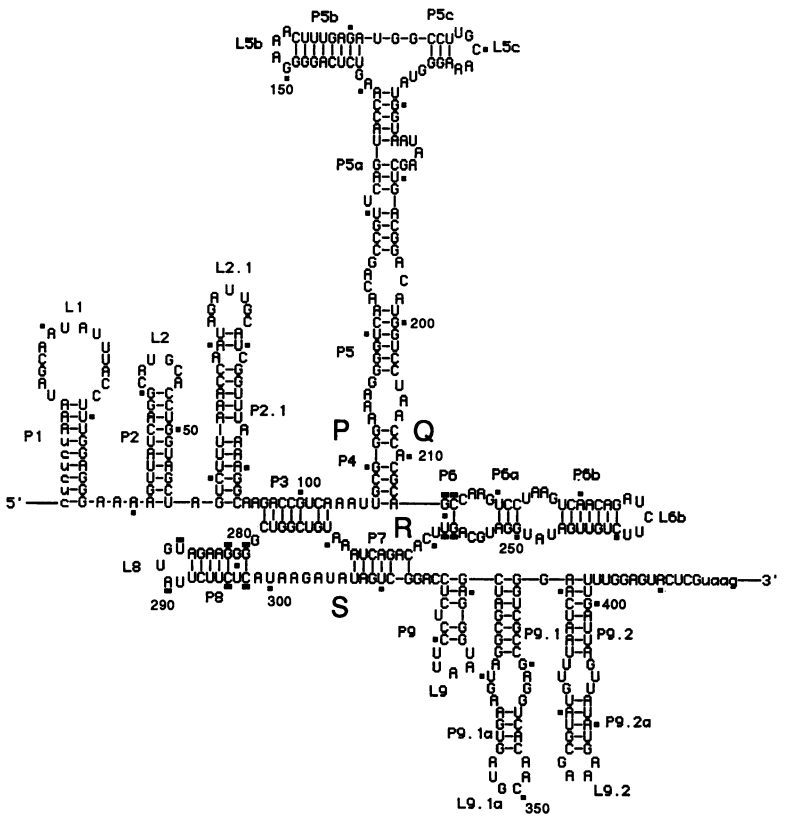

Fig. 2. Secondary structure model of *Tetrahymena* rRNA intron. Intron sequences are indicated by uppercase letters; exon sequences by lowercase letters. Locations of conserved sequence elements are as follows: element P, bases 104-115; element Q, bases 206-216; element R, bases 257-270; element S, bases 301-312. Bars indicate mutation sites.

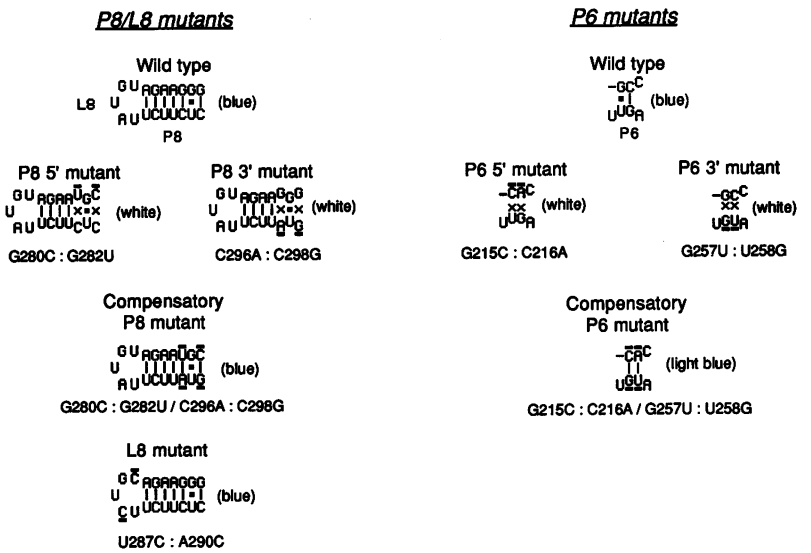

Fig. 3. Mutants constructed. Bars indicate base substitutions relative to the wild type sequence. Colony colors on X-gal plates of clones harboring pTZIVSU and mutant derivatives are indicated in parentheses. This assay gives an estimation of splicing activity in *E. coli* (see legend to Fig. 1 and Results).

restore the putative pairings (compensatory P8 mutant, G280C:G282U / C296A:C298G; compensatory P6 mutant, G215C:C216A / G257U:U258G). In addition, a two base mutation in L8 (L8 mutant, U287C:A290C) was constructed and analyzed. Colony color was scored on X-gal plates, and self-splicing and *trans*-splicing activities were determined *in vitro*.

Compensatory Mutants in Secondary Structure Element P8

Two-base mutations in P8[5'] and P8[3'] each resulted in a substantial decrease in splicing activity in *E. coli*, as evidenced by white colonies on X-gal plates (Fig. 3). In sharp contrast, clones containing the compensatory P8 mutation, which contains both of the two-base changes in P8[5'] and P8[3'], are blue (Fig. 3). These results indicate that compensatory changes in the opposite strand of the P8 helix restore splicing activity to both the P8[5'] mutant and the P8[3'] mutant in *E. coli*.

Self-splicing experiments demonstrate that P8 is required for the RNA-mediated reactions *in vitro* (Fig. 4). The P8[5'] and P8[3'] mutants each result in a large reduction in intron processing activity in the self-splicing assay. The P8[5'] and P8[3'] mutations block splicing at the first step, guanosine attack at the 5' splice site. The compensatory P8 mutant restores intron processing activity to 70% of the wild type level at the earliest time point (2 min.). Thus, the P8 helix is required for self-splicing activity *in vitro*.

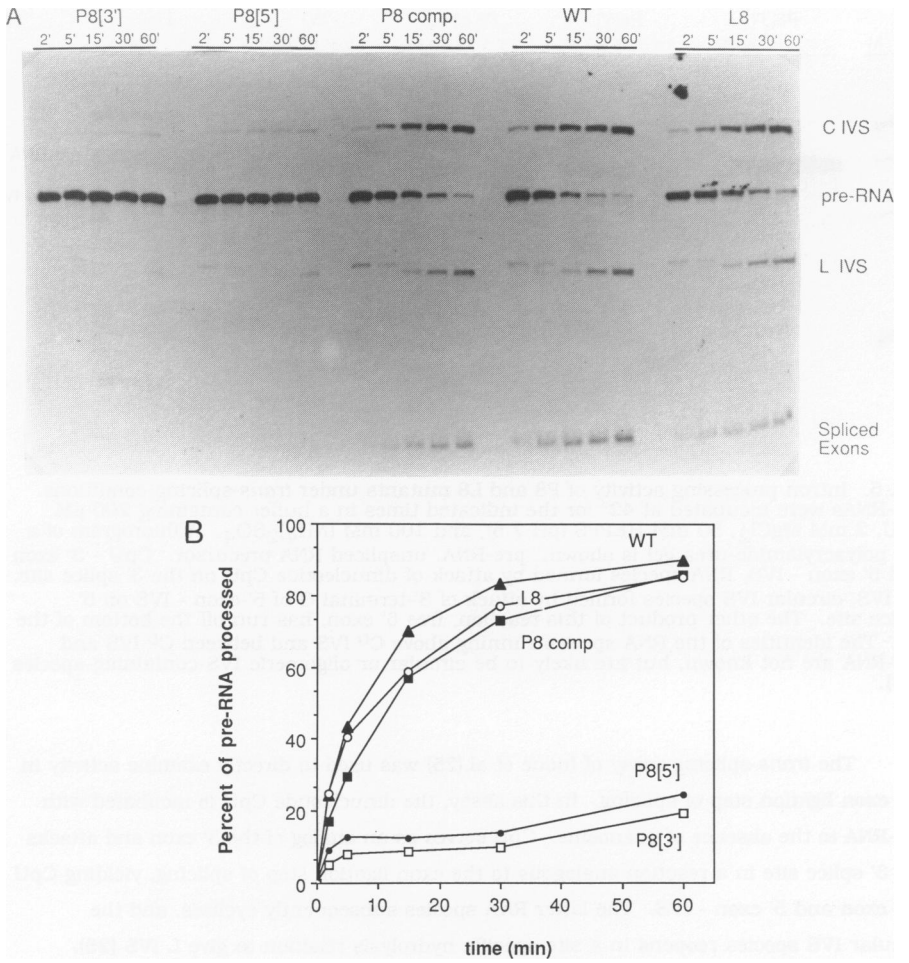

Fig. 4. Intron processing activity of P8 and L8 mutants under self-splicing conditions. Uniformly ^{3}H-labeled pre-RNAs were generated from BglI - linearized wild-type and mutant plasmids *in vitro* using T7 RNA polymerase as described (10). Gel-purified pre-RNAs were incubated at 42°C for the indicated times in a buffer containing 200 μM GTP, 2 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.5), and 100 mM $(NH_4)_2SO_4$. The reaction mixture was analyzed by electrophoresis on a 4% polyacrylamide urea gel. Processing products were visualized by fluorography (A) and quantitated by liquid scintillation counting (B), both carried out as described (9,10). Regions of the gel below the pre-RNA were excised and counted to determine levels of radioactivity due to nonspecific RNA degradation; background counts were subtracted from the reported results. pre-RNA, unspliced RNA precursor. C IVS, circular IVS RNA species formed by cyclization reactions of linear IVS. L IVS, linear IVS species. Slower migrating species is 414 base G-IVS species formed during exon ligation. Faster migrating band contains L - 15 and L - 19 IVS formed by hydrolysis of C IVS. In part B, percent of pre-RNA processed is determined as the ratio of radioactivity in RNA processing products (L IVS + C IVS + spliced exons) to that in all discrete RNA species (pre-RNA + L IVS + C IVS + spliced exons).

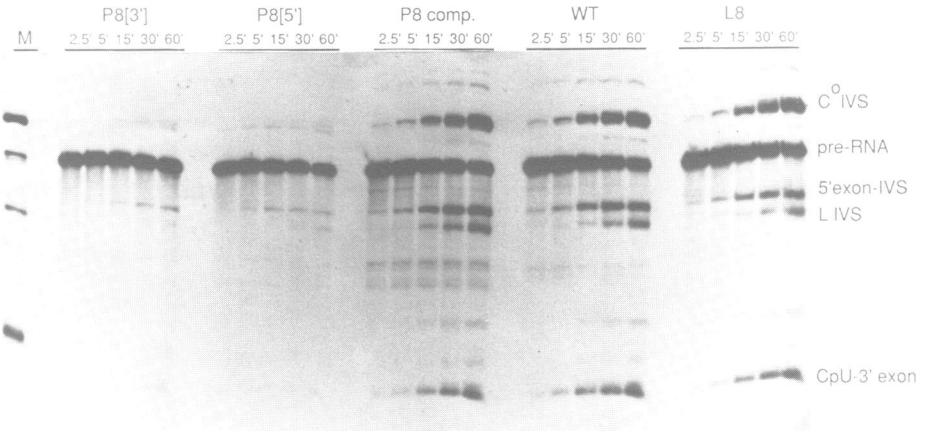

Fig. 5. Intron processing activity of P8 and L8 mutants under *trans*-splicing conditions. Pre-RNAs were incubated at 42° for the indicated times in a buffer containing 200 μM CpU, 2 mM $MgCl_2$, 50 mM HEPPS (pH 7.5), and 100 mM $(NH_4)_2SO_4$. A fluorogram of a 4% polyacrylamide-urea gel is shown. pre-RNA, unspliced RNA precursor. CpU - 3' exon and 5' exon - IVS, RNA species formed by attack of dinucleotide CpU on the 3' splice site. C^o IVS, circular IVS species formed by attack of 3'-terminal G of 5'-exon - IVS on 5' splice site. The other product of this reaction, free 5' exon, has run off the bottom of the gel. The identities of the RNA species running above C^o IVS and between C^o IVS and pre-RNA are not known, but are likely to be circular or oligomeric IVS-containing species (28).

The *trans*-splicing assay of Inoue et al.(25) was used to directly examine activity in the exon ligation step of splicing. In this assay, the dinucleotide CpU is incubated with pre-RNA in the absence of guanosine. CpU serves as an analog of the 3' exon and attacks the 3' splice site in a reaction analogous to the exon ligation step of splicing, yielding CpU - 3' exon and 5' exon - IVS. The latter RNA species subsequently cyclizes, and the circular IVS species reopens in a site-specific hydrolysis reaction to give L IVS (25). Results of the *trans*-splicing assay are shown in Fig. 5, and indicate that the P8[5'] and P8[3'] mutants are defective in CpU attack on the 3' splice site, and that the compensatory P8 mutant restores this activity. Together, these experiments show that base-pairing between the 5' and 3' segments of P8 is a requirement for both steps of the splicing reaction, guanosine attack at the 5' splice site and exon ligation.

Mutation in Loop L8

A two-base mutation in L8 was constructed to provide a preliminary test of the functional importance of this loop sequence. The mutation creates a unique AatII restriction site in the plasmid DNA. Clones harboring the L8 mutant IVS gave blue colonies, indicating that efficient splicing occurs in *E. coli*. *In vitro* analysis showed that

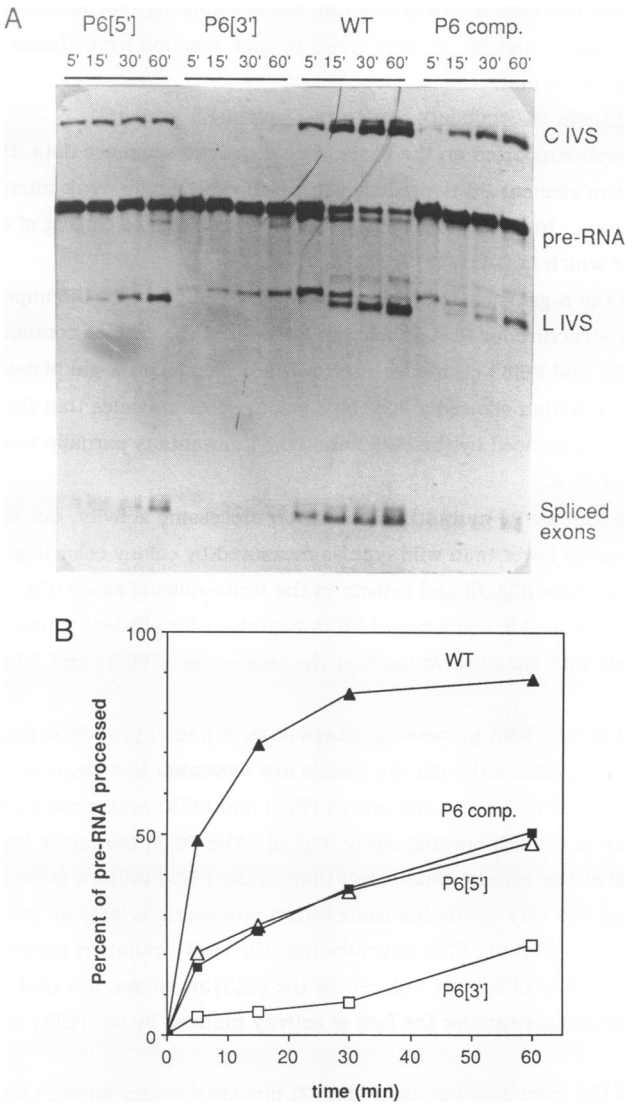

Fig. 6. Intron processing activity of P6 mutants under self-splicing conditions. A, fluorogram of a 4% polyacrylamide-urea gel. The band migrating slightly faster than pre-RNA is G - IVS - 3' exon. The band migrating slightly slower than L - 19 IVS is 5' exon - IVS. B, Quantitation of results from A, obtained by scintillation counting. For further details, see legend to Fig. 4.

the L8 mutation did not have a strong effect on the self-splicing reaction or the *trans*-splicing reaction (Figs. 4 and 5). Activity levels in each reaction were similar to those of the compensatory P8 mutant.

Compensatory Mutants in Secondary Structure Element P6

Although well-supported on the basis of phylogenetic sequence data, the putative secondary structure element P6 is predicted to be an exceedingly weak interaction in many group I introns. In the *Tetrahymena* IVS, P6 is predicted to consist of only two base-pairs, one of which is G-U (Fig. 2).

Results of the ß-galactosidase color assay provide support for the importance of the P6 secondary structure for RNA splicing in *E. coli* (Fig. 3). Clones containing two-base mutations in P6[5'] and P6[3'] each gave rise to white colonies on X-gal plates, while the compensatory P6 mutation showed a light blue color. This indicates that the loss of ß-galactosidase activity induced by the P6[5'] and P6[3'] mutants is partially restored by the compensatory mutation.

The compensatory P6 mutant retains intron processing activity, but the level of activity is significantly lower than wild type as measured by colony color (Fig. 3), activity in the self-splicing assay (Fig. 6) and activity in the *trans*-splicing assay (Fig. 7). This suggests that the nucleotide sequence of P6 is important for efficient intron processing activity, consistent with the observation that the sequences of P6[5'] and P6[3'] are well-conserved (2).

Results of *in vitro* RNA processing assays (Figs. 6 and 7) provide support for the P6 secondary structure model, although the results are somewhat less clearcut than for the P8 series of mutants. Two base mutations in P6[5'] and P6[3'] each reduce intron processing activity in the self-splicing assay (Fig. 6). The P6[3'] mutant is less active (10% of wild type at the earliest time point) than is the P6[5'] mutant (40% of wild type). The P6[5'] mutant has very nearly the same intron processing activity as the P6 compensatory mutant (Fig. 6). This indicates that the P6[5'] mutation partially compensates for the loss of activity induced by the P6[3'] mutation, but that the P6[3'] mutation does not compensate for the loss of activity induced by the P6[5'] mutation in this assay.

Results of the *trans*-splicing assay (Fig. 7) provide stronger support for the existence and functional importance of P6. The mutations in P6[5'] and P6[3'] each reduce activity to very low levels. As in the self-splicing assay, the P6[5'] mutant is somewhat more active than the P6[3'] mutant. The compensatory P6 mutant has significantly greater activity than either the P6[5'] mutant or the P6[3'] mutant. Since each mutation partially compensates for the loss of activity induced by the other, results

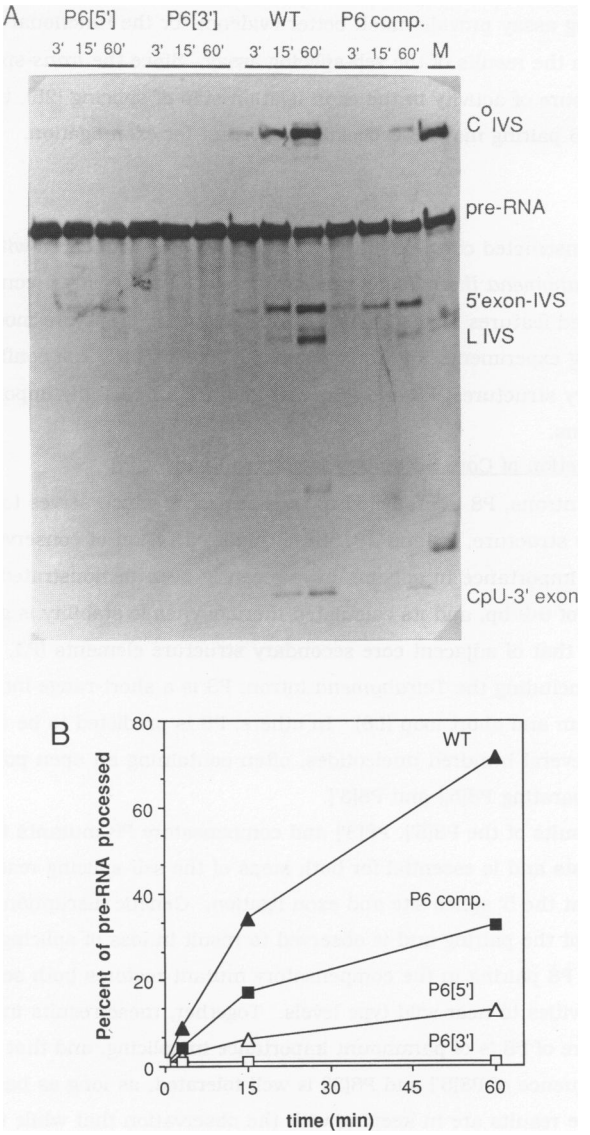

Fig. 7. Intron processing activity of P6 mutants under *trans*-splicing conditions. A, fluorogram of a 4% polyacrylamide-urea gel. B, Quantitation of results from A, obtained by scintillation counting. In part B, percent of pre-RNA processed is determined as the ratio of radioactivity in RNA processing products (CpU-3' exon + L IVS + C^{o} IVS) to that in all discrete RNA species (pre-RNA + CpU-3' exon + L IVS + C^{o} IVS). For further details, see legend to Fig. 5.

of the *trans*-splicing assay provide much better evidence for the functional importance of the P6 pairing than the results of the self-splicing assay. Since the *trans*-splicing assay is taken to be a measure of activity in the exon ligation step of splicing (25), these results suggest that the P6 pairing may be especially important for exon ligation.

DISCUSSION

We have constructed compensatory mutations in core sequences within the group I intron of the *Tetrahymena thermophila* nuclear large ribosomal RNA precursor in order to test two predicted features of the group I intron secondary structure model. Results of *in vitro* self-splicing experiments and *in vivo* splicing screens in *E. coli* confirm that the predicted secondary structures, P8 and P6, exist and are functionally important for intron processing reactions.

Structure and Function of Core Secondary Structure Element P8

In group I introns, P8 is predicted to be a structure which serves to link two regions of the core structure, P3 and J8/7 (the unpaired region of conserved sequence element S) whose importance in splicing have recently been demonstrated (10,13). P8 generally consists of 6-9 bp, and its calculated thermodynamic stability is generally equal to or greater than that of adjacent core secondary structure elements (P3, P4, P5, P6). In some introns, including the *Tetrahymena* intron, P8 is a short-range interaction comprised of a stem and short loop (L8). In others, P8 is predicted to be a long range interaction with several hundred nucleotides, often containing an open polypeptide reading frame, separating P8[5'] and P8[3'].

Splicing results of the P8[5'], P8[3'] and compensatory P8 mutants indicate that the P8 pairing exists and is essential for both steps of the self-splicing reaction, guanosine attack at the 5' splice site and exon ligation. Genetic disruption of P8 is predicted to disrupt the pairing and is observed to result in loss of splicing activity. Restoration of the P8 pairing in the compensatory mutant restores both self-splicing and *trans*-splicing activities to near wild type levels. Together, these results indicate that the secondary structure of P8 is of paramount importance in splicing, and that variation in the nucleotide sequence of P8[5'] and P8[3'] is well tolerated, as long as base-pairing is maintained. These results are in keeping with the observation that while the potential for forming the P8 secondary structure is universal among group I introns, no nucleotide sequence conservation in P8 has been noted.

Mutational data on P8 have been obtained in only one other group I intron. Belfort and coworkers have isolated a mutation in P8[3'] of the intron within the *td* gene of bacteriophage T4. This mutation is a G to A transition which disrupts a GC pair in the center of P8, and results in a splicing-deficient phenotype (26).

The mutational and phylogenetic data on P8[5'] and P8[3'] suggest that the primary

function of these sequences is to provide a base-paired structure which serves as an essential component of the intron folding required for splicing. These data are in many respects similar to those obtained for P3. Like P8, little sequence similarity is observed for P3[5'] and P3[3']. Mutational data strongly support the functional importance of the P3 pairing for splicing *in vivo* (13) and self-splicing *in vitro* (10). If secondary structure is maintained, compensatory mutations in P3 have little or no effect on self-splicing activity (10). Results on P8 and P3 are in distinct contrast with those on P7 (9,10). P7, the base-pairing segment of sequence elements R and S, shows strong conservation of both primary and secondary structure. Individual mutations in P7 cause a loss of splicing activity which is restored by compensatory mutations. However, the P7 compensatory mutants show activity levels which are on the order of 5% of wild type (10), supporting the concept that while the P7 pairing is essential for splicing, the wild type sequence is advantageous.

Structure and Function of Core Secondary Structure Element P6

Comparative sequence data indicate that all group I introns have the capacity to form P6. However, P6 is generally the shortest of the nine conserved pairings and probably the least stable, being most commonly comprised of only two or three base-pairs. Cech has recently pointed out that the nucleotide sequence of P6 is more highly conserved than was previously appreciated (2). The first two bases of P6[5'] are those at the 3' end of conserved sequence element Q (GC in the *Tetrahymena* intron). The first base is G in 51 of the 64 introns surveyed by Cech; the second base is either C or G in 61/64 cases. They are predicted to pair with the last two bases of P6[3'] (GU in the *Tetrahymena* intron), which are the two bases at the 5' end of conserved sequence element R. The first base is a G or C in 61/64 group I introns, the second is U or C in 56/64. In 62 cases these nucleotides can form two base pairs (including G-U pairs); in both of the exceptions one G-C and one A-C pair may form.

In particular, P6 predicted for the *Tetrahymena* intron appears to be exceedingly weak, involving one G-C pair and one G-U pair (Fig. 2). The occurrence of such weak interactions can be predicted from comparative sequence data, but would generally not be identified by computer programs designed to identify minimum free-energy secondary structures.

Splicing results for the P6[5'], P6[3'] and P6 compensatory mutants indicate that the conserved bases of P6[5'] and P6[3'] are important for splicing and support the existence of the P6 pairing. Screening for splicing activity in *E. coli* indicated that the P6[5'] and P6[3'] mutants reduced splicing, and that the activity of each mutant was partially restored by compensatory changes in its pairing partner. *In vitro* self-splicing and *trans*-splicing experiments showed that both the P6[5'] and P6[3'] mutants reduced intron processing activity considerably. Restoration of potential base-pairing in the

compensatory P6 mutant significantly increased activity in the *trans*-splicing assay with respect to the P6[5'] and P6[3'] mutants. We conclude that P6 base-pairing exists and is important for intron processing in the *trans*-splicing assay, and suggest that P6 may be particularly important in exon ligation. The role of the P6 pairing in guanosine attack on the 5' splice site is less clear. The increase in activity of the compensatory P6 mutant relative to the P6[3'] mutant suggests that the P6 pairing is important in guanosine attack; however the compensatory P6 mutant shows no significant increase in activity relative to the P6[5'] mutant. Possibly features of the RNA structure stabilize P6 pairing even in the presence of the base changes in the P6[5'] mutant. For example, an alternative P6 pairing may take place in the P6[5'] mutant, in which A216 and C217 pair with G257 and U258.

It is significant that the activity of the compensatory P6 mutant in both self-splicing and *trans*-splicing assays was substantially lower than wild type. This suggests that the wild type sequences of P6[5'] and P6[3'] are important for efficient splicing. This is consistent with the conserved sequences of P6[5'] and P6[3'] in group I introns.

Recently compensatory mutations in P6 of the group I intron of the bacteriophage T4 *td* gene have been constructed using *in vitro* techniques. Results of *in vivo* thymidylate synthase expression and *in vitro* self-splicing experiments indicate that the P6 pairing exists and is important for splicing of the *td* intron (27).

Crucial Role of Conserved Sequence Element R in Splicing

Sequence element R (GUUCACAGACUAAAUG in the *Tetrahymena* intron) represents the longest contiguous sequence of conserved nucleotides within group I introns. Based on the secondary structure model and mutational data, we conclude that element R is comprised of four segments endowed with distinct functions. The first two nucleotides (GU) base-pair with the last two nucleotides of element Q. This pairing (P6) is important for splicing, particularly in the exon ligation step. The next three bases (UCA) form J6/7, an unpaired region which may contribute to the guanosine binding site of the IVS (10). The seven bases of the central portion (CAGACUA) form a characteristic pairing (P7) containing a bulged A (underlined) with six bases of element S. This pairing is essential for all IVS reactions (9,10). The last two nucleotides of element R (UG) form the beginning of P3[3']. We have shown that P3 is essential for all IVS reactions (10). Thus, the sixteen nucleotides of element R participate in three distinct secondary structure elements and contribute to the intron's binding site for guanosine substrate.

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